

AFM Imaging Artifacts due to Bacterial Cell Height and AFM Tip Geometry

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Atomic force microscopy (AFM) has become an important tool for investigating various biological materials, and it is now being applied more routinely for imaging bacteria. By imaging bacteria in water, AFM can provide in-situ images of viable cells and be used to measure interaction forces between the AFM tip (or a colloid probe) and the cell surface. However, the relatively large height and compliance of the bacterium can also lead to imaging artifacts. AFM images of *Escherichia coli* K12 were consistently found to contain image shadows that were oriented in parallel lines 27° from the direction of the cantilever tilt, regardless of the scan direction. Similar image shadows were also observed for 1 μm diameter polystyrene latex microspheres. Using a simple geometric model for the interaction of the tip and the bacterium, it is demonstrated here that these lines observed for bacteria are image artifacts produced by the pyramidal shape of the tip, the 10° tilt of the cantilever, and the height of the bacterium relative to the size of the tip. Such image artifacts disappear when we image dehydrated bacteria that are lower in height, or bacteria that become damaged and deflated during imaging in water. The interaction of the edge of the tip with the bacterium is also shown to result in inconsistent shapes of force curves unless the force curve is centered on the crest of the rounded bacterial surface.

Introduction

The development of atomic force microscopy (AFM) or scanning probe microscopy, has opened the door for researchers to obtain topographical images and force measurements on living cells. Examples of living cells imaged with AFM include Madine–Darby canine kidney (MDCK) cells,¹ monkey kidney cells,² and various strains of bacteria including *Buckholderia cepacia* G4, *Pseudomonas stutzeri* KC,³ *Shewanella putrefaciens* strain CN-32,⁴ *Staphylococcus aureus*,⁵ and *Escherichia coli*.^{6,7} AFM has also been used as a tool to probe cell morphology and the forces responsible for cell adhesion.^{6,7} The main advantages of AFM over other techniques are that images and force curves can be obtained in-situ and can provide quantitative morphological information and particularly high vertical resolution.

There are a number of challenges in using AFM to examine living cells. One is that the cells are deformable under the force applied by the AFM tip. This leads to a reduction in the lateral resolution and complicates the deconvolution of surface forces and deformation in the force curve analysis.^{2,7–10} Another challenge is that cells

can move in response to the lateral movement of the tip and the cantilever, and can be pushed across the surface during imaging. Therefore, the cell must be anchored to the surface by forming covalent or electrostatic bonds between the bacterium and the surface.^{11,12}

Another difficult situation that has arisen in the imaging of bacteria using AFM, but that has not been explained previously in the literature, is the appearance of unknown “material” on the edge of large cells or bacteria when they are imaged in water. Such “material” has been observed in tapping mode images of *Pseudomonas chrysosporium* spores,¹³ images of a fixed liver endothelial cell (LEC),¹⁰ and images of *E. coli* bacteria.⁷ In contrast, images of other smaller bacteria (100–300 nm in height) such as *B. cepacia* G4 and *P. stutzeri* KC, do not show this “material”.³

The regular appearance of this “material” on the edge of live cells was of concern to us, as it suggested that molecules were being pulled off the surface of the cells and deposited on the substrate. The long length of the “material” also made the overall shape of the cells appear much different than we expected in AFM images. On further examination of these bacterial images, however, we noticed that the “material” consistently formed lines not in the direction of the tip scan but at an angle approximately ±27° from the normal direction of the scan (scan angle = 0°). However, the location of the artifact did not change when the scan direction was changed. This

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suggested to us that there was no “material” in the image and that what we were seeing was an imaging artifact.

Although previous papers have discussed the convolution of the AFM tip with the specimen under investigation, none of these directly explain the artifact we describe here. For example, for samples with dimensions that are smaller than those of the AFM tip, the bluntness of the tip can cause distortion of the image so that the objects appear much larger. This can also cause the reverse tip image effect.^{14,15} The artifacts observed here also appear to have a reverse tip image effect, but the dimensions of bacteria are much larger than the radius of the tip. Other studies have mentioned a “shadowing” effect due to interaction of the object with the sides of the tip.^{16–18} This “shadow” effect is primarily due to the scan direction (or scan angle) and can be minimized by using a slower scan speed or by changing the scan angle. However, in our work, we have found that the location of the artifact on the bacterium does not change with the scan direction or the scan speed.

