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HUMIDITY EFFECTS ON ATOMIC FORCE MICROSCOPY OF GOLD-LABELED DNA ON MICA

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Abstract

Recent work in atomic force microscopy (AFM) of deoxyribonucleic acid (DNA) has relied on immobilizing DNA molecules by drying a small volume of buffered DNA solution onto cleaved mica. When imaging in air, relative humidity has been known to affect both the resolution and measured height of the DNA strands. We present data of measured height versus humidity for DNA and attached gold labels, and we propose a model for this data based on swelling of coadsorbed buffer salts upon exposure to moisture. In this model, small particles (e.g., DNA) stay near the top of the swelling salt layer, whereas larger particles (e.g., gold spheres) tend to be anchored down to the substrate until a moderate humidity is reached. At high humidity (around 65%), the salt layer becomes fluid-like and susceptible to tip-induced motion; the salts are either removed from the scan area or aggregate into island structures, depending on initial salt concentration on the surface.

Key Words: Atomic Force Microscope, mica, salt, DNA, colloidal gold particle, humidity.

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Introduction

Several authors have recently shown the ability to use the atomic force microscope to image DNA in air [2, 7, 14, 15, 18, 19] and in liquids [5, 6, 8, 16]. In these papers, the measured heights of the DNA, lying flat on the substrate, have been reported to vary between 0.5 and 3.0 nm. The complicated forces between the tip and sample, as well as instrumentation noise, make accurate nanometer scale measurements of biomolecules difficult to obtain [17]. Furthermore, one author has shown that the apparent height of the DNA can change as a function of relative humidity [14]. These authors and others [11, 17, 20] have proposed that these phenomena can be attributed to a frictional effect due to cantilever bowing.

By superimposing the trace and retrace scan lines, it is possible to obtain images that retain positive contrast at any humidity until the DNA comes off the surface. An important question is what actually happens to the DNA as the humidity is increased. Part of the difficulty in addressing this problem is that the height of the DNA is almost buried in the noise level of the scanning signal. Also, DNA is a biomolecule that is flexible and may compress under the loading forces typically operated in an AFM [2, 18]. By imaging a larger, incompressible, co-adsorbed particle, we hope to give a more detailed explanation of the humidity effect.

Colloidal gold is frequently used in optical and electron microscopy as a marker for imaging specific sites of biomolecules [3]. Several authors have shown that gold and silver-enhanced particles can be used as a marker on cells and DNA in atomic force microscopy [9, 10, 12, 13]. In this report, we present data for measured heights of gold-labeled DNA as a function of relative humidity and propose a model to account for the changes in height.

Methods

Sample preparation for AFM imaging of the 5 nm gold labeled DNA follows that of Shaiu et al. [12, 13]: Supercoiled plasmid DNA (pUC119) was prepared by alkaline lysis and $CsCl_2$-EtBr gradient purification. DNA was 5' end-labeled with bio-dUTP (Enzo Biochem) by

using Klenow fragment of *E. Coli* DNA polymerase I to fill in 5' overhanging ends generated by digestion with Hind III (Figure 1) or nick-translated with biotin-dUTP (Enzo Biochem) DNase I and *E. Coli* DNA polymerase I for 20 minutes at 37°C (Figure 4). Unincorporated biotin-dUTP was removed 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_4OAc, 1 mM EDTA and incubated with 2 ml streptavidin-gold conjugate (Amersham) for 60 minutes 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. Fractions containing DNA were pooled and ethanol precipitated. Biotin-streptavidin-gold-DNA in 20 mM Tris-HCl (pH 7.2), 100 mM NaOAc and 5 mM $MgCl_2$ was deposited directly onto freshly cleaved mica (Ted Pella, Inc.), rinsed thoroughly with distilled, deionized water and blown dry with nitrogen before imaging in the AFM. It is assumed that quick drying with nitrogen uniformly spreads the residual buffer salts, as well as gold-labelled DNA, over the mica.

