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SCANNING TUNNELING MICROSCOPE IMAGES OF ADENINE AND THYMINE AT ATOMIC RESOLUTION

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Abstract

The scanning tunneling microscope has been used to obtain images of DNA that reveal its major and minor grooves and the direction of helical coiling, but sufficient resolution has not yet been achieved to identify its bases. To determine if this technology is capable of identifying individual DNA bases, we have examined the molecular arrangements of adenine and thymine attached to the basal plane of highly oriented pyrolytic graphite. Both molecules form highly organized lattices following deposition on heated graphite. Lattice dimensions, structural periodicities, and the epitaxy of adenine and thymine molecules with respect to the basal plane of graphite have been determined. Images of these molecules at atomic resolution reveal that the aromatic regions are strongly detected in both molecules while the various side-groups are not well-resolved. These studies provide the first evidence that tunneling microscopy can be used to discriminate between purines and pyrimidines.

Key Words: DNA bases, adenine, thymine, purines, pyrimidines, scanning tunneling microscopy, heated graphite, adsorbate epitaxy, pyrolytic graphite.

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Introduction

The scanning tunneling microscope (STM) is a powerful tool which can achieve atomic resolution on a number of surfaces (Hallmark *et al.*, 1987; Mate *et al.*, 1989). Despite this, it has not been clearly shown that the STM can resolve the atoms of biological macromolecules. Numerous laboratories have reported STM images of DNA (Driscoll *et al.*, 1990; Dunlap and Bustamante, 1989) and protein (Guckenburger *et al.*, 1989). While the images of polyadenine reported by Dunlap and Bustamante have suggested that individual bases may be resolvable with the STM, deposition techniques have not been developed to the extent that DNA can be imaged reproducibly. In contrast, images of a bacterial coat protein (Guckenburger *et al.*, 1989) were obtained reproducibly but resolution was well below that needed to resolve individual amino acids. In an effort to determine whether the STM can resolve and discriminate between purines and pyrimidines, adenine and thymine (two of the four bases that encode genetic information in DNA) have been imaged with the STM at atomic resolution.

Materials and Methods

Preparation and Imaging of Adenine and Thymine Lattices

A 10μl droplet of distilled water containing 1 mg/ml of adenine or thymine was applied to a freshly-cleaved, highly oriented pyrolytic graphite surface that was heated to approximately 80 °C. During the heating process the droplet beaded on the hydrophobic surface of graphite. Immediately before drying, it spread and wet the surface. Vaporization of the droplet occurred in 20-30 seconds and the sample was then immediately air-cooled. This sample preparation method results in a very thin, clean adsorbate. Optically, the graphite appears bare, except for a ring of sample material which has deposited along the perimeter of the initial droplet. The STM tip was positioned in the center (bare) area of the sample and imaging was performed in air at room temperature using the Nanoscope II (Digital Instruments,

Santa Barbara, CA). The STM tips used for these experiments were Pt-Ir (90% Pt) and either hand-cut or mechanically prepared. Achieving a high-quality tip, capable of high resolution imaging and free of noise was essential to obtaining images of the adsorbates.

Adsorbate Epitaxy

In order to remove the adsorbate layer, the tunneling gap resistance was typically lowered by a factor of 50, bringing the tip much closer to the substrate. At a bias of 50 mV and tunneling current of 1 nA the tip removed the adsorbate layers and the underlying graphite could then be imaged. The adsorbate was again imagable after the tunneling gap resistance was increased back to its original setting. A defect in the adsorbate lattice could be observed in the area where adsorbate had been removed. In addition, applying a voltage pulse between the STM tip and sample (4 V per 10 μsec) could also remove a limited portion of the adsorbed patch directly beneath the STM tip.

Molecular Modeling

The adenine model was generated using the program ENCAD, a molecular dynamics program developed by Michael Levitt (Levitt, 1983). Prior to minimization, the adenine and water molecules were initially placed on the graphite atoms at positions suggested by STM measurements, with water molecules given random rotational orientations. The adenine molecules were moved on the graphite lattice until the aromatic rings of adenine stacked onto the graphite in an ABAB-like configuration. (Subsequent layers of hexagonally co-ordinated carbon atoms stack in a ABAB configuration; since the six and five membered rings of adenine are approximately similar in shape and size to the graphite hexagons, adenines may be expected to stack above the graphite substrate in an ABAB-like manner). The molecules then assumed their most stable positions after several iterations of energy minimization.