In this paper, we explain the presence of artifacts in AFM images of bacteria and particles that are approximately 1 μm in height. Using a simple geometric model, we show that the artifacts are due primarily to the tilt of the cantilever and the height of the object being imaged. We also demonstrate that force curves obtained on such large and highly curved surfaces must be systematically obtained at the crest of the curved surface in order to obtain accurate force curves.

Methods

Preparation of Bacteria-Coated and Polystyrene-Coated Slides. *E. coli* K12 (obtained from the *E. coli* Genetic Stock Center at Yale University) were grown in Luria broth at 37 °C for 2 h to midexponential growth phase and washed three times in 1 mM Tris buffer or 1 mM NaCl, unless otherwise stated. Bacteria were irreversibly attached to a polyethylenimine (PEI) (750 000 Daltons)-coated glass slide, as previously reported.⁷ Glutaraldehyde, used to fix bacteria for some images, was stored at –70 °C and thawed immediately before use. Fluoresbrite Carboxylate YG 1.0 μm microspheres (Polyscience Inc.) were washed in MQ water and also attached to the glass slide using PEI.

Atomic Force Microscopy Imaging and Force Measurements. A Bioscope atomic force microscope (Digital Instruments) was used to image cells in aqueous solutions (unless otherwise stated) using a fluid cantilever holder. The cantilevers, with a spring constant of either 0.045 or 0.25 N/m, were cleaned using a BioForce UV/ozone cleaner prior to obtaining an image. All topographic images were obtained in tapping mode.

Force measurements, obtained in contact mode, were made on a location of the bacterium selected from the tapping mode image. During force imaging, the tip was brought into contact with the bacterium by vertical scanning with a scan size and frequency of the cantilever of 1 μm and 1 Hz, respectively. Force curves obtained on the PEI-coated silica surface were used to convert cantilever and tip deflection in millivolts to nanometers.

During AFM imaging, the side of the AFM tip will come into contact with the side of the bacterium (Figure 1). The

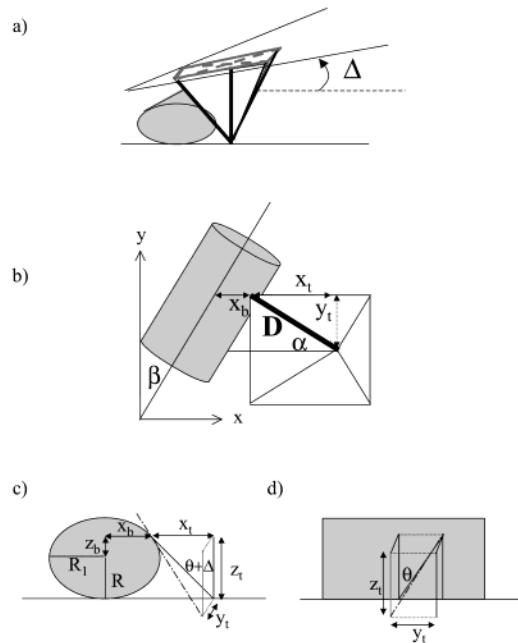


Figure 1. Geometric diagram of an AFM tip with a half-cone angle of θ interacting with a bacterium of radius R . The tip and cantilever are tilted at an angle of Δ from the x - y plane, and the bacterium is placed at an angle β from the y -axis on the x - y plane. The edge of the tip (---) interacts with the side of the bacterium at a distance D and an angle α from the apex of the tip. Parts b, c, and d are views of the tip and bacterium from the x - y plane, the x - z plane, and the y - z plane, respectively. Note: The dash-dot line in parts c and d is the edge of the tip and is not coplanar with the plane shown.

distance, D , and the angle from the horizontal, α , between the apex of the tip and the point of contact can be obtained from the tip and bacterium geometry. Standard AFM tips obtained from DI are pyramidal with a half-cone angle (θ) of 35°. The cantilever and tip are placed in the AFM head at a tilt angle (Δ) of 10° from the horizontal (Figure 1a). As the AFM tip traverses the surface, it encounters a bacterium oriented at an angle, β , from the y -axis (Figure 1b).

To support our hypothesis concerning the image artifact, we have modeled the approach of the AFM tip toward the bacterium. For the model we assume that the bacterium is a cylinder with a radius, R . D (the x - y distance between the apex of the tip and the contact point) and α (the angