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CRITICAL POINT MOUNTING OF KINETOPLAST DNA FOR ATOMIC FORCE MICROSCOPY

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Abstract

Atomic force microscope (AFM) images of intact kinetoplast DNA were obtained from samples prepared utilizing critical point drying. These images are compared with AFM images obtained using conventional methods for DNA deposition. Although the images obtained on chemically pretreated mica show more details than on unmodified mica, images obtained with critical point drying were superior. Kinetoplast networks with expected sizes and structures were routinely observed with critical point drying. The resolution of individual strands of DNA was greatly improved, and image artifacts associated with air dried samples were eliminated. Samples prepared using mildly sonicated kinetoplast DNA show isolated minicircles.

Key Words: Atomic Force Microscopy, kinetoplast DNA, critical point drying, chemical treatment.

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Introduction

The atomic force microscope (AFM) [2] has enormous potential as a structural probe for biological samples. The AFM can be operated in air or in a biologically relevant medium, and the sample can be a conductor or an insulator. The AFM operates by recording the mechanical interactions of a sharp tip mounted on a cantilever as it is scanned over the sample surface [17, 18]. In the contact-mode AFM, the cantilever deflection is typically held constant by a servo-circuit controlling the sample height. In another mode of operation, called "tapping mode" (Digital Instruments Inc., Santa Barbara CA), the servo maintains a constant amplitude of cantilever vibration as the tip gently touches the surface at the cantilever excursion maximum. Although factors controlling resolution and contrast are not completely understood, the AFM is a high resolution instrument capable of resolving atomic details on flat surfaces [3]. One distinct advantage of AFM as compared to electron microscopies is the simplicity of sample preparation and operating environment.

AFM images of DNA have been routinely obtained by different sample preparation techniques [5, 9-11, 12, 22, 28, 30, 32]. By one technique, in which DNA is adsorbed on chemically modified mica, reproducibly higher quality images of DNA are produced compared to DNA adsorbed on unmodified mica [5, 9]. Chemical modifications of mica increase the bonding of DNA to the substrates and make it stable against removal by rinsing in ethanol-water mixture [23]. Since the DNA adsorbed on chemically treated mica can be rinsed, artifacts arising from salt deposition are minimized in these sample preparations. However, pretreating the mica surface with cations is a time consuming procedure and can be completely eliminated by adding 5-10 mM of magnesium directly to the DNA solution prior to deposition [10]. The role of this cation in improving the adhesion of DNA to the mica surface is not known. Better sample preparation techniques that may improve resolution and eliminate artifacts caused by air drying are essential to obtain high quality images. This is especially the case for imaging kinetoplast DNA (kDNA), which is a network of thousands of interconnected DNA circles.

Kinetoplast DNA is the mitochondrial DNA of parasitic protozoa such as those of the genera Trypanosoma, Leishmania, and Crithidia [4, 6-8, 16, 21, 22]. Each kDNA network is composed of interlocked DNA circles forming a structure that is about 10 μm in diameter. Two kinds of double stranded DNA circles constitute the network. The most abundant are minicircles which vary in size from 0.5 to 2.8 kbp. Less abundant maxicircles have a size of 30 kbp. Each kinetoplast DNA network contains approximately 104 minicircles and approximately 50 maxicircles. The kDNA used in this work from Trypanosomatid Crithidia faciculata is composed of approximately 5000 minicircles and about 25 maxicircles. The lengths of minicircles and maxicircles in this network are 2.5 kbp and 37 kbp, respectively, as shown by electron microscopy [16].

Understanding the structure of any biological system is an important first step in understanding its function. AFM images of kDNA obtained with routinely used techniques show reduced resolution and drying artifacts. However, kDNA samples prepared using critical point drying show improved resolution and total absence of drying artifacts. Critical point drying, which is routinely used for spreading biological samples for electron microscopy, involves exchanging of an ethanol environment with that of liquid carbon dioxide and then passing the system through the liquid-gas phase at the critical point. In this report, we compare images of intact kDNA adsorbed on mica using critical point drying with images of kDNA obtained with routinely used techniques. We also show that resolution can be improved by using the tapping mode AFM.