Results and Discussion

We have found that aqueous-phase adenine and thymine molecules adsorb strongly to a heated graphite surface and reliably form highly ordered surface layers. Due to the packing of the molecules within the surface layer, these structures are sufficiently stable to withstand imaging with the STM. Single bases or small adsorbates containing only a few adenine or thymine molecules, on the other hand, were not observed. The imaged surface layers appear as patches, typically several thousand angstroms in diameter, and are surrounded either by the bare graphite substrate or by another contiguous adsorbate layer of a different orientation. The layers appear to be monomolecular, since their height extends only 1-2 angstroms above the surrounding graphite. We assume, just as others have observed for alkylcyanobiphenyl molecules (Smith *et al.*, 1990), that the STM only images the molecular layer in contact with the surface, with excess sample being pierced or pushed by the tip. We have also been able to image the adenine adsorbate patches on graphite using the atomic force microscope but with resolution insufficient to identify individual molecules.

Although we have observed other lattice configurations on rare occasions, the lattice structures shown here (Figs. 1 and 2) are exceedingly favored and have highly characteristic dimensions (specific for adenine and for thymine) that show little variation from one preparation to another. Images of these lattices are obtained in approximately nine out of ten preparations. These structures have never been observed in negative control experiments where distilled water was vaporized on heated graphite.

Adenine lattices consist of bimolecular rows running head to tail and roughly parallel (Figs. 1a and 1b). We measure the peak-to-peak length of each adenine molecule to be 5.4 Å (standard deviation, s.d. = 0.9 Å) with a peak-to-peak separation of 3.2 Å (s.d. = 0.7 Å) between molecules along one monomolecular row. The average center-to-center distances between adenines in the same row (positioned head to tail) and between molecules in adjacent rows of a single bimolecular row are 8.8 Å (s.d. = 1.4 Å) and 5.8 Å (s.d. = 0.7 Å) respectively. These distances allow no room for the placement of water molecules between the purines within the row and correspond to tight van der Waals packing.

Regularly spaced channels, located to either side of each bimolecular row (Figs. 1a and 1b), appear to be imposed by water molecules bound within the lattice. The N6 atom is the most charged atomic species of adenine with the greatest affinity for water (Renugopalakrishnan *et al.*, 1971). Therefore, we suggest that the amino groups project out into these channels where they can interact with and form hydrogen bonds to water. Although the heating process during sample preparation (to 80 °C) may drive off a large percentage of available water, the ambient humidity could easily supply enough water to account for the channels observed in the adenine lattices. To check for the presence of water within the adenine lattice, the film was analyzed by laser ionization time-of-flight mass analysis (data not shown). With the power density used, the adenine molecules fragmented to nitrogen and various hydrocarbons. In addition, oxygen was detected, which suggests that water molecules may also be present in the adenine layer adsorbed to graphite (oxygen is not detected when using bare graphite). While the observed nitrogen to oxygen ratio is consistent with the presence of one water molecule per adenine molecule in the lattice, these results by themselves do not provide conclusive evidence that water is actually a component of the adenine lattice being imaged by the STM tip. On the other hand, these results are consistent with previous X-ray data which show that water is present in crystals of adenine-hydrochloride (Broomhead, 1948) and other organic compounds.

The center-to-center distance between bimolecular

rows is 22 Å with the proposed water channel measuring 11.3 Å in width. These water channels are not always observed in all areas of the lattice, suggesting that the N6 position can undergo dehydration leading to a more densely packed configuration of adenine molecules.

The individual aromatic rings within a single adenine molecule appear to be resolved (Figs. 1b and 1c). The peak-to-peak diameter of the larger ring is 3.1 Å (s.d. = 0.7 Å) and the smaller ring measures 2.2 Å (s.d. = 0.3 Å). These measurements agree with X-ray crystallography data on adenine hydrochloride (Broomhead, 1948). The amino group is not detected other than for a smear which appears near the N6 position in but a few images. As with the alkylcyanobiphenyls, the hydrogen atoms are not clearly resolved.

Thymine lattices (Fig. 2) are more densely packed and lack the channels observed with adenine. The molecules usually appear evenly spaced and have a center-to-center separation of 7.1 Å. The pyrimidine ring is 2.9 Å (s.d. = 0.3 Å) wide and the presence of a single ring (Fig. 2) distinguishes it from the purine (double ring) structures observed in adenine lattices. As with adenine, the side-groups of thymine are not clearly resolved.

In order to identify the relative positions of the adenine and thymine molecules with respect to the underlying carbon atoms of the basal plane of graphite, the gap resistance between the tip and sample was reduced (moving the tip closer to the substrate) and the adsorbate layers were removed by the tip during subsequent scans. The underlying graphite lattice could then be imaged at atomic resolution. These experiments revealed a very specific surface/sample interaction between the molecules in the lattice and the atomic structure of graphite. The bimolecular rows of adenine show a 30 degree angle of separation to the 3-fold symmetry axes of the graphite lattice while thymine rows appear to stack just slightly off the 3-fold symmetry axes of graphite with a 3-4 degree mismatch. For thymine, the base-substrate interactions appear to be strongest at the edge of the lattice, where the bases align best with the underlying graphite atoms (Fig. 2). In addition, the observation that two contiguous lattices always have lattice vectors rotated by 60 and 120 degrees also indicates that both adenine and thymine molecules interact with the underlying graphite lattice. Moreover, these results suggest that the adsorbed layers nucleate and form on the graphite surface as opposed to forming in solution during dehydration.

Figure 1d shows a computer model of the adenine lattice on graphite. The organization of the molecules in this lattice represents a purely hypothetical, minimum energy configuration that is consistent with numerous STM images of adenine lattices and the underlying graphite. This structure is stabilized almost exclusively by van der Waals interactions. The positions of the adenine molecules were adjusted only slightly by minimization, while each water molecule oriented itself within hydrogen bond distances with respect to the N6 donor and N7 acceptor of each adenine molecule. The small dots and streaks which appear to either side of the bimolecular row in Fig. 1b correspond to water molecules hydrogen-bonded to these two nitrogens. For the most part, the adenines maintained their original epitaxy with respect to the underlying carbon lattice (which is quite similar to biphenyl positions observed on graphite [Smith *et al.*, 1990]). This is common, since the algorithms used to minimize the energy of the system only find local energy minima and large changes in the positions of molecules are not achieved without performing extensive molecular dynamics.

Because only a single ring is present in thymine and the side groups are poorly resolved in current STM images of thymine lattices, insufficient information is presently available for us to generate a plausible computer model of the thymine lattice. While additional studies must be performed using other techniques to confirm the structures of these lattices, the present results demonstrate that the STM can be used to discriminate between purines and pyrimidines if the bases are optimally oriented (i.e., laid flat) on the surface of graphite. Whether this approach can be used to differentiate between each of the four bases in DNA remains to be demonstrated. Perhaps the most significant outcome of this work is that we have succeeded in preparing lattices of each base (guanine and cytosine also form lattices) that can be imaged routinely and used in future studies to identify a spectroscopic method that may be coupled with STM imaging to identify the four bases in a polynucleotide chain and to sequence DNA.

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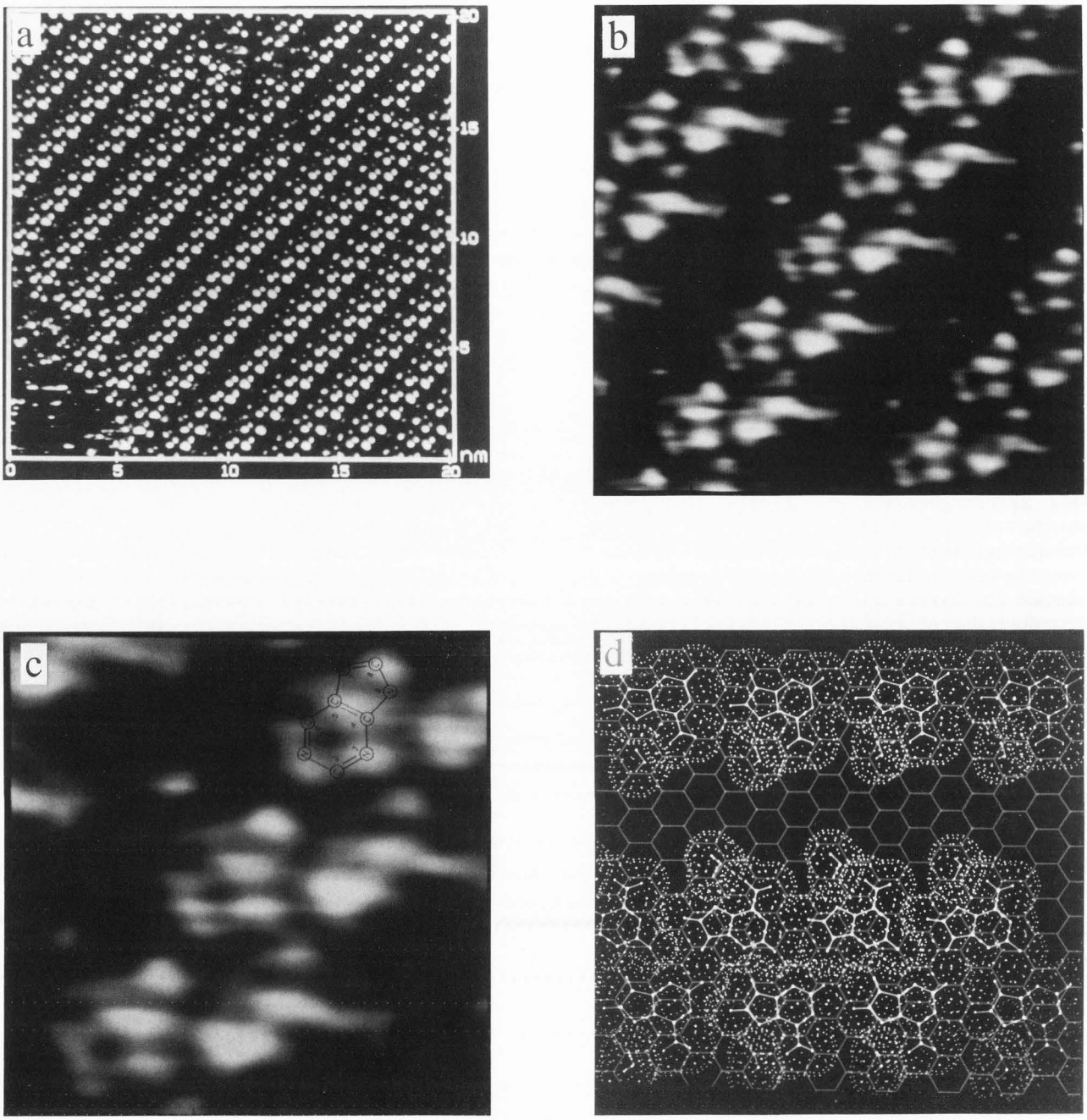

Figure 1. STM images of the adenine lattice. **a)** 200 x 200 Å image showing the bimolecular row configuration of the adenine lattice on graphite. The adenine molecules all appear to be oriented in the same direction along the bimolecular rows. The channels which separate the bimolecular rows of adenine most likely contain the water component of the lattice. The adenine lattice terminates into surrounding graphite in the lower left of the image. Bias 305 mV. Current setpoint 0.3 nA. **b)** 48 x 48 Å image of adenine molecules within the two-dimensional lattice. Both the five- and six-membered aromatic rings of adenine are clearly detected in molecules along the left side of each bimolecular row while molecules on the right side appear slightly distorted. Bias 274 mV. Current setpoint 0.7 nA. **c)** Enlarged image (25 Å x 25 Å) showing the atomic-level detail of individual adenine molecules. Given the placement of the adenine model overlay, nitrogen atoms do not appear to be detected at the N6, N7 and N9 positions. **d)** Computer model of the adenine lattice at an energy minimum that is consistent with the STM data. The lattice is stabilized primarily by tight van der Waals packing of hydrated adenines to each other and to the underlying graphite substrate. van der Waals surfaces are shown only for the adenine and water molecules.

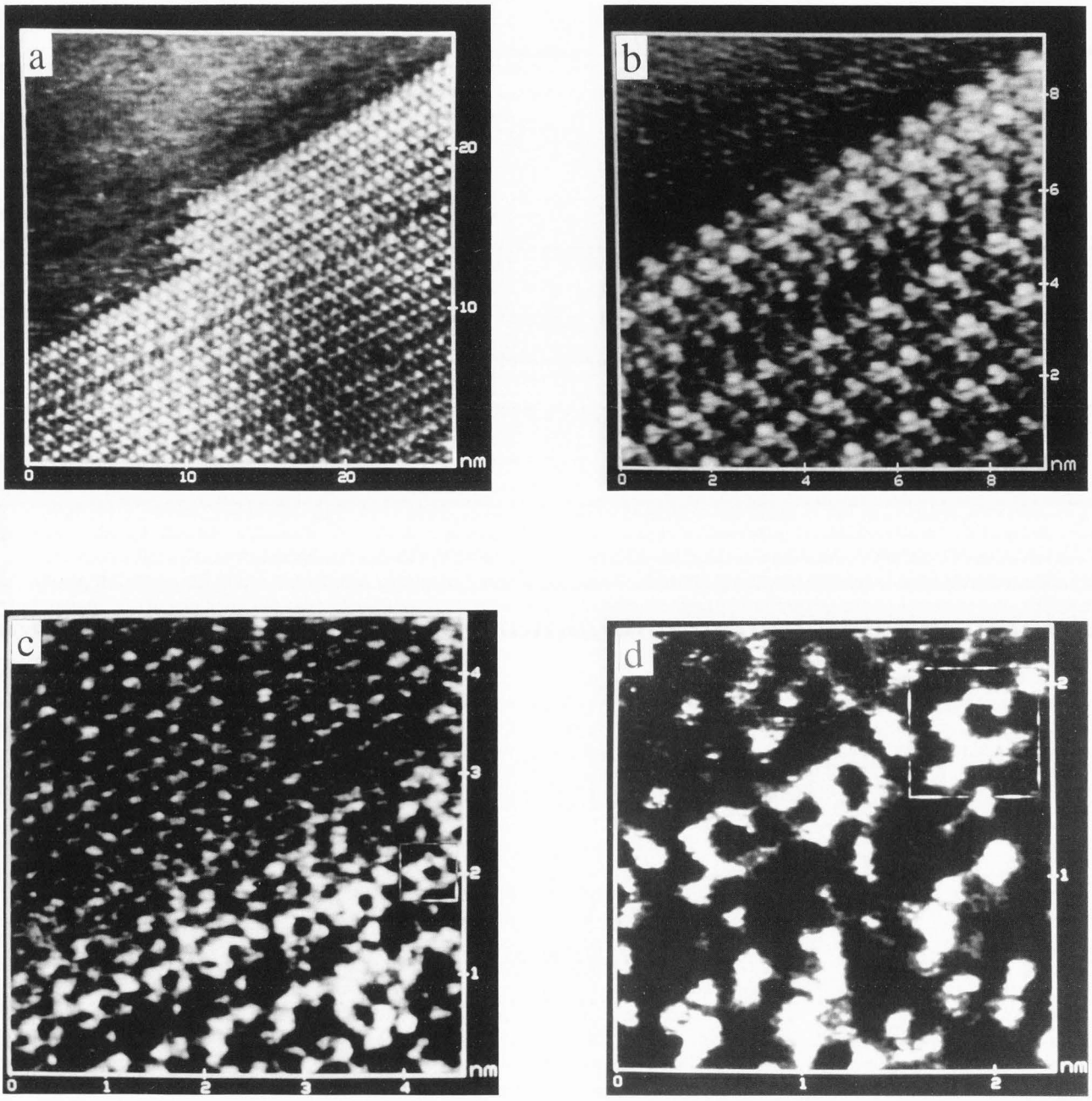

Figure 2. STM images of the thymine lattice. **a)** Low resolution scan (268 x 268 Å) of the thymine lattice adsorbed to the underlying graphite. A graphite/thymine boundary is seen from the lower left to the upper right of the image. Unlike adenine lattices, straightened edges are characteristic of thymine lattices. The scanning STM tip frequently removes thymine molecules situated along the edge of the lattice, whereas internal molecules are better stabilized and thereby resistant to removal. Bias 308 mV. Current setpoint 0.35 nA. **b)** 90 x 90 Å image showing individual thymine molecules organized into linear rows separated by narrow channels (3-4 Å wide) too small to accommodate water molecules. Single ring structures were most clearly resolved along the straight lattice edges, while internally positioned molecules generally could not be imaged with the same clarity, possibly due to tight packing. The lattice terminates into surrounding graphite in the upper left of the image. Bias 221 mV. Current setpoint 0.35 nA. **c)** 45 x 45 Å image of seven thymine molecules (the box outlines a single molecule) clearly visible along the edge of a lattice. Graphite atoms are visible in the top portion of the image. Bias 412 mV. Current setpoint 0.35 nA. **d)** A different high resolution image (23 x 23 Å) of three thymine molecules. The single ring of thymine is detected while the oxygen and methyl groups are not clearly distinguishable. Bias 445 mV. Current setpoint 0.26 nA.

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Discussion with Reviewers

D.P. Allison: In the author's proposed model of the adenine lattice, composed of bimolecular rows of adenine separated by channels, only one of the rows of adenine is resolved in the images they have presented. This could be due to electronic interference of one molecular row of adenine on the other and might depend on the direction of the scan. Have the authors ever been able to resolve the ring structure of both chains of adenine perhaps by scanning directly along a biomolecular row?
Authors: Yes, other images of adenine resolve the ring structure of both rows of molecules. Because we do not reproducibly detect the distortion shown in Figure 1b, it is difficult to guess what might have caused it. We cannot completely rule out the possibility of a scan direction related artifact. The subtle differences observed between molecules in different rows in other images could be related to an electronic effect in which one row of adenines interact with the underlying graphite in a slightly different registry. We have also considered possible topographic influences such as amino groups that may have rotated above the lattice plane. The distortion might also be caused by interactions between these amino groups and the adsorbate layers above the surface monolayer that the tip apparently pushes aside as imaging occurs.

J.D. Baldeschwieler: Have the authors measured the dependence of the tunneling current on the distance between the tip and surface? If so, what is the effective function derived from this relationship?
Authors: No, we have not yet determined how changes in the tunneling current relate to the distance between the tip and the surface for adenine and thymine.

J.K.H. Hörber: I like the idea very much to prepare these molecules in a similar way as liquid crystals. This makes it possible to get more information about the involved imaging mechanisms for DNA. The images are very encouraging for further investigations. On the other hand, I have some doubts about the interpretation of the images. I cannot agree that the ring structure of the molecules fits to the structure of the graphite lattice, they differ a little bit and are too rigid to be deformed. We learned with the liquid crystals that the chains interact much more with the graphite, and the ring positions are mainly determined by the intermolecular interactions. These molecule calculation programs can behave quite unfriendly if they are forced by the starting conditions into a wrong direction.

I would also prefer more information about the defects produced by the tip mentioned in "**Adsorbate Epitaxy**". Possibly these defects can provide some hints about the ordering of the molecules.
Authors: We agree that the structure of the ring is too rigid to be deformed to align precisely with the underlying graphite atoms. However, our results suggest that the sample must interact with the surface graphite atoms. The registry experiments show that both adenine and thymine align in a regular, reproducible fashion with the three fold symmetry of the graphite. In addition, the exact alignment differs consistently for the two different bases. It is unlikely that each base in the entire lattice aligns in the same precise manner with the underlying graphite atoms, but what may be occurring is that only certain rows or certain bases within the rows align and maintain the registry.

Regarding the defects in the lattice induced by pulsing, we did not (unfortunately) scan the defects at high enough resolution to obtain useful information about the ordering of the molecules with respect to the graphite.