Scanning Microscopy

Volume 9 | Number 3 Article 9

6-22-1995

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Scanning Microscopy, Vol. 9, No. 3, 1995 (Pages 729-739) 0891-7035/95$5.00+.25
Scanning Microscopy International, Chicago (AMF O'Hare), IL 60666 USA

SCANNING FORCE MICROSCOPY OF CHROMATIN

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(Received for publication March 5, 1995, and in revised form June 22, 1995)

Abstract

Scanning force microscopy (SFM) is a new method to obtain the topography of surfaces with nanometer-resolution. The ability to image under liquids makes the technique attractive for biological applications, especially for the determination of the ultrastructure of biomolecules under native conditions. One growing field of interest is the investigation of chromatin and chromatin-related structures. Different levels of chromatin condensation were the subject of several previous SFM investigations, from the nucleosomal chain, to the 30-nm fiber, ending with the metaphase chromosome. The SFM yielded new information on such fundamental problems as the core spacing of the nucleosomal chain, the internal structure of the 30-nm fiber and the banding mechanism of metaphase chromosomes. Other investigations dealt with the SFM characterization of polytene chromosomes. This paper reviews the state-of-the-art in SFM chromatin research and discusses future developments in this field.

Key Words: Atomic force microscopy, chromatin fiber, chromosome, nucleosome.

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Introduction

Chromatin, the nucleo-protein complex consisting primarily of DNA and histones, influences life processes by storing and processing genetic information. Fundamental processes such as transcription, translation and replication are closely coupled to chromatin structure. The access of different enzymes to the genetic information, influenced by chromatin packing as well as by associated proteins, regulates cell metabolism. Better understanding of the relationship between chromatin structure and function is the goal of high resolution microscopy of chromatin.

There are several levels of chromatin packaging (for a review see, Van Holde, 1988). The first level of folding is achieved by wrapping the DNA around histone cores (nucleosome core), thereby creating the nucleosomal chain (Kornberg, 1974). The beads-on-a-string substructure, resolved originally in the electron microscope (Olins and Olins, 1974), is very sensitive to salt concentration (Christiansen and Griffith, 1977; Thoma and Koller, 1981). With increasing ionic strength, compaction of the extended nucleosomal chain to a 10 nm fiber occurs. Additional compaction results in formation of a ~30 nm fiber. During the cell cycle, the 30 nm fiber is further condensed, together with various proteins, building up the metaphase chromosome.

Many efforts have been undertaken to resolve the structural details at different levels of chromatin condensation state using electron microscopy (EM), crystallography, spectroscopy, and molecular biological techniques (for a review see, Van Holde, 1988). However, to date, it has not been possible to resolve the DNA path between nucleosomes in complex native chromatin assembly by microscopic or diffraction techniques. Methods with high lateral resolution (e.g., EM) require pre-treatment of the sample, introducing potential artifacts. On the other hand, techniques for investigating chromatin in a native aqueous environment (e.g., diffraction) sacrifice some resolution by providing averaged properties. Thus, a microscopic techniques with high lateral resolution operating under aqueous conditions is needed. In addition to the X-ray microscope (Rudolph *et al.*, 1992),

the recently introduced scanning force microscope (Binnig *et al.*, 1986); also known as atomic force microscope, AFM) couples, in principle, nanometer resolution with the ability of working under aqueous conditions. Initial attempts toward the SFM investigation of chromatin have been promising, illustrating the potential of SFM as a research tool for structural biology.

Substrate for chromatin preparation

For reasonable microscopic imaging, the contrast of the sample should be considerably higher than the background. Therefore, topographic contrast of the SFM demands that the sample height exceeds the substrate surface roughness. For imaging biomolecules with heights in the nanometer-range, surface roughness in the Å-range is required. Mica is often the substrate of choice because of its easy cleavage resulting in an atomically flat surface.

The SFM images of a nucleosomal chain were obtained after adsorption of the sample to magnesium-treated mica (Vesenka *et al.*, 1992b). In another study, chromatin fibers were visualized after adsorption from magnesium-containing buffer on mica {Fig. 1b; (Martin *et al.*, 1995)}. For the magnesium-mediated adsorption of DNA and chromatin onto mica, a mechanism based on Coulomb interactions mediated by magnesium ion "bridges" between the negative charges of mica and the DNA has been proposed (Bustamante *et al.*, 1993; Sogo *et al.*, 1975; Vesenka *et al.*, 1992a). Spermidine-treatment of mica also facilitates adsorption of isolated chromatin fibers (Zlatanova *et al.*, 1994).

Chromatin shows a reasonable adsorption onto glass, as reported by previous light microscopic investigations of spread chromatin (Schlammadinger, 1990; Trendelenburg and Puvion-Dutilleul, 1987). Nucleosomal chains were prepared according to this protocol, including centrifugation of the sample onto glass (Fritzsche *et al.*, 1994). In other studies, reconstituted nucleosomal chains {Fig. 1a; (Allen *et al.*, 1993)} and chicken erythrocyte chromatin fibers in different states of condensation (Fritzsche *et al.*, 1995; Leuba *et al.*, 1994) were adsorbed to glass. The local mean surface roughness of cover glass was determined to be about 1.5 Å (Allen *et al.*, 1993), demonstrating that glass can be a highly suitable substrate for investigations in the nanometer-range. Glass is the standard substrate for cytogenetic metaphase chromosome preparation and has been successfully used for mounting metaphase chromosomes (De Grooth and Putman, 1992; De Grooth *et al.*, 1992; Fritzsche *et al.*, 1994; Musio *et al.*, 1994; Rasch *et al.*, 1993) and polytene chromosomes (Jondle *et al.*, 1995; Mosher *et al.*, 1994; Puppels *et al.*, 1992) for SFM investigations.

Hydration State Influences Viscoelasticity and Height of Biological Specimens

Chromatin preparation for SFM imaging includes air drying of the samples. Drying can induce structural alterations by flattening (Engel, 1991). Native metaphase chromosomes are cylinder-shaped and contain ~90% water (Hearst, 1994); after air drying, they appear flat in scanning electron microscopy (SEM) (Christenhuss *et al.*, 1967) or SFM (De Grooth and Putman, 1992). Omitting this drying step results in decreased stability of imaging in aqueous solution, as shown in the case of chromatin fibers (Fritzsche *et al.*, 1995). This instability is partially due to a change in viscoelasticity of biological samples during rehydration. During imaging, dried biological specimens, for example, metaphase chromosomes, show no response to changes in the applied vertical force giving the appearance of no elasticity. On the other hand, the same chromosome exhibits a high elasticity after rehydration, resulting in significant changes of the apparent topography due to changes in the applied vertical force (Fritzsche *et al.*, 1994).

Another reason for decreased imaging stability of wet samples is the lack of specimen-substrate adhesion. The forces applied by the scanning tip in contact SFM imaging mode overcome this adhesive interactions and the sample is moved or swept away. At a given deflection, the applied vertical force depends on the force constant of the cantilever, so the development of more flexible cantilevers, by using polymeric material instead of Si_3N_4, is an interesting alternative to avoid sample damage and is currently under investigation (Pechmann *et al.*, 1994). Moreover, new imaging modes, such as tapping (Zhong *et al.*, 1993), lowers the applied vertical and lateral forces, and have been successfully used for chromatin investigations on air (Leuba *et al.*, 1994; Martin *et al.*, 1995; Zlatanova *et al.*, 1994). The application of tapping mode under liquid to soft biological specimen (e.g., cells) was demonstrated (Putman *et al.*, 1994), opening the way for gentle chromatin investigations under native conditions.

Rehydration of previously dried samples partially reverses the flattening process of biological samples. Raising the relative humidity influences the height of polytene chromosomes (Mosher *et al.*, 1994). The effect of full rehydration of chromatin is a significant swelling of the dried structures. The increase in height after rehydration in physiological buffer was determined to be 400-500% for metaphase chromosomes (De Grooth and Putman, 1992; Fritzsche *et al.*, 1994) and polytene chromosomes (Mosher *et al.*, 1994). Reversible changes in the three-dimensional structure were observed upon exposure of metaphase chromosomes to solutions of different ionic strengths (Fritzsche *et al.*, 1994). A

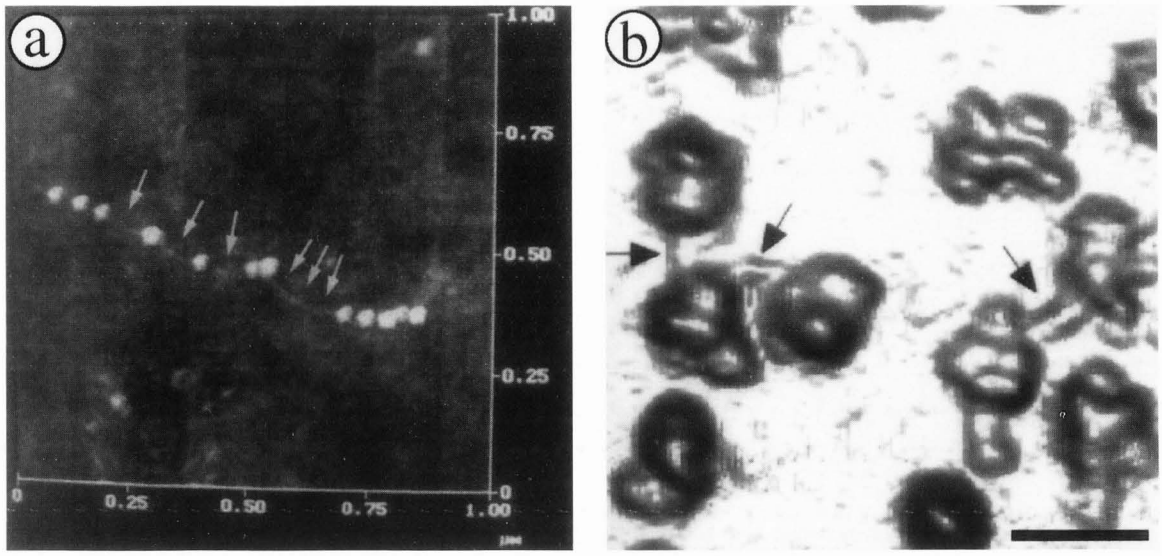

Figure 1. Scanning force micrographs of the nucleosomal chain. (**a**) Chicken histone octamers reconstituted onto a linear DNA fragment and adsorbed on glass. The arrows indicate unfilled nucleosome-positions. Reprinted from Allen *et al.* (1993) with permission. (**b**) Tapping mode image of nucleosomal chains (illuminated perspective view). Structures resembling DNA are marked. Scale bar = 50 nm. Reprinted from Martin *et al.* (1995) with permission; (**a**) and (**b**) copyrighted by the American Chemical Society.

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similar effect was found for chromatin fibers of spread lymphocyte chromatin {(Fritzsche *et al.*, 1995), Fig. 2}. The increase in viscoelasticity of rehydrated metaphase chromosomes causes structural changes as function of the exerted scanning force (Fritzsche *et al.*, 1994). This effect could be used for determination of the local viscoelasticity of chromatin structures with high lateral resolution, as shown for other biological samples (e.g., cells (Hoh and Schoenenberger, 1994)).

The Apparent Sample Width in SFM

The width obtained by SFM is exaggerated due to the tip-sample interaction which is a function of tip geometry and local fluid environment. The contact between tip and sample is not always at the apex of the tip (especially at steep structures) causing apparent structural broadening. Deconvolution procedures, assuming a regular tip geometry (Keller, 1991; Keller and Franke, 1993; Vesenka *et al.*, 1993), have been developed. While useful, the application of this assumption to commercially made tips is unrealistic because of their asymmetrical shape. To better determine the actual tip shape, calibration standards with defined geometry (e.g., colloidal gold) have been employed (Vesenka *et al.*, 1994; Xu and Arnsdorf, 1994). The determination of the full-width-at-half-maximum height (Fritzsche *et al.*, 1994) provides a practical and simple compromise between the apex and edge detection modalities of a conical tip in SFM. Subtracting the tip diameter from the apparent width should result in an approximation for the true value. Another deconvolution approach uses the square root of the product of tip and sample radius as the effective size (Vesenka *et al.*, 1992a; Zenhausern *et al.*, 1992). Most common tips used for chromatin investigations are etched Si tips {e.g., Ultralever, Park Scientific Instruments (PSI), Sunnyvale, CA; Nanoprobes, Digital Instruments (DI), Santa Barbara, CA} and electron beam deposited tips {EBD; (Keller and Chou, 1992)} with tip diameter in the order of ~10 nm {PSI; (Vesenka *et al.*, 1992b)}.

"Dry" is a relative term. Martin *et al.* (1995) and Leuba *et al.* (1994) have shown residual moisture on the mica surface stabilizes chromatin structure even at low ambient humidity. It is this level of moisture that severely affects sample adhesion in contact mode SFM, but is much less effected by tapping mode imaging (data not published), which, in effect, measures the capillary layer surrounding a biomolecule.

Nucleosomal Chain

The nucleosome, a protein octamer encircled by DNA loops, has a disk-like shape with a thickness of 5.5 nm and a diameter of 11 nm according to X-ray data (Richmond *et al.*, 1984). Heights determined by SFM

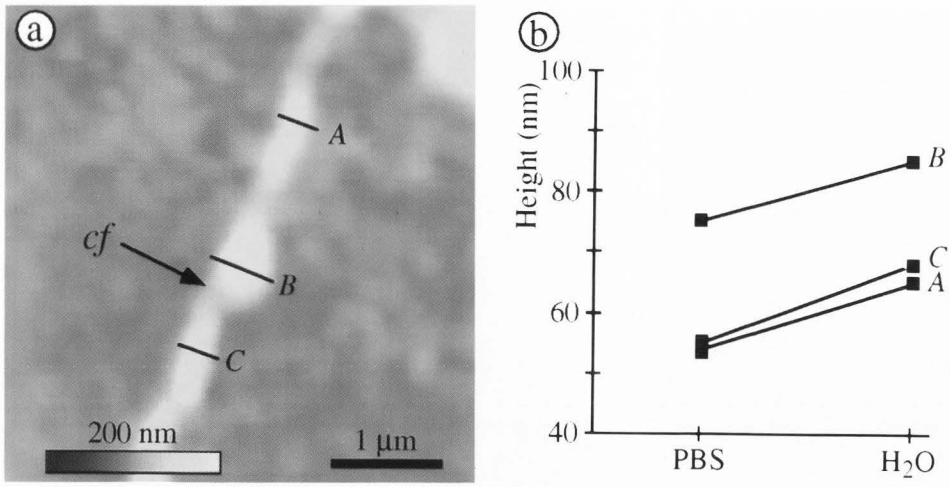

Figure 2

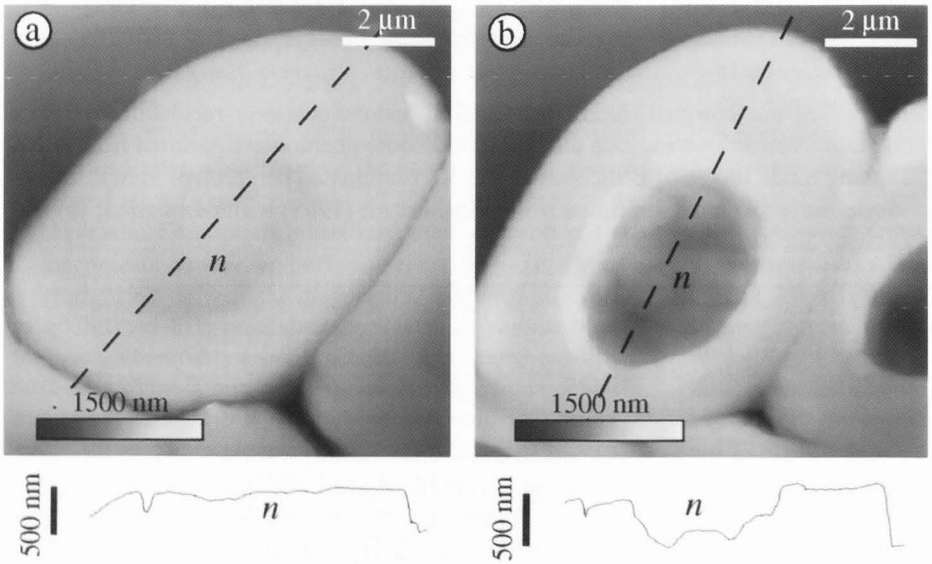

Figure 3

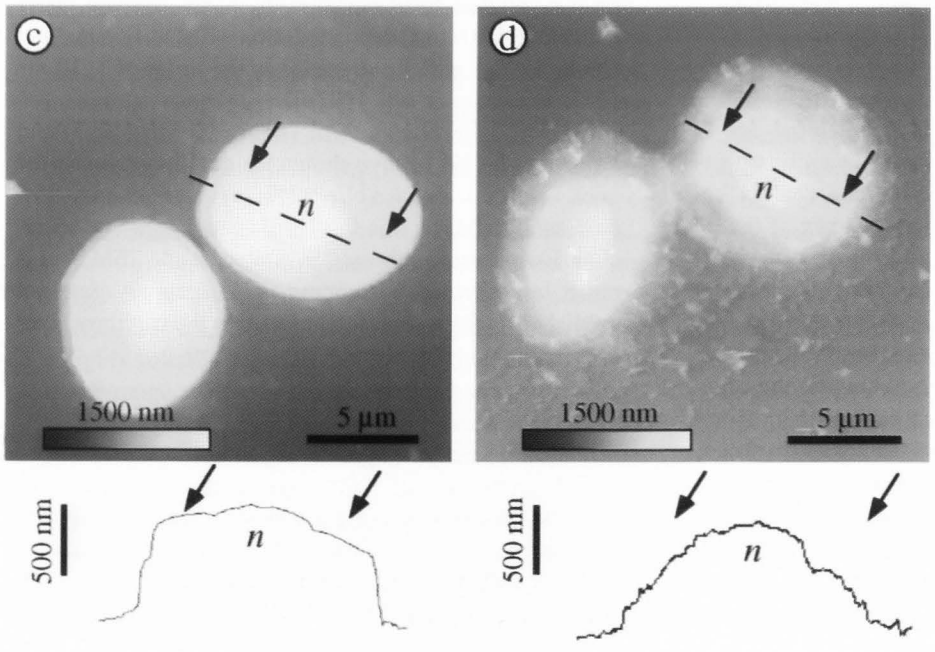

Figure 2 (on the facing page 732 at top). Salt-dependent swelling of a chromatin fiber investigated by SFM. (a) Human b-lymphocyte chromatin was spread by a modified droplet diffusion method as described in (Fritzsche *et al.*, 1995). The sample was rehydrated and a chromatin fiber (*cf*) was visualized in PBS (phosphate buffered saline, a) and in water (not shown). (b) The maximum height of the chromatin fiber in (a) measured on three different locations (A, B, C) in PBS and in water along the fiber.

Figure 3 (on the facing page 732 at bottom). The selectivity of detergent extraction of cellular components visualized by SFM. Chicken erythrocytes adsorbed on glass were visualized in air before (a and c) and after detergent treatment with 1% SDS (b) or 0.1 % Triton X-100 (d). The lines in the SFM images indicate the location of the cross-sections shown below each panel. The nuclear region of the cell (indicated with n) is selectively extracted after SDS treatment (b). It remains after Triton X-100 treatment, in contrast to the peripheral regions of the cell, which were extracted (d).

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were ~2.5 nm (Vesenka *et al.*, 1992b) and ~4.5 nm (Fritzsche *et al.*, 1994) for spread chicken erythrocyte chromatin, up to 5.5-6.0 nm for reconstituted nucleosomes {Fig. 1a; (Allen *et al.*, 1993)}, and ~4.5 nm for rDNA chromatin {Fig. 1b; (Martin *et al.*, 1995)}. As discussed above, the apparent height of samples in SFM is usually less than the value for the native structure, probably due to flattening during air drying. Previous transmission EM investigations of negatively stained (Finch *et al.*, 1975) or metal shadowed nucleosomes (Oudet *et al.*, 1975) yielded widths of 8.5-13.0 nm. Nucleosome widths from spread chromatin measured by contact mode SFM yielded base to base full widths of ~30 nm (Vesenka *et al.*, 1992b) and ~31 nm (Martin *et al.*, 1995), and full width at half maximum height of ~21 nm (Fritzsche *et al.*, 1994). Allen *et al.* (1993) reported a range of 27-40 nm for the full widths of reconstituted nucleosome cores by contact mode SFM, whereas tapping mode measurements of spread chromatin result in ~23.2 nm for the base to base full nucleosome width (Martin *et al.*, 1995).

In contrast to numerous SFM reports of plasmid DNA with clearly visualized contours (Bustamante *et al.*, 1993), the linker DNA between the nucleosomes was not readily resolved from the background. Measured heights were below 1 nm (Allen *et al.*, 1993; Fritzsche *et al.*, 1994; Martin *et al.*, 1995). The difficulty in tracing the DNA could be due to residue from the sample preparation increasing surface roughness, e.g., sucrose in the case of hypotonically spread chromatin (Fritzsche *et al.*, 1994).

The SFM has been used for the determination of the DNA compaction and the nucleosome spacing in reconstitution experiments {Fig. 1a; (Allen *et al.*, 1993)}. The closest achievable core-to-core distance was about 37.2 nm. Erythrocyte chromatin exhibited a core-to-core distance of adjacent nucleosomes of ~30 nm in SFM (Fritzsche *et al.*, 1994), in agreement with values of 18-36 nm in similar EM investigations (Zentgraf *et al.*, 1980).

Chromatin Fiber

Several preparation techniques have been developed for rendering chromatin fibers accessible to structural investigations by microscopy {for a review see, Zentgraf *et al.* (1987)}. Isolated chromatin can be obtained through mechanical, osmotic, or chemically-facilitated rupture of the surrounding membranous components and transferred onto solid substrates by direct adsorption or by centrifugation through a fixative {for reviews see, Bakken and Hamkalo (1970); Sogo and Thoma (1989)}. For SFM analysis, glutaraldehyde-fixed isolated chromatin fibers from chicken erythrocyte chromatin were adsorbed onto mica in the presence of different NaCl concentrations (0-80 mM) (Zlatanova *et al.*, 1994). Fiber compaction due to higher salt concentrations was observed. Additional digestion accessibility studies of the visualized structures were conducted to obtain information about the location of the linker DNA (Zlatanova *et al.*, 1994). Another study of the salt dependent chicken erythrocyte chromatin structure used hypotonic spreading prior to centrifugation through a formaldehyde-sucrose cushion on a glass substrate (Fritzsche *et al.*, 1995). Different compaction states of the chromatin fiber were visualized. Investigation of unfixed air-dried extended chromatin fiber by tapping mode SFM (Zhong *et al.*, 1993) revealed their three-dimensional structure (Leuba *et al.*, 1994). The observed structure correlated well with a recently proposed model for the chromatin fiber (Woodcock, 1994). An SFM study of isolated chromatin reported visualization of individual histones within dissociated nucleosome cores and associated linker DNA {Fig. 1b; Martin *et al.* (1995)}. Three-dimensional information gathered from these chromatin fibers supports a variable "zig-zag" conformation for the fiber, as recently proposed (Horowitz *et al.*, 1994).

Cell lysis performed at an air-water (hypotonic or isotonic salt concentrations) interface yields largely intact chromatin in contrast to the isolated single chromatin fibers discussed above. The cells release their contents which can be picked up by contacting the interface with a solid support (Gall, 1963; Zentgraf *et al.*, 1987). SFM investigations of material adsorbed according to

this protocol showed a complex fibrous structure. The definite identification of this material as chromatin was achieved by DNA-specific fluorescence-labeling prior to optical and SFM imaging of the same structure (Fritzsche *et al.*, 1995). The adsorption of the chromatin fibers onto the glass substrate was strong enough to visualize rehydrated samples in an aqueous environment. A reversible and salt-dependent swelling behaviour of the samples was observed (Fritzsche *et al.*, 1995). Due to its high spatial resolution, the SFM allowed the investigation of the swelling behaviour of a single chromatin fiber along its long axis (Fig. 2). Height changes in the nanometer-range could be detected dependent upon the salt concentration of the aqueous environment (Fig. 2b). Single natively hydrated (which have been never dried) and unfixed chromatin fibers could be visualized by SFM, but the stability of the sample was decreased, probably due to insufficient adsorption onto the substrate (Fritzsche *et al.*, 1995).