Experiment

Sample preparation

In this study, we have used kinetoplast DNA isolated from Trypanosomatid Crithidia fasciculata [6]. The DNA was dissolved in 0.01 M ammonium acetate buffer (Fisher Analytical reagent grade) at pH 7.2. The mounting substrates were freshly cleaved muscovite mica (New York Mica Co. NY).

Three different sample preparation methods were used in this study. The first technique involved depositing a drop of kDNA on freshly cleaved mica. Rinsing such samples in water or ethanol-water mixture was found to remove all kDNA from the surface indicating poor adhesion of DNA to the substrate. Therefore, in this procedure, a 20 μl drop of kDNA solution, at a concentration of 10 μg/ml, was deposited and allowed to air dry on a freshly cleaved mica surface prior to imaging. In the second technique, the kDNA was adsorbed on mica surfaces chemically modified by treatments with either barium or magnesium ions [23] to increase adhesion. DNA mounted in this fashion was stable against rinsing in ethanol-water. The rinsing step removes buffer salt that otherwise would dry along with DNA introducing artifacts. On chemically pre-treated mica, 100 μl kDNA solution at a concentration of 35 μg/ml was placed on the surface for five minutes. The DNA solution was then removed by wicking with filter paper, and the surface was then rinsed gently by dipping in a mixture of water and ethanol (1:1 by volume). Samples were blown dry with nitrogen and stored overnight in a desiccator prior to imaging with the AFM. In the third technique, 10 μl of 0.1 μg/ml of kDNA containing 5-10 mM magnesium acetate was placed on freshly cleaved mica for 5 minutes. The samples were first rinsed in a stream of distilled water. The samples were then rinsed by plunging into ethanol-water mixture (1:1 by volume) five times followed by rinsing five times in ethanol. The samples were placed in 100% ethanol in an Autosamdri-810 critical point dryer (Tousimis, Rockville, MD) and dried using CO_2. This procedure takes approximately 30 minutes and replaces ethanol with liquid CO_2 that is passed through the liquid gas-phase at the critical point preventing drying artifacts.

Atomic force microscopy

AFM images were collected using commercially available contact and tapping mode instruments (Nanoscope, Digital Instruments, Santa Barbara, CA). The contact scanning tips used were commercially available silicon nitride (Si_3N_4) cantilevers, 200 μm long with a spring constant of 0.12 N/m (Digital Instruments, CA). The tapping mode tips used were rectangular silicon cantilevers with a spring constant of 30 N/m (Digital Instruments, Santa Barbara, CA). The contact AFM images were obtained in a constant force mode (3 to 10 nN net repulsive force on the cantilever) and are presented here as raw data except for flattening. Since AFM images of DNA show contrast and width variation as a function of relative humidity, the AFM was placed in a low humidity chamber (dry nitrogen filled) during imaging [20]. Friction effects on contrast (contact mode) were minimized by recording scans at 180 degree. The tapping mode AFM was operated in helium atmosphere at a scan rate of 1.5 Hz. Typical resonant frequencies were about 310 kHZ with a quality factor around 600.

Results and Discussion

AFM images

AFM images of kinetoplast DNA networks adsorbed on freshly cleaved mica by depositing a drop and air drying appear to be thin circular discs approximately 5-7 μm across. This is smaller than the reported kDNA size of 15μm by 10μm obtained with electron microscopy [16]. This decrease in size of kDNA network adsorbed on freshly cleaved mica is likely caused by capillary forces acting on the network during drying. A typical AFM image showing two separate networks lying close to each other on a freshly cleaved mica is shown in Figure 1a. A patch of DNA circles protruding from one of the networks (right) can also be seen in this image. This may be a broken patch of DNA network that is partially overlapping a complete network. Similar patches of interconnected DNA circles and linear DNA chains that may have broken away during sample preparation

were often seen on the substrates along with intact networks. Figure 1b shows an edge of a kDNA network. The strand that extends from the network is approximately 9 μm long and appears to be a broken maxicircle. There is a noticeable lack of structural resolution within the kDNA network adsorbed on freshly cleaved mica.

Figure 2 shows an edge of a kDNA network adsorbed on mica pre-treated with magnesium ions. The widths of strands observed were always thinner than those prepared by droplet drying. The images of kDNA adsorbed on mica pre-treated with magnesium show networks with expected diameter of about 15 μm indicating that the drying artifacts and codeposition of buffer salts can be much reduced by this technique. However, Figure 2b, taken at the center of a network, shows circles elongated in one direction that may be due to blow drying of the sample [19]. Thinner strands and identifiable DNA circles, as shown in Figure 2a, were absent on images obtained when the networks were adsorbed on freshly cleaved mica (Figure 1). One distinct minicircle can be seen in Figure 2a. However, most isolated minicircles observed on pre-treated mica were twisted around themselves, possibly due to surface tensional forces acting during drying. Unlike electron micrographs of kDNA, the contact AFM images, obtained both on freshly cleaved and pre-treated mica, did not show any isolated or intact images consisting of two or three interlocked minicircles. Even when sonicated kDNA were used, the images of DNA circles obtained showed deformations due to drying.

The lack of resolution of kDNA, when adsorbed on freshly cleaved mica by drop drying technique, may be directly related to sample preparation. Since these networks were deposited on the surface from a drop of kDNA solution, as the solution dries, the drying forces compress the strands of the network. This can also explain the shrinking of kDNA network size observed with AFM. Similar results are observed for circular plasmid DNA when deposited by drying a drop containing DNA [24]. Even on chemically treated mica, where DNA structures that are relatively free from salt codeposition and drying artifacts are found, open minicircles were rarely found. We attribute the lack of completely open minicircles and intact networks in AFM images as due to drying forces during sample preparation.

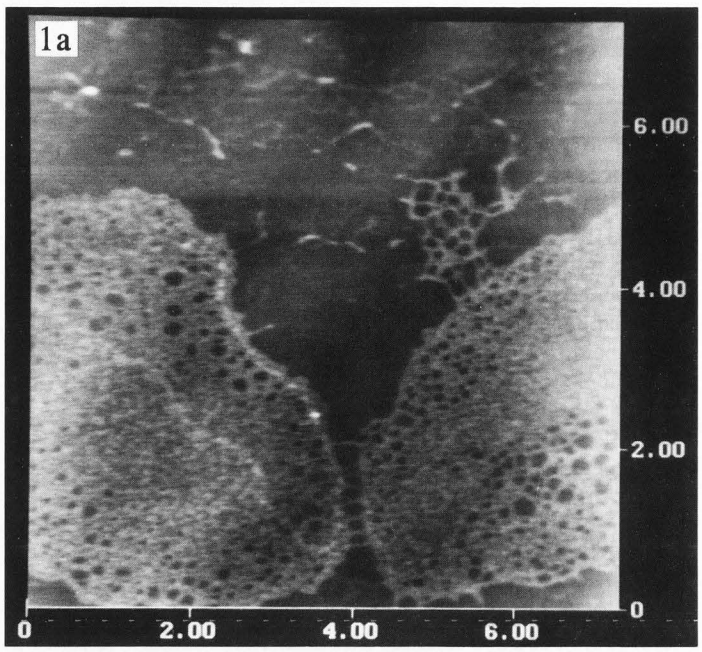
These problems were eliminated by using critical point drying. Figure 3 shows a kDNA network prepared using critical point drying. Isolated strands of intact minicrcles that are free from drying artifacts can be found near the network edge. The size of the network is approximately 10-15 μm, in good agreement with the expected size of the network. It is also interesting to observe that the DNA strands in these images do not overlap or twist around themselves. Though the network contains approximately 25 maxicircles, open maxicircles were found rarely in the AFM images. This result is consistent with that of electron microscopy studies [16]. Away from the kDNA networks prepared by critical point drying, large numbers of completely open minicircles can be seen. Samples prepared by gentle ultrasonic agitation show increased number of completely open minicircles (Figure 4). However, after sonication, many broken DNA strands were found in AFM images.

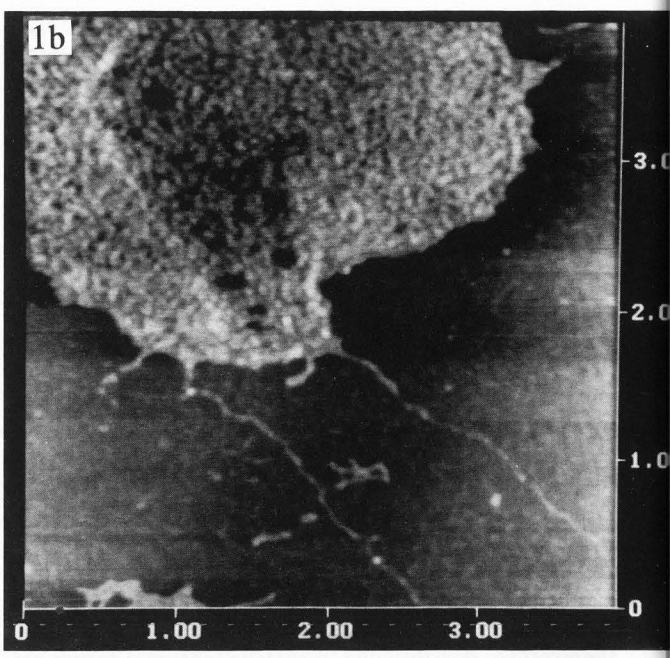
Contrast and width of DNA strands

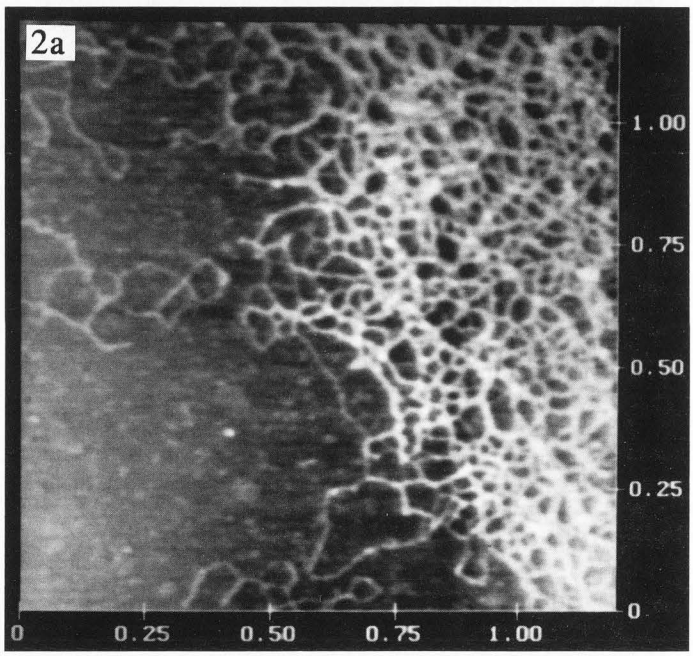
The heights of kDNA networks above the substrate, as measured from AFM images obtained by drying kDNA on freshly cleaved mica, varied between 1.5 and 6 nm (Figure 1). This variation in the height measurements is due to variation in the number of layers overlapped due to collapsing of the complete network. The height of isolated strands of DNA measured was around 1.2 nm. This is less than the intrinsic diameter (2 nm) of bare DNA. The factors that give reduced heights are not well understood at present but may be influenced by deformation of DNA under tip forces or conformational changes of DNA caused by drying [5]. Recently, Vesenka *et al.* [28] have shown that the reduced heights of DNA in AFM are due to codeposition of buffer salts.

The apparent widths of kDNA strands measured from AFM images on samples prepared by drying a drop of DNA on mica was approximately 50 nm. The images taken on pre-treated mica and rinsed in ethanol-water show thinner strands (about 20 nm wide and approximately 1.2 nm high) at relative humidity lower than 10%. This difference in dimension is clearly due to deposition of buffer salt around DNA strands during drying. For DNA samples prepared by critical point drying, the width and heights were approximately 12 nm and 0.7 nm, respectively.

The measured widths of isolated strands larger than the 2 nm are probably due to geometrical broadening due to finite tip radius [1, 5, 9, 11, 22, 23, 25, 28, 30, 32, 33]. The presence of large adhesion forces may also increase the DNA width larger than that predicted by geometrical broadening [13, 26, 30]. The adhesion force is a sum of the attractive van der Waal's force, the capillary force, and the electrostatic interaction. The adhesion force can be measured as the force required to break the tip away from the surface in a force-distance curve. Whenever larger DNA widths were observed, larger adhesion forces were also observed even at low humidity. For a clean tip, the adhesion force should decrease with decreasing humidity reversibly. Observation of large adhesion force at low humidity is probably due to tip contamination [24]. Large adhesion force implies bigger contact area between tip and the sample, and thus decreased resolution. Therefore, lowering the humidity alone may not guarantee thinner DNA strands. It is possible to reduce the geometrical broadening of DNA by using tips with small radius of curvature. Broadening caused by large adhesion force of contact AFM can be reduced by using clean tips under low relative humidity or under buffer solution. Large adhesion force may also reduces image contrast [15]. Most of the problems that limit the resolution, which are scan related, can be avoided by using the tapping mode where the lateral forces are completely eliminated.







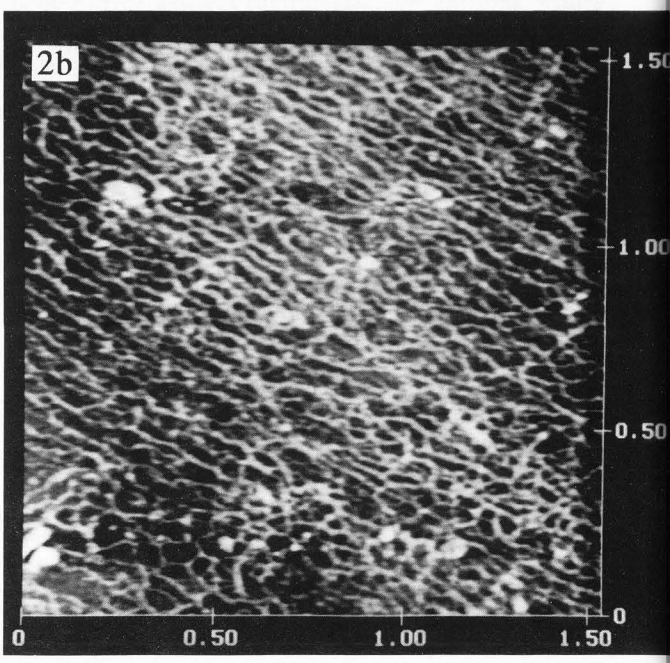
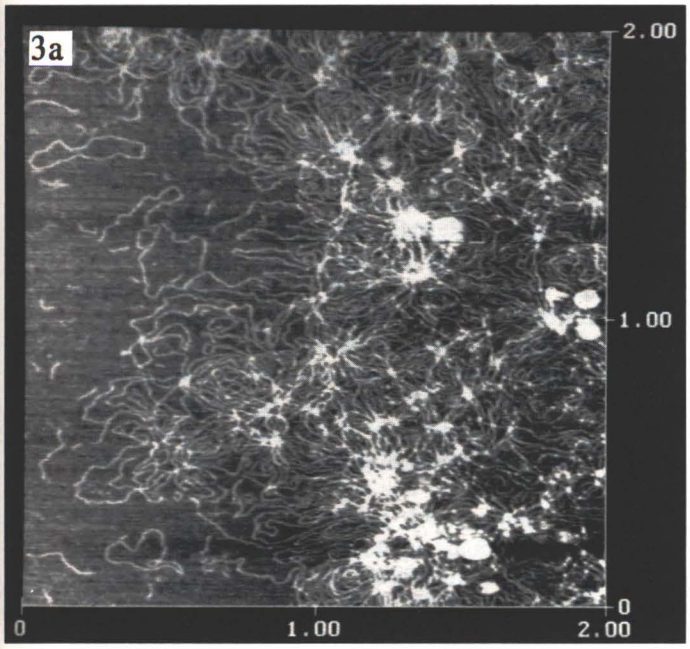
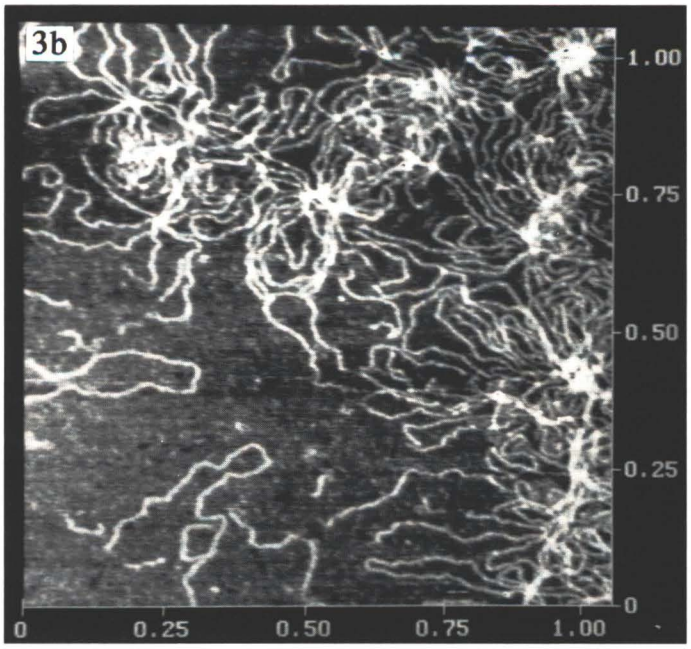
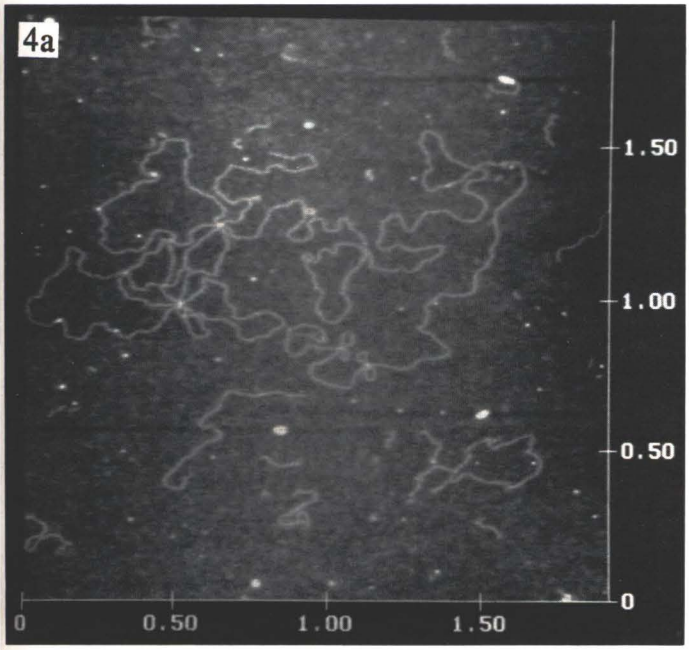


Figure 1. AFM images kDNA networks prepared by depositing kDNA solution on freshly cleaved mica followed by air drying: **(a)** low resolution image showing two kDNA networks. The average height of the network is 6 nm. **(b)** Edge of a kDNA network. The strand that is extending from the network is a broken maxicircle. Even at high resolution these images do not show any detail because of salt codeposition. The average height and width of isolated DNA strands are 1.5 nm and 50 nm respectively.

Figure 2. AFM images of edges of kDNA networks adsorbed on mica pre-treated with magnesium. The quality of images is greatly improved and DNA circles can be distinguished clearly. Average height and width of DNA strands are 1.2 nm and 20 nm respectively. **(a)** Edge of a network showing interconnected minicircles. **(b)** Center of a kDNA network where DNA circles appear to be elongated due to drying. Imaging these samples using the tapping mode AFM did not improve resolution or contrast.







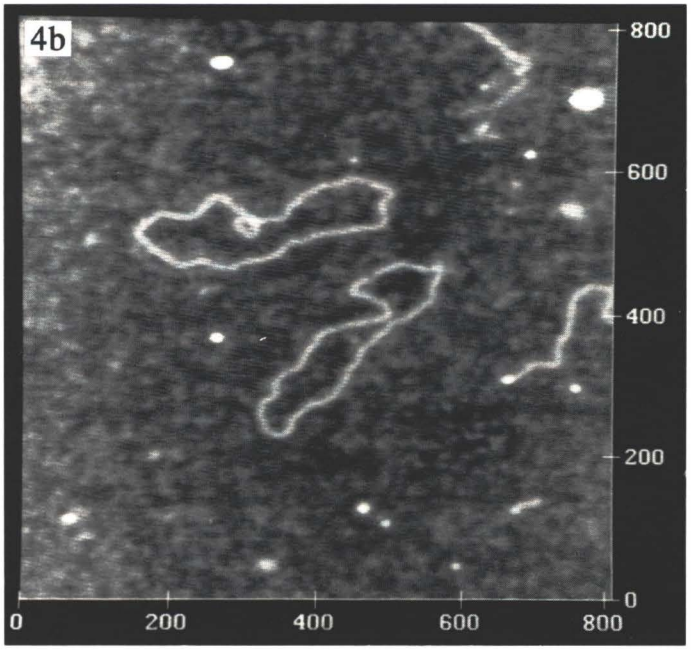


Figure 3. Tapping mode AFM images of kDNA networks obtained by critical point drying. The quality of images obtained on samples prepared by critical point drying was enhanced due to lack of drying artifacts. **(a)** Edge of a kDNA network showing isolated minicircles. **(b)** Higher resolution images of kDNA network.

Figure 4. Tapping mode AFM images of gently sonicated kDNA prepared by critical point drying. **(a)** Broken patch of minicircle network. **(b)** Isolated strands of minicircles that are free from drying artifacts.

Sample preparation for AFM that is sample specific is a challenge for obtaining high quality AFM images. Critical point drying is especially suited for complex DNA samples like kDNA but should also improve resolution on isolated DNA molecules. Critical point drying reduces artifacts associated with sample drying and produces intact kDNA networks. Also, the use of critical point drying yields increased resolution, perhaps due to a reduction in buffer salt contamination. Tapping mode operation reduces the lateral forces significantly and enhances imaging of small DNA fragments. Presently, experiments are under way to develop sample preparation techniques that will enable us to image kDNA under biologically relevant media at higher resolution.

Conclusions

Improved images of kinetoplast DNA networks were obtained when samples were prepared by depositing kDNA solution along with magnesium acetate on freshly cleaved mica followed by critical point drying. This sample preparation technique reduces time required for sample preparation in addition to producing consistently high quality images. It is shown that critical point drying is superior to other sample preparation techniques such as air drying DNA solution on mica or using chemically modified mica. Images obtained by critical point drying show increased resolution and greatly reduced drying artifacts. Large numbers of minicircles in the network can be easily seen in the AFM images.

Acknowledgment

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Discussion with Reviewers

N.J. Tao: Are the DNA molecules on the sample prepared with the critical point drying method stable in aqueous solution?
Authors: We were unable to obtain any images of kDNA under solution using silicon nitride tips.

N.J. Tao: Were the contact mode images of critical point dried samples better?
Authors: The contact mode images of critical point dried samples, when taken with small capillary forces, were better than samples prepared by simple drying. Using tapping mode reduces the lateral forces that tend to decrease the quality of images.

J. Vesenka: Would the authors like to speculate as to why the height of the DNA seems to decrease after critical point mounting compared to other sample preparation schemes?
Authors: Decreased height of DNA observed with critical point mounting is probably due to reduced salt. The other sample preparation techniques tend to concentrate residual salts on and around the DNA strands during the drying process.

S.M. Lindsay: I have argued that co-adsorption of ionic layer is essential for stability of molecules in scanning probe imaging [12]. Work by this group (and others) have shown that some of the chemical methods used by us [13] do not work as advertised: it is the drying step rather than specific chemistry that seems to be important.
Authors: We have pointed out in earlier papers that drying often introduces artifacts [23]. This is especially true for complicated networks such as kDNA.

S.M. Lindsay: I do not understand the comment about "recording scans at 180 degree". Please elaborate.
Authors: When a large adhesion force is present, contact mode imaging produces negative images due to cantilever buckling. This can be avoided by taking images at 180 degrees (for example, left-to-right rather than right-to-left). A discussion about this can be found in Ref. 24.

H.J.K. Hörber: How can 20 μl of kDNA of 10 μg/ml in one method be compared with 100 μl kDNA at 35 μg/ml and to 10 μl of 0.1 μg/ml of other two preparation techniques?
Authors: In this paper, we do not compare the density of DNA observed. Because of the varying nature of the substrates, different sample volumes and concentrations are used to save time in finding a uniform coverage of DNA on the substrates. Freshly cleaved mica is hydrophilic and only a small volume of sample solution is needed to cover the surface. On the other hand, mica surfaces modified with Ba or Mg cations are hydrophobic and need more solution to wet the surface. Adding Mg ions to buffer solution increases DNA adhesion and stability against rinsing, and therefore, the DNA concentration must be reduced to maintain an optimal surface coverage.

H.J.K. Hörber: Please provide a description of barium and magnesium activation used in the second sample preparation technique.
Authors: Details of that technique have been published before [5, 14, 23, 27]. Briefly, freshly cleaved mica was soaked overnight in 5 mM solution of Ba or Mg cations, rinsed throughly in distilled water, cleaned using ultrasonic agitation, and dried at 100°C oven for three hours. The increased adhesion of DNA on mica surface activated by barium or magnesium is probably due to increased surface roughness.

L.A. Bottomley: Throughout the manuscript the authors refer to the chemical modification of mica. Several different strategies are available: chemisorption of divalent metal ions, chemisorption of tetraalkylammonium ions and silanization reactions. Have the authors evaluated the suitability of their critical point drying method on these chemically modified mica surfaces?

C. Rabke: Was the critical point drying used only with preparation method (3) (where Mg ions were added directly to buffer solution)?

Authors: We have tried critical point mounting of DNA only on mica surfaces chemically modified using divalent metal ions (either pretreating mica with divalent cations or adding Mg ions directly to buffer solution).