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We have imaged gold-labeled DNA molecules with the atomic force microscope (AFM). Circular plasmid DNA was labeled at internal positions by nick-translation using biotinylated dUTP. For visualization, the biotinylated DNA was linked to streptavidin-coated colloidal gold spheres (nominally 5 nm diam) prior to AFM imaging. Reproducible images of the labeled DNA were obtained both in dry air and under propanol. Height measurements of the DNA and colloidal gold made under both conditions are presented. The stability of the DNA-streptavidin colloidal gold complexes observed even under propanol suggests that this labeling procedure could be exploited to map regions of interest in chromosomal DNA.

I. INTRODUCTION

The potential capability of the atomic force microscope (AFM) to generate atomic resolution images of biological surfaces has motivated immense interest in imaging of DNA molecules.[1–11] Currently, AFM images of DNA in air and under solutions routinely display resolution comparable to that obtained by electron microscopy. The AFM can also be used to manipulate DNA. Individual plasmid DNA molecules have been cut by the AFM scanning tip and the excised fragments moved.[3,5,8] The high resolution imaging and manipulation capabilities of the AFM portend its utility as an instrument for chromosome mapping, gene manipulation, and possibly DNA sequencing.[12]

We have investigated the possibility of imaging DNA tagged with modified nucleotides that could serve as site specific markers for mapping of chromosomes and, potentially, as physical handles for manipulating DNA. In previous work, a method for imaging linear DNA molecules with gold spheres attached specifically at their ends was developed.[13] This was achieved by incorporating biotinylated nucleotides into DNA and reacting the biotin-containing DNA with a streptavidin-gold conjugate (SAG). In this report, we demonstrate that circular DNA molecules can be labeled with gold at internal positions and reproducibly imaged both in air and under propanol. Differences in the measured heights of DNA and gold spheres under different imaging conditions and the implications of these results for reliable imaging of biological samples in the AFM are discussed.

II. MATERIALS AND METHODS

A. DNA labeling

Supercoiled plasmid DNA (pUC119) was prepared by alkaline lysis and CsCl–EtBr gradient purification. Purified DNA was nick-translated with biotin-dUTP (Enzo Biochem, NY) DNase I and *E. Coli* DNA polymerase I for 20 min at 37 °C. Unincorporated biotin-dUTP was re-

moved by ethanol precipitation, and the bio-dUTP labeled DNA was resuspended in 10 mM tris-HCl (pH 7.2), 5 mM MgOAc, 50 mM NH$_4$OAc, 1 mM EDTA (TMNE) and incubated with 2 μl streptavidin-gold conjugate (SAG; Amersham) for 60 min at 25 °C. The DNA-bound gold particles were separated from unbound gold conjugate by chromatography through Bio-gel A-50 (Biorad) in 20 mM tris-HCl (pH 7.5), 100 mM NaOAc (TN). Fractions containing DNA were pooled and ethanol precipitated.

B. Sample deposition and AFM imaging

Biotin-streptavidin-gold-DNA (BSG-DNA) in 20 mM tris-HCl (pH 7.2), 100 mM NaOAc and 5 mM MgCl$_2$ (TNM) was deposited directly onto freshly cleaved mica (Ted Pella, Inc., Redding, CA) for imaging in the AFM, or further concentrated by ethanol precipitation. Samples were prepared for imaging as previously described.[13] All images were collected on a Nanoscope II (Digital Instruments, Inc., Santa Barbara, CA) using Si$_3$N$_4$ tips (Nanoprobes). All images displayed were collected in error signal mode[14] (ESM). In ESM images the error signal generated as the piezo attempts to compensate for cantilever deflection is displayed, rather than the true sample height. In height mode (HM) images, a gray scale typically represents the sample height with lighter features being taller. All height and width measurements were made on height mode images. The Z-piezo was calibrated using step edges on graphite.

III. RESULTS AND DISCUSSION

We have previously reported a procedure for labeling linear DNA molecules specifically at the ends with biotin-streptavidin-gold (BSG).[13] In this study, a nick-translation protocol was used to incorporate label at random internal positions in circular plasmid DNA molecules (see Materials and Methods). Figure 1 shows a typical field and gallery of internally labeled DNA molecules imaged in dry air (relative humidity < 10%). The nonrandom orientation of the molecules is the consequence of the gold labeling and drying procedure and has been discussed elsewhere.[13] Most

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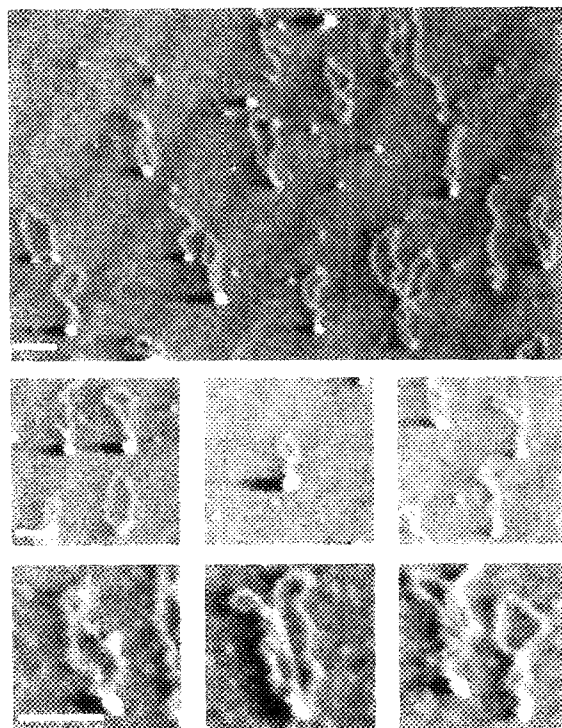[a]Author to whom all correspondence should be addressed.

FIG. 1. Typical field and gallery of circular DNA molecules labeled at internal positions with biotin-streptavidin-gold (see Sec. II). All images were taken in the error signal mode (Ref. 14) in dry air (<10% relative humidity). Bar = 250 nm.
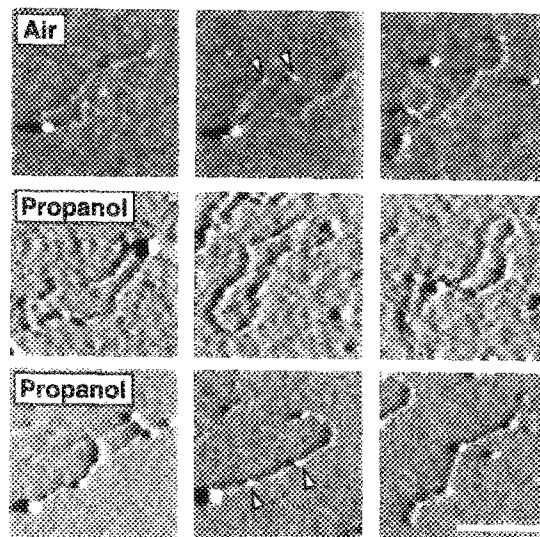

FIG. 2. Gallery of individual internally labeled DNA molecules. The top two panels show examples of DNA molecules from the same sample preparation, imaged first in dry air and subsequently under propanol, using the same scanning tip. The bottom panel shows images obtained from the same DNA sample imaged under propanol with a different scanning tip. The rodlike appearance of the circular DNA molecules in the bottom panel is probably due to harsher drying conditions used during spreading (see the text). White arrows indicate positions in which unconjugated streptavidin protein appears to be bound to DNA. All images were taken in the error signal mode. Bar = 250 nm.

of the labeled molecules are circular, as expected, with a single gold sphere attached. Under nick-translation conditions used in this experiment, approximately 30% of the DNA molecules appear to contain BSG, although much higher labeling efficiencies were achieved by increasing the length of the labeling reaction. In such cases, however, the labeled DNA often contained so many gold spheres that it was difficult to visualize the DNA (data not shown). Despite the brief DNA labeling protocol used in this study, it was initially surprising to observe the majority of molecules bound by a single gold particle. However, upon closer scrutiny it was apparent that many of the DNA molecules had unconjugated streptavidin protein attached to them at multiple sites (Fig. 2, white arrowheads). EM studies have demonstrated that commercially available colloidal gold-protein conjugates often contain a large proportion of free protein.[15] Thus, the efficiency of streptavidin-biotin complex formation was probably significantly higher than that calculated based on the number of gold particles observed bound to DNA.

Unlike the EM, in which many contaminants are transparent, the AFM detects stable features it encounters on a surface with little prejudice. Therefore, AFM samples must be very clean. In this and the previous study,[13] two purification steps were essential for obtaining reliable images of BSG-DNA with low background: (1) removal of unincorporated streptavidin-gold conjugate by column chromatography and (2) removal of salts (components of the labeling reaction buffer) by ethanol precipitation (see Sec. II).

AFM imaging of DNA under propanol has been shown to give better resolution and narrower apparent widths than imaging in air.[2,3,8,9] Figure 2 shows a gallery of images collected from the same labeled DNA sample, imaged with the same tip in dry air (top row) and subsequently under propanol (middle row). In this case, differences in the apparent width are small, with the air images appearing slightly thinner. However, DNA imaged in air with different tips often appeared much thicker (see Fig. 1). While innate differences in individual tip geometry clearly affect the apparent DNA thickness, an explanation for observing this phenomenon more frequently in air than in propanol may be that the tip is more susceptible to becoming charged and picking up surface contaminants (e.g., salt residue) in air than in propanol where static electrical charges may be more easily dissipated and the tip is constantly immersed in a cleansing solvent. In addition to tip shape, other imaging parameters can alter the apparent width. For example, in error signal mode[14] the apparent width of the DNA is dependent upon the gain settings. Finally, the appearance of DNA in the AFM is critically dependent upon the sample preparation method. BSG-DNA samples imaged in dry air showed a higher frequency of circular DNA whereas the same sample imaged under propanol sometimes appeared to contain primarily linear molecules (Fig. 2, third row). This is probably due to the use of excessive air force during drying of the sample on the mica surface, since a higher proportion of circular molecules was observed under propanol when care was taken to dry the DNA sample under very gentle air flow (Fig. 2, middle row). Apparently, most of the molecules

DNA Height in Propanol
Points = 101
Mean = 1.11 ± 0.47

DNA Height in Air
Points = 148
Mean = 0.59 ± 0.18

SAG Height in Propanol
Points = 109
Mean = 3.9 ± 1.8

SAG Height in Air
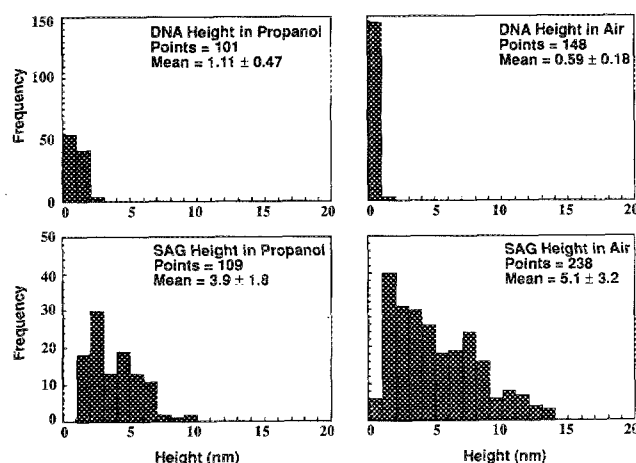Points = 238
Mean = 5.1 ± 3.2

FIG. 3. Histograms showing the apparent height distributions for the DNA and SAG used in this study. Measurements made under propanol are shown in the panels on the left; those made in dry air are shown in the panels on the right. All measurements were taken from height mode images.