The specimens were imaged and analyzed using a commercially available atomic force microscope (Nanoscope II from Digital Instruments). We used triangular silicon nitride cantilevers 120 μm long and 22 μm wide (nominal spring constant 0.2 N/m). The direction of scan with respect to the cantilever (scan orientation) was adjusted until the forward and reverse traces superimposed, an imaging condition that reduces the frictional distortion to specimen height [17]. The calibration of the z-direction of the piezoelectric translator was independently determined (to within 20%) by imaging the 0.26 nm steps on graphite. The gold-labeled DNA specimens were imaged in the height mode at a repulsive force (tip pushing down into the sample to ensure constant contact) of 3 nN for all humidity levels, as the height and width of the DNA is known to be affected by excessive loading forces [2, 18]. The total force was measured from the bottom (pull-off point) of the force-distance curve such that the adhesion force component (tip pulling away from the sample) ranged from 7 to 100 nN as humidity increased from 10 to 65%. Relative humidity (RH) was measured by a calibrated capacitance-driven digital hygrometer (VWR Scientific). Analysis of the height was carried out with the Nanoscope software and plotted using Kaleidagraph software.

Results and Discussion

Figure 1 shows AFM images of gold-labeled DNA as a function of relative humidity at a repulsive force of 3 nN. The linearity of the DNA is due to the specimen spreading technique and is explained in detail elsewhere [12]. The asymmetry of the gold particles (the egg-shaped structures at the top of each linear portion of DNA) is a tip shape artifact [1, 4] due to the tip radius exceeding the particle size. There are four gold particles in these frames, with initial heights from 3.1 to 6.7 nm at RH = 10% (Figure 1A). Although nominally 5 nm, the diameter of the gold balls can in fact vary from 3 to 7 nm or more [12, 13] due to chemical variations during synthesis of the colloid. The smallest observed particles (height 1.4 nm) may be streptavidin rather than gold and were disregarded for the analysis of Figure 2. In Figures 1B-E, the same samples were imaged at increasing humidity. Notice that in Figures 1A and 1B the background is rough due to the presence of residual buffer salts [15], but in Figures 1C-E the surface is smoother, signifying a decrease in surface roughness.

It was possible to monitor the height of the DNA above the surface to about RH = 55%, though these heights were near the level of surface roughness at all humidity levels (see Fig. 2 and the proposed model in Fig. 3). The gold particles could be monitored almost all the way until their removal from the imaged area at a surface saturation humidity (for this sample) of 64%. This saturation level is partly determined by residual salts from the rinsing step.

Figure 1F is a view of the previously scanned region at slightly lower magnification after returning to the initial humidity of 10%. Note that the original scan area has been swept clean of the salts, DNA, and gold, which have been deposited on the borders. Outside the borders, several undisturbed circular and linear DNA molecules can still be seen.

Figure 2 shows height versus humidity plots for four gold particles and for average DNA height. These are typical height decrease responses from colloidal gold particles in both Figure 1 (solid circles) and Figure 4 (open circles). Unlike the results of Thundat et al. [14], we did not observe reversal of contrast with increasing humidity. We attribute this to the reduction of frictional effects on height by adjusting the scan orientation between the tip and sample, typically around 90°, until trace and retrace signals are superimposed [12, 17]. Once this scan angle has been determined, it is used throughout the experiment. Another factor that could possibly effect image heights are chemical interactions between tip and sample [6] though we did not attempt these experiments with silicon or carbon-coated tips. In Figure 2, the height of the DNA is just detectable above the background roughness and changes little as the humidity increases, disappearing completely above RH = 55%. However, the heights of the gold particles diminish rapidly during the initial increase in RH, then more gradually from about RH = 40 to 65%, when the particles are swept from the surface.

We suggest an improvement to previous efforts to explain meniscus-layer imaging for this particular type of substrate preparation [16]. Figure 3 shows a model of DNA (end-on view), a gold particle and the scanning tip in the presence of dried buffer salts. We propose that the salts on the mica swell in response to increased moisture, retaining a solid-like surface. As the humidity increases, small molecules, such as DNA, stay on near the top of the surface in this medium, because of surface

tension and buoyancy, while the anchored gold particles tend to remain on the mica surface initially, probably through the incidental streptavidin linkage to the mica [12]. At moderate humidity (about 40%) even the gold particles, which are attached to the DNA through the biotin-streptavidin link, begin to destabilize. Smaller gold particles seem to destabilize at lower RH than larger particles; in Figure 2 note that the height versus RH of the smallest gold particle levels off around RH = 30%, whereas that of the largest particle levels off around RH = 50%. The important consideration here is that the salt layer, although probably "spongy," still has enough three-dimensional integrity to keep the smaller structures from moving. As the humidity increases further, the treated surface becomes fluid-like and all gold and DNA particles are swept aside by the lateral force of the scanning stylus.

Figure 4 shows another sample of gold-labeled DNA and buffer salts, which illustrates the swelling of salt particles with increasing humidity. At low humidity (Figures 4A and B), gold-labeled DNA is visible along with patches of buffer salts. At RH = 50% (Fig. 4C), the swelling of salt clusters (to around 5.0 nm height) nearly obscures the gold-labeled DNA. However, these clusters are still immobilized and can be imaged indefinitely without aggregation. At RH = 80% (Fig. 4D), the clusters begin to aggregate quickly, resulting in an island structure which remains after the humidity is reduced (Fig. 4E). The island thickness reduces from 7.9 $\pm$ 1.2 nm to 3.0 $\pm$ 0.3 nm as RH is reduced from 80% to 10%, illustrating the swelling effect of moisture. A low-magnification scan (Fig. 4F) at low humidity shows that aggregation has occurred only in the area scanned at high humidity and not in the surrounding region. Evidently the salt clusters do not aggregate spontaneously by diffusion at high humidity but are susceptible to motion induced by the imaging tip. (Note that the islands are elongated along the fast scan axis.)

For all samples tested with the Tris-HCl/NaOAc/$MgCl_2$ buffer, the surface saturation humidity generally fell between 60 and 70%. This value was not much affected by tip shape or scan rate and may be an inherent property of the buffer-substrate combination.

Conclusion

AFM measurements show that the apparent heights of DNA and gold particles, adsorbed onto mica from buffer, decrease with increasing humidity until the particles are swept from the scan area at a saturation humidity between 60 and 70%. We propose that this phenomenon is due to the swelling of dried buffer salts upon exposure to moisture. As the humidity reaches the saturation value, the salt layer assumes fluid-like properties; though there is little spontaneous diffusion of adsorbed particles in this state, these particles are vulnerable to motion induced by the scanning tip.