Metaphase Chromosomes

Cell-cycle dependent chromatin condensation culminates during metaphase. The chromosome is formed by a series of chromatin packing hierarchies utilizing both loop and coil folding motifs. The 30 nm fiber condensed into a structure with a diameter of 225-250 nm, referred to as the chromatid fiber (Rattner, 1992). The coiling and condensation of this fiber give rise to the final form of the arms of a metaphase chromosome.

SFM imaging of metaphase chromosomes spread on glass revealed the typical x-like shape. Substructures were visible on the air-dried chromosomes with dimension of ~50 nm (De Grooth *et al.*, 1992; Fritzsche *et al.*, 1994). These structures can be interpreted as loops of the 30 nm fiber, as revealed by EM (Adolph *et al.*, 1986). Other explanations for the observed features include salt or cell debris from the preparation procedure, which dried onto the chromosome. Our SFM investigations confirm the existence of a thin layer covering the areas with spread metaphase chromosomes, as reported elsewhere (De Grooth *et al.*, 1992). The layer is occasionally disrupted and exhibits holes some hundreds of nanometers in diameter. The diameter of the covered area reaches 30 μm. In phase contrast, no layer could be resolved, but it could be visualized by scanning electron microscopy by virtue of its different surface roughness in contrast to the uncovered substrate. We applied detergents to obtain information about the composition of this coating. Detergents such as Triton X-100 and SDS (sodium dodecyl sulfate) are widely used in microscopy for selective removal of cell components (Penman *et al.*, 1983; Pietrasanta *et al.*, 1994; Webster *et al.*, 1978). In such studies, the detergent application occurs before the drying step. Drying of biological samples could result in denaturation of their components (e.g., proteins), therefore, we tested the effect of detergents on dried samples by treatment of air-dried cells (human lymphocytes and chicken erythrocytes, cf. Fig. 3). We used the ability of the SFM to image specimen without special treatment (e.g., coating or staining) to visualize samples before and after detergent treatment. SDS application resulted in selective removal of nuclear material (Figs. 3a and 3b), whereas Triton X-100 solubilized the peripheral parts of the cell leaving the nuclear (central) part unaffected (Fig. 3c and 3d). The Triton X-100 treatment of chromosome samples results in a removal of the coating, suggesting that it consisted of non-nuclear membranous or cytoplasmic material. Digestion of samples by trypsin (a proteinase) caused collapse of the chromosome structure, but had no effect on the layer. In support of the membranous nature of the layer was the observation that biological membranes exhibit heights of ~10 nm in SFM/EM investigations (Stemmer *et al.*, 1987); this is in agreement with the determined layer height of 7-9 nm.

The metaphase chromosome is longitudinally heterogeneous in structure and function and this is the basis for the specific banding patterns produced by various chromosome staining techniques for optical microscopy (Craig and Bickmore, 1993). A chromosome banding map can be connected with the genetic linkage map, unifying genetic and microscopical results (Francke, 1992). The underlying chromosomal order responsible for the banding phenomena has been discussed (Saitoh and Laemmli, 1994), but not completely understood. Differences along the arms of treated chromosomes in optical or fluorescence contrast could also be visualized using transmission or scanning EM (Harrison *et al.*, 1981; Xu and Wu, 1983). In the topographic contrast of SFM, G-banded chromosomes exhibit a pattern similar to the bands described in optical microscopy. Heights of ~50 nm and ~300 nm have been reported (De Grooth *et al.*, 1992; Rasch *et al.*, 1993). The reason for different heights is probably that the volume of the chromosome strongly depends on the prior history of the sample, i.e., on the specific cell growth conditions, on the procedure for growth inhibition, on the methods of isolation, fixation and washing (Burkholder, 1988), and on the banding procedure. The main difference between these investigations was a washing step with acetic acid in the first study, which removes the above mentioned layer (De Grooth *et al.*, 1992), and possibly also some of the chromosomal proteins.

It has been hypothesized that staining to generate banding patterns enhances a pre-existing pattern corresponding to the chromomeres of meiotic pachytene chromosomes (Comings, 1978). Untreated chromosomes

exhibit no banding pattern in optical microscopy or EM (Burkholder, 1974). However, the SFM was able to resolve height modulations of ~5 nm in untreated metaphase chromosomes similar to the pattern observed after banding treatment in optical microscopy (Musio *et al.*, 1994). Thus, the SFM is capable of obtaining information that is difficult or impossible to acquire by other methods.

In situ hybridization, the marking of defined DNA sequences in the chromosome, can allow correlation of chromosome ultrastructure with the genetic information. Topographic labeling by enzyme-catalyzed crystal growth was used to visualize specific chromosomal regions by SFM (Putman *et al.*, 1993; Rasch *et al.*, 1993). The precision of probe detection was in the same range as the diameter of the signal (possibly down to ~50 nm). A further increase in resolution demands the development of SFM specific probes, overcoming the limitations in lateral resolution of fluorescent or topographic labeling.

Polytene Chromosomes

Polytene chromosomes are an amplified form of interphase chromosomes found in the nuclei of specific, often genetically well mapped, cells {for a review see, Hill and Rudkin (1987)}. The SFM resolves their internal structure of bands separated by interbands (Jondle *et al.*, 1995; Mosher *et al.*, 1994; Puppels *et al.*, 1992; Vesenka *et al.*, 1995). The application of tips with high aspect ratio (EBD tips; Keller and Chou, 1992) results in high resolution (better than 30 nm) but at the cost of compromising the structural integrity of the sample (Jondle *et al.*, 1995). Pyramidal shaped tips showed no obvious perturbation of the sample, but provided relatively low resolution. A reasonable compromise was achieved through the use of etched Si tips. These tips could be used for imaging as well as sample dissection, as a function of applied vertical force. This effect was used for microdissection of polytene chromosomes, an preliminary step toward DNA isolation for gene mapping with high lateral precision (Mosher *et al.*, 1994; Vesenka *et al.*, 1995).

The ultrastructure of polytene chromosomes was investigated by a combined Raman microspectroscopic and SFM study (Puppels *et al.*, 1992). Spectroscopy yielded information about the distribution of DNA and protein between bands and interbands, and was complemented by SFM structural information about the ~100 and ~25 nm chromatin fibers that were typically observed. A combined inverted optical microscopy / SFM study enabled selective staining of the banded structure and identified topographic correlation with DNA and protein content (Vesenka *et al.*, 1995).

Conclusions

After the first years of chromatin studies by SFM the potential of this new microscopic method has become clear. SFM provides a unique data set including some information difficult or impossible to obtain by conventional methods. A crucial point for SFM studies is the sample preparation, including choice of substrate and the adsorption, immobilization, fixation and/or drying of the sample. A special challenge for future development of the SFM as a structural biology tool is the approach to measurements under native conditions. The use of sharper tips, in conjunction with new imaging modes (e.g., tapping), or the development of weaker cantilevers (softer materials) for the contact mode imaging, should improve SFM resolution further. The demand for specific labeling techniques is apparent, perhaps combining the highly developed molecular-biological probes (e.g., DNA probes, antibodies) with an SFM specific stain. This should allow full exploration of the SFM technique as a powerful tool for the direct observation of chromatin structure, and establish new perspectives in imaging complex biological structures.

Acknowledgments

This work was supported by the German Academic Exchange Service. Journal Paper No. J-16403 of the Iowa Agriculture and Home Economics Experiment Station, Ames, Iowa. Project No. 3064.

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Discussion with Reviewers

H. Hansma: You hypothesize that cell growth conditions might affect the height of chromosomes in the SFM. What is the evidence for this?
Authors: We have no direct evidence for this suggestion. Beside DNA, chromosomes contains a complex set of proteins. The structure of a chromosome is influenced by these proteins, as demonstrated by structural changes induced by proteinase treatment. We believe that, for example, the growth medium composition could affect the composition of the protein set, therefore, inducing structural changes. These changes are probably too small to distinguish from other factors, so it is only a hypothesis.

E. de Harven: Could you please identify, in chromatin studies, one structure visualized under the SFM at higher resolution than with other ultramicroscopic methods?
Authors: In case of chromatin investigations, the techniques of conventional ultramicroscopic methods were developed and optimized over a long period, and are capable of a level of lateral resolution not yet achieved by SFM. In terms of vertical resolution, there is no comparison since SFM obtains Å resolution and EM requires non-real time reconstruction. We see the advantage of the SFM for chromatin studies today in measurements in liquid environments, and exploiting the high vertical resolution as demonstrated in the case of the rehydrated single chromatin fibers under different ionic strength (cf. Fig. 3). Another interesting application for chromatin structures is the ability of SFM of determination of viscoelastic properties of samples with a high lateral resolution.

E. de Harven: You state that the main advantage of the SFM is its operation under aqueous conditions. Still, you quote many recent SFM publications in which air drying was used. Please clarify.
Authors: By its contrast mechanism, the contact SFM needs to apply a mechanical force to the local surface which is in contact with the tip. Therefore, in early studies, immobilization of the specimen on a surface is necessary. In solution, the adsorption of chromatin to substrates was usually not strong enough to resist the applied forces, as a consequence, the imaging stability without air drying was substantially decreased {cf. SFM images of undried chromatin fibers under PBS in (Fritzsche *et al.*, 1995)}. The air drying step improved the stability of imaging, allowing the first reasonable SFM imaging of chromatin. Improvements in immobilization methods coupled with the recent introduction of shear-force reduced imaging modes (e.g., tapping mode) should result in a growing number of studies of native chromatin in aqueous conditions.

In the case of metaphase chromosomes, the standard spreading technique, includes air drying, which is also one step in several SEM studies {e.g., (Harrison *et al.*, 1981; Sumner, 1991; Sanchez-Sweatman *et al.*, 1993)}. These studies used air drying prior to rehydration and critical point drying, which implies that the structural alterations induced by air drying are at least to some extent reversible. Allen *et al.* (1985) found no difference between the structure of natively hydrated chemically isolated chromosomes (which have been never dried) and air dried metaphase spreads by SEM. Sanchez-Sweatman *et al.* (1993) speculated that nucleic acids may have an inherent resistance to damage by air drying. Based on these studies we feel that, while not truly native, rehydrated specimen retain some of their native structural attributes.

On the other hand, gentle drying techniques, such as the critical point drying, which have been used for a long period in EM, could be used for SFM specimen preparation, as recently shown for the case of DNA (Thundat *et al.*, 1994). Our own experiments with these technique in drying cells and chromosomes resulted in decreased imaging quality (unpublished results). This was presumably due to preserved fine structures which are not stable against the forces exerted by the scanning tip in contact mode SFM; experiments with modes of reduced force (e.g., tapping) will further test the applicability of this technique for SFM.

H. Ris: Scanning Force Microscopy is a promising new tool for structural analysis of the basic level of chromatin organization, the interaction of DNA with the nucleosome and its changes during replication and transcription. It has nanometer range lateral resolution, provides topographical information and can operate in a natural physiological environment. There are still serious obstacles for specimen preparation in the choice of the substrate, the immobilization of the specimen on the substrate, and the interaction of the tip with the specimen.

This manuscript, and the images provided, convince this reviewer that, so far, no new information on chromatin structure has been obtained which justifies publication. It is inconceivable that today anybody would take images of air dried chromatin seriously, even when obtained by a new technique that is advertised to give nanometer resolution of biological structures in a normal physiological environment!
Authors: Other than the answers given above to Prof. de Harven and below to Dr. Malecki, we have no additional comments on this review.

M. Malecki: Chromatin is organized into a complex architecture. This architecture is inevitably destroyed dur-

ing air drying aimed at anchoring to a substrate. Organization after rehydration may not represent its organization in the native state. Furthermore, for studies with transmission or scanning electron microscopy, the chromatin organization is stabilized through deposition of metals and/or carbon. The great promise of atomic force microscopy in biology is its ability to attain molecular resolution in liquids. However, the basic requirement to attain such resolution is a mechanical stability of the specimen under investigation. What advances in the preparation of a specimen or in the design of an atomic force microscope can you foresee allowing us to study the architecture of the chromatin in its native state?

Authors: We believe, that high resolution SFM studies under native conditions will be made feasible by the development of SFM modes with minimized vertical force applied by the tip (compared to the conventional contact mode). The mentioned tapping mode is one step in this direction. The application of this mode allowed, for the first time, the observation of adsorption of DNA molecules on mica under aqueous conditions (Hansma *et al.*, 1995) or the assembly of the DNA-polymerase complex (Guthold *et al.*, 1994). These studies demonstrated, that electrostatic interactions of biomolecules provide sufficient mechanical stability for SFM imaging in the natively hydrated state (i.e., without intermediate air-drying). Chromatin adsorbs to glass substrates without any treatment. This has been shown by fluorescence microscopy and contact mode SFM of the natively hydrated specimen (Fritzsche *et al.*, 1995), although the latter was hampered by low stability (probably due to its high mechanical forces exerted by the tip). Therefore, based on the successful application of low force imaging of biomolecules, it should be possible to collect aqueous images of chromatin that has never been subjected to air drying.

M.J. Allen: It is clear from our own AFM work on reconstituted and native chromatin that occasionally the fibers are obviously "stretched-out" along the substrate and that the majority of nucleosomes are oriented "face-down" on cover glass and mica. Is this your own observation, and, if so, to what extent do you see these types of interactions with the substrate surface during adsorption? Please comment on the possible alterations of the chromatin's 3D solution structure during sample adsorption.

Authors: In the case of the extended nucleosomal chain (beads-on-a-string), the dimensions of the beads we observed leads to the conclusion of a face-down orientation of the nucleosomes on the substrate. This aligned orientation reflects the influence of adsorption, not the 3D solution structure. With increasing condensation, the height of the fiber increased, but we could no longer resolve single nucleosomes to get information about the orientation. The adsorption onto the substrate could induce a stretching of the fibers. This would explain the observation that on the same specimen of hypotonically spread chicken chromatin, clusters of fibers exhibit a more condensed state, but single fibers appear often stretched (Fritzsche *et al.*, 1994). Other factors causing such an behaviour could be inhomogeneous spreading conditions or mechanical forces during centrifugation of the specimen.

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