with a linear appearance under propanol are supercoiled and/or nicked circular molecules in which two halves of the circular DNA molecules have aggregated locally, or "collapsed" to form half-sized linear structures. Others have observed similar effects of drying and spreading conditions on the appearance of circular DNA molecules in the AFM.[16]

The height of DNA molecules and gold spheres was measured both in air and under propanol (Fig. 3). The average height of DNA was 1.11 ± 0.47 ($n = 101$) measured under propanol and 0.59 ± 0.18 nm ($n = 148$) measured in air. The average height of streptavidin-colloidal gold spheres was 3.9 ± 1.8 nm ($n = 109$) measured under propanol and 5.1 ± 3.2 nm ($n = 238$) measured in air. T tests performed on these data indicated that the differences in heights measured in air versus propanol were statistically significant (for DNA, $T = 12.12$, $p = 0.0000$; for gold spheres, $T = 3.75$, $p = 0.0002$). Although the apparent height of the gold under both imaging conditions was close to that expected, the average height in propanol was somewhat less than in air. The DNA showed a striking deviation from the expected height (~ 2 nm) under both conditions and had an apparent height in propanol nearly double that observed in air.

Bustamante and colleagues[1] have shown that increased scanning force leads to decreased apparent height in repulsive contact mode imaging (~ 100 nN) in air. Interestingly, the measurements reported here were made in the attractive contact mode in air (with negative cantilever deflection, due to meniscus forces[17]) and at much lower forces (~ 10 nN), yet the DNA heights measured were still less than expected. Several authors have shown that apparent sample height can vary as a function of humidity[7] and frictional effects.[11] Although we have optimized the scan orientation so that we only obtained positive contrast images,[13] cantilever bending under frictional load could have contributed to the differences in apparent heights ob-

served in air and in propanol. Different adhesive interactions between the tip and the sample in the two imaging environments may alter the frictional effect and/or result in physical compression of the sample, even in the attractive contact imaging mode. Finally, salt residue surrounding the sample or accumulated on the tip due to electrostatic charges could play a role in the height differences observed.

IV. CONCLUSIONS

This article demonstrates the capability of AFM to image DNA molecules tagged at internal positions with biotinylated nucleotides. The tagged positions were visualized by reacting them with a streptavidin-colloidal gold complex. A critical point is that these complexes were stable during imaging both in dry air and under propanol. This approach could be exploited for physical mapping of genes or regulatory regions within chromosomal DNA. For example, in EM studies of DNA replication, sites of nascent DNA synthesis have been observed by incorporation of biotinylated nucleotides into DNA *in vivo*, followed by visualization of the sites of incorporation using streptavidin-colloidal gold or streptavidin-ferritin conjugates.[18] Our results suggest that a similar protocol could be used to map replication origin regions using the AFM, with the advantages of much simpler sample preparation and potentially higher resolution. Moreover, since the AFM can distinguish unconjugated protein bound to DNA, satisfactory mapping results may be obtained using free protein rather than protein-colloidal gold conjugates. Finally, these results imply that the AFM may be a useful tool to investigate many types of DNA–protein interactions.

The use of incompressible gold spheres as internal height standards may provide a means to assess tip geometry and the degree of specimen damage by the scanning tip during data collection.[19] Unfortunately, the gold conjugates currently available are very heterogeneous in size and therefore relatively poor height standards. If gold spheres are used as height standards only, homogeneous preparations of unconjugated gold that are commercially available are the method of choice. However, direct attachment of gold spheres (even heterogeneous preparations) may prove very useful as a physical mechanism for manipulating DNA molecules to which they are attached.[13]

Note added in proof: Since submission of this manuscript, Murray *et al.* (Proc. Natl. Acad. Sci. USA, in press) have reported AFM imaging of DNA fragments modified by the attachment of biotin–streptavidin complexes, further demonstrating the capability of the AFM to image DNA molecules biochemically tagged at specific positions.

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