Acknowledgments

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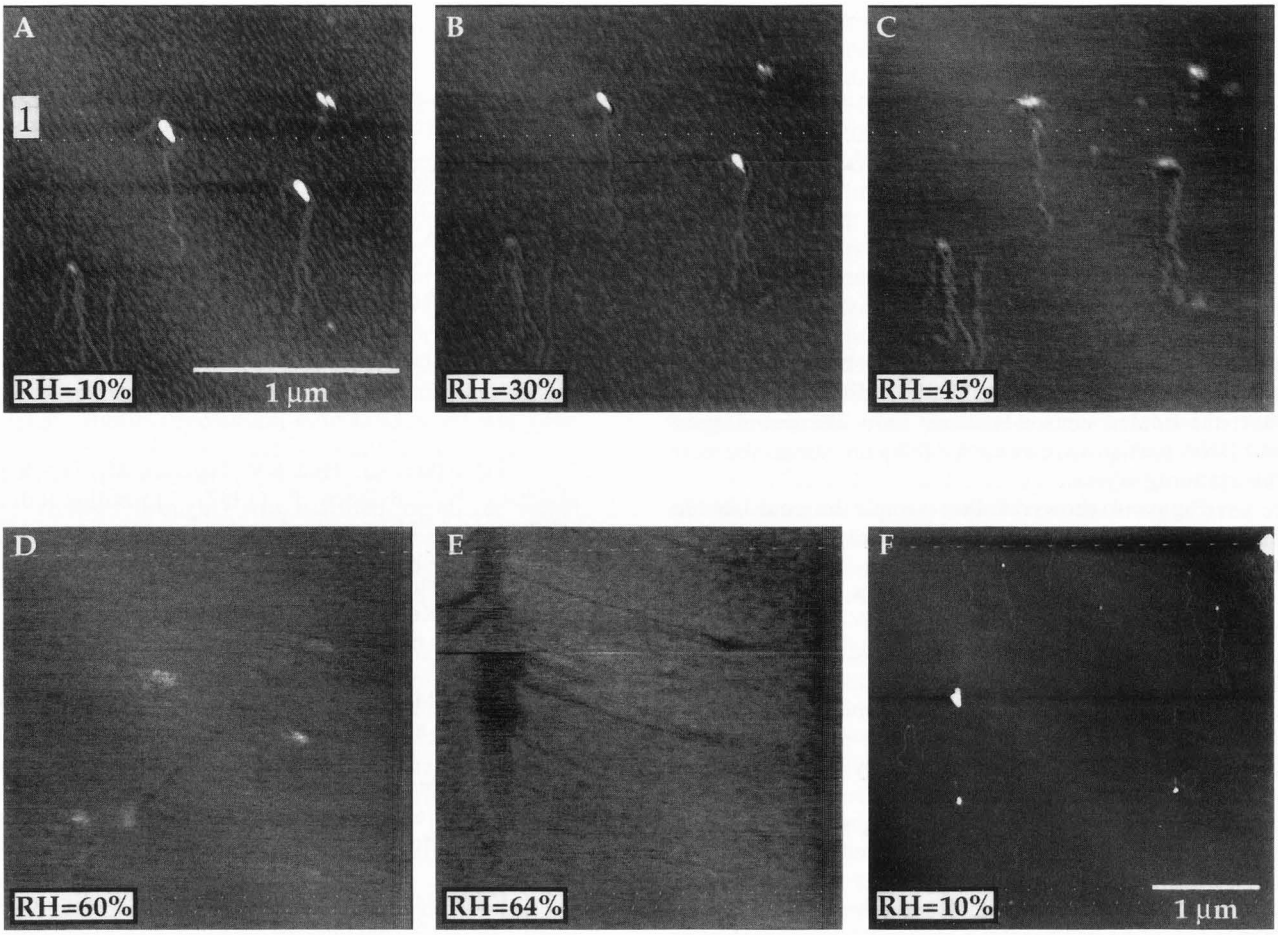

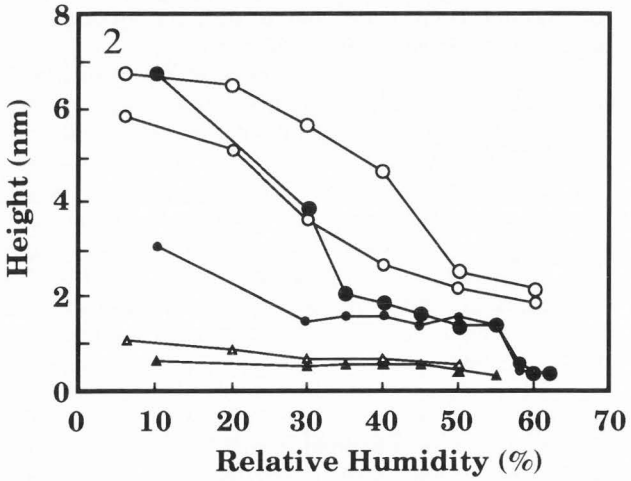

Figure 2. Height of four gold particles (circles) and average DNA heights (triangles) versus relative humidity levels from 10 to 64% (error bars are the size of the plot symbols). Data for filled and unfilled symbols are from Figures 1 and 4 respectively. The plot shows heights of all surface structures diminishing with increasing humidity, with the larger particles "shrinking" much more, relative to their original heights, than smaller particles.

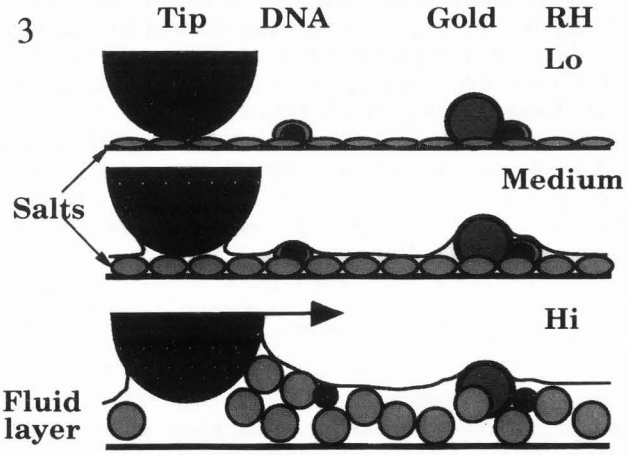

Figure 3. Model describing the effect of humidity on sample height. At low humidity, all molecules are tightly bound to the surface. As the humidity increases, smaller structures float upwards with the swelling buffer salts. At higher humidity levels, even larger particles become free to move about the surface. Salt particles tend to build up on the leading edge of the tip. Tip, particle and meniscus sizes are approximately to scale (tip radius was estimated using the apparent lateral size of the gold particles; see Ref. 17).

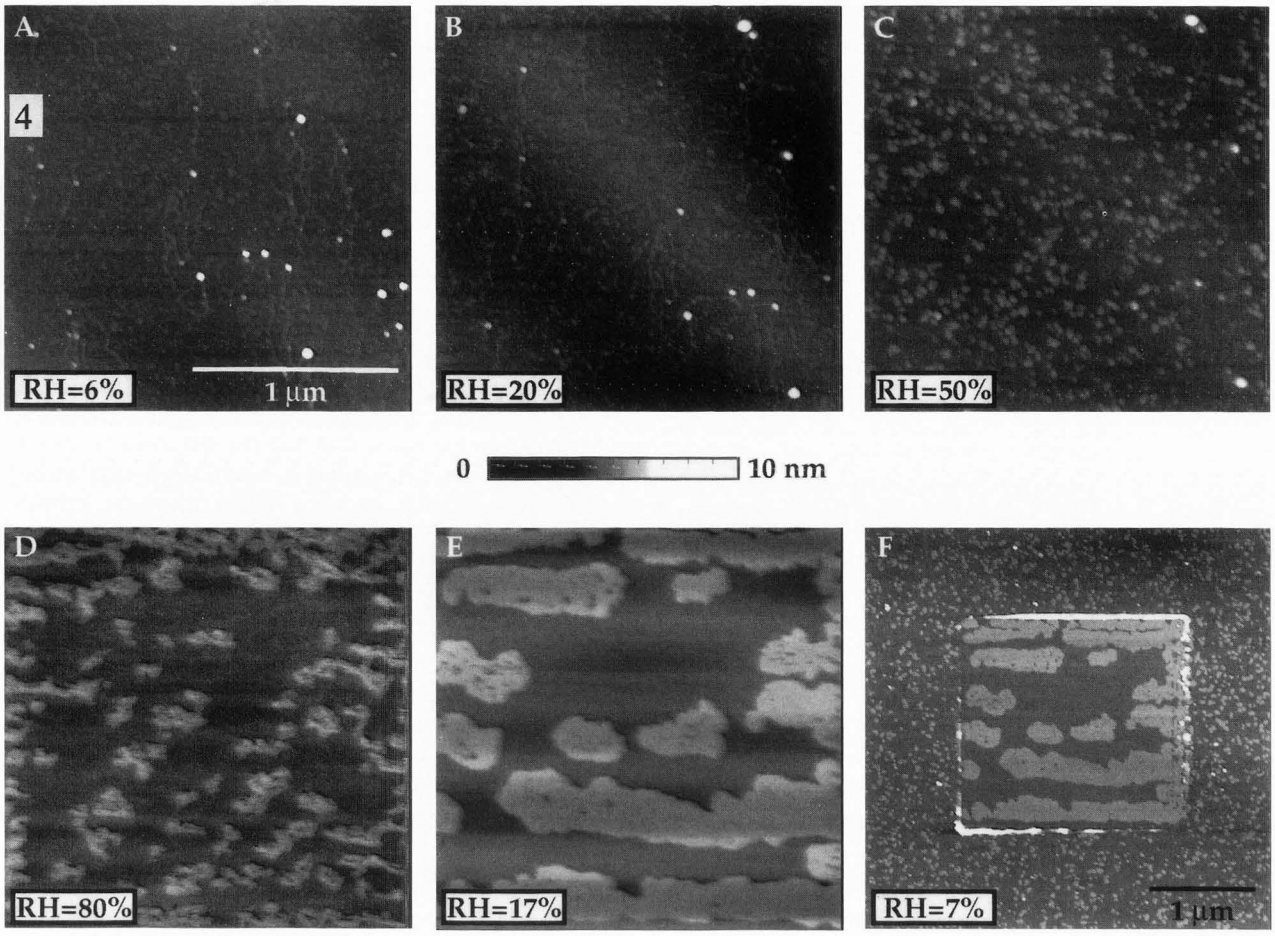

Figure 1 (facing page, top). Six images of gold-labeled DNA at different humidity levels. Images **A**-**E** are of the same 2 μm area at increasing humidity. Image **F** is a 4 μm scan, encompassing the previous scan area, imaged after restoring the humidity to 10%. The repulsive imaging force was maintained at 3 nN for all humidity levels. The vertical range is determined by the gray scale in the center of the panel, with lighter features higher than darker features.

Figure 4 (above). Six images at varying humidity of a sample prepared as in Figure 1, but with higher residual salt concentration. At low RH (**A** and **B**), the gold-labeled DNA is easily distinguished from salts. As RH increases (**C** and **D**), salt islands appear and cluster at RH = 80%; these islands remain but shrink in height as RH is reduced back to 10% (**E**). A lower magnification image (**F**) encompassing the previous scan area shows tip-mediated island formation within the original scan area. All images were obtained at the same repulsive imaging force (3 nN).

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Discussion with Reviewers

R. Balhorn: The concept that molecules can float to the surface (movement in z) of the salt layer at a given humidity (below 40%) level and yet still be immobilized sufficiently on the surface to be imaged by the tip (lack of movement in x and y) seems inconsistent.

B.L. Blackford: In relation to Figure 3, what is meant by: "As the humidity increases, smaller structures float upwards..."?

Authors: This important point of our model needs reiterating. We are suggesting that, at low to intermediate humidity, the residual salts hydrate as the ambient humidity increase and become "spongy", providing enough two dimensional integrity for the trapped gold and DNA molecules to fend off lateral motion induced by the tip until the residual salts saturate. As long as the gold or DNA is trapped near the surface they will provide a relief that can be detected in the vertical dimension. Upon saturation, at high humidity, the surface becomes a liquid layer that can be swept clean at low salt concentrations, or swept into piles at higher salt concentrations, by the scanning tip.

The measured gold height decreases because the residual buffer salt swell around the gold which is anchored to the mica, possibly through the streptavidin linkage. Streptavidin binds to bare mica at low humidity very strongly, but can also be pried loose at higher humidity (Wen Ling Shaiu, unpublished data). On the other hand, colloidal gold particles do not stick to mica at **any** humidity without previous substrate treatment [17] or, as in the present case, through proteinatious attachment. DNA stays near the surface of the swelling salt layer primarily through surface tension, and to a lesser extent due to buoyancy. The DNA in these experiments did have a tendency to disappear from the scanning area prior to the gold particles, suggesting a less strong attachment to the mica.

P. Mulhern: There is an inconsistency in the **Results** after the humidity is reduced back to low values. The size of the salt island in Figure 4F are clearly larger than the islands in Figure 4A. The claim that aggregation only occurred in the region scanned is too strong, as some aggregation also occurred elsewhere on the substrate. This leads to another question about the initial sample preparation of the sample: The sample preparation procedure says wet samples were blown dry as the last step. This procedure seems equivalent to reducing the humidity. Why then were the original salt crystals so much smaller than those at the end of the experiment?

J. Yang: It is puzzling that the authors measured patches of 3 nm thick after the humidity cycle, that appeared (at least from Fig. 4F) a net increase of the total mass on the surface. Please comment on these issues.

Authors: We believe these observations are consistent with our argument in the text. The quick drying procedure seems to spread and dry the residual salt concentration uniformly over the entire sample. However, through the mechanism of surface diffusion at high humidity, the residual salt crystals will aggregate, through Brownian motion, to form larger clusters, probably mediated by Coulombic forces outside the originally scanned area. These salt islands appear taller because of less uniform coverage over the mica surface. This explains why the salt crystals are larger at the end of the run than at the beginning. We have observed (unpublished data) the same effect taking place when similar samples are prepared and left in ambient room conditions. The key point we were trying to make is not the difference between the Figures 4A and 4F in the region outside the scanned area, but within the scanned area where larger islands were formed. Since the only difference between the two areas is contact by the scanning tip the larger islands must result from that contact.

R. Balhorn: Have the authors used any technique to determine the amount of Na or Mg on the mica surface before and after DNA deposition to ensure that there really is a significant amount of salt present on the surface?

Authors: Yes. Iowa State University has access to excellent facilities at Ames Laboratories including X-ray photoelectron spectroscopy (XPS), an elemental and concentration analysis tool. Using the same sample preparation procedures as in this paper, for a thoroughly rinsed sample (similar to the one in Figure 1) we observed **no** increase in C, Mg or Na signature by XPS. However, for more obviously coated samples imaged in the AFM (as in Figure 4) a carbon peak, probably from Tris salt residue, was evident (J. Vesenka, unpublished results). A concentration series of buffer dried directly onto mica without rinsing was also examined and gave a strong C and cation peaks. But, when the samples were rinsed with distilled water, the XPS signature reduced to that of plain mica. The implication of these experiments was that AFM can be more sensitive to residual buffer salts than XPS. Auger electron spectroscopy was also attempted but charge buildup destroyed the mica samples before any useful analysis could be performed.

J. Yang: A large part of this paper deals with humidity effect on DNA imaging. How would the authors explain

apparent difference of their result from those that have already been published. For example, in ref. 18, it was found that the humidity did not affect both the resolution and the adhesion force with a different specimen preparation method.

Authors: We believe the key difference lies in the specimen preparation method of ref. 18, a cytochrome-C spreading technique on top of carbon-coated mica. This combination, together with a carefully grounded substrate, effectively neutralizes surface charges on mica, as pointed out in ref. 18. These surface charges are thought to be responsible for the weak-binding employed by cationic attachment of DNA to mica [15]. The most interesting consequence of our observation of possibly high salt concentrations at the mica surface concerns their potential effect on biomolecular structure, a repercussion that deserves greater scrutiny.

T. Thundat: Did the authors keep the scanning angle constant while performing the experiments at different humidities? If my understanding is correct, the angle has to be changed continuously with humidity to superimpose the trace and retrace. If the scanning angle is continuously changed, negative contrast will never happen.

F. Zenhausern: As shown in Figure 1, the asymmetry of gold particles is due to tip convolution. Could you explain why its direction is different from Figure 1B to Figure 1C: Did you rotate the scan direction or is it an effect of increased RH on frictional forces?

Authors: Once a scanning angle was determined, all data were taken at this angle. Still, we did not observe contrast reversal even at high humidities. Though we cannot rule out the possibility of frictional force changes effecting the tip asymmetry, we are inclined to believe that the change in gold particle shape is another manifestation of a hydrating salt layer. We might be seeing widening of the gold particles in the direction of scan (left to right) in the AFM images due to greater freedom of the colloidal gold to move in the hydrating salt medium, possibly mediated by a streptavidin tether to the mica surface. A final possibility that we cannot discount is that the tip is being contaminated by salt or sample, giving rise to a different asymmetry of the gold particles. Tip contamination is a dynamic process and the roundness of the particles in the final frame suggest this indeed might have occurred.

J. Yang: It has been known that a major obstacle for in air AFM in biology is the existence of the adhesion force. Did the authors investigate the relationship between the humidity and the adhesion force with their specimen preparation method?

Authors: No. We did not recognize this relationship when these experiments were made. The adhesion/humidity relationship was not the major impetus of this work but has been examined by Thundat et al. on plain mica [Thundat T, Zheng X-Y, Chen GY and Warmack RJ (1993) Role of relative humidity in atomic force microscopy imaging. Surface Science Letts. **294**: L939-L943].

J. Yang: In Figure 4, the authors claim a specimen with higher residue salt concentration. If they prepared the specimen the same way as that described in the **Method** section, I do not see it possible after thoroughly rinse with distilled and deionized water.

R. Balhorn: In text (under **Results** in discussing Figure 1) the authors state: "The gold particles could be monitored almost all the way until their removal from the imaged area at a surface saturation humidity (for this sample) of 64%. This saturation level is partly determined by residual salts from the rinsing step". This should be explained in more detail or experimental results should be provided.

Authors: Through empirical evaluation, the optimal rinsing condition for our sample preparation scheme uses 1 ml of distilled deionized water, pipetted over the specimen at 45° to the normal, followed by immediate drying with dry nitrogen. The purpose of the rinse is to get rid of excess salt without removing too much sample. Though these steps can be routinely reproduced, there is still one step in the process that seems to defy reproducibility about 25% of the time, which is cleavage of the mica. Our criteria for mica cleavage is that the fresh, cleaved mica be free of any obvious surface roughness "to the eye", which is not a rigorous criteria. Different "mirror-smooth" pieces of mica do not always give rise to the same specimen spreads, even when all the remaining steps can be faithfully reproduced. This response is probably due to the varying amount of excess negative charges on the mica surface that can bind the DNA (through the cations in the buffer salt). Still, in principle, it can be controlled by the amount of rinsing, and this is why we attribute the amount of residual salt to rinsing.

The purpose of including the spread on Figure 4 was to show the exaggerated effect of excess salt on the surface as a function of humidity. Such a spread would not usually be used for a publication when the authors are interested in a specimen with low-background noise. However, in this case, we were trying support the idea of a hydrated salt layer to explain the change in height of the colloidal gold particles, a salt layer that is obvious in Figure 4 but more subtle in Figure 1.

F. Zenhausern: How can you be sure that DNA molecules were not removed by the scanning tip? Do you have an image at lower magnification of the same sample or a TEM replica of your to confirm data?

Authors: Yes, we do have a lower magnification AFM images of the sample before and after going through the entire change from low to high humidity back to low again. At about the 65% humidity level, all gold and DNA always seem to be swept to the edges of the scanned area, as has been confirmed elsewhere [12, 13]. Though we can not pick out individual pieces of DNA from the salt in the scan boundary, one can see taller

gold particles after low humidity was again attained. No TEM replicas have been made of these samples.

F. Zenhausern: Did you have some calculation of adsorption energies or forces?
Authors: No. In view of the complicated nature of the binding mechanism in the gold-streptavidin-mica linkage trapped within a sea of residual buffer salts, we have not attempted to quantify these forces.

Reviewer: In the text, under **Methods**, you mention a nominal spring constant of 0.2 N/m. Did you measure the spring constant?
Authors: We made no attempt to measure the spring constant of the cantilevers used, since the exact value of the force was unimportant for these experiments as long as it was maintained constant. Measured spring constant values in the literature [Cleveland JP, Manne S, Bocek D, Hansma PK (1993) A nondestructive method for determining the spring constant of cantilevers for scanning force microscopy. Rev. Sci. Instr. **64**: 403-405] range from 0.10 to 0.36 N/m for the type of cantilever we used, from which we take a nominal value of 0.2 N/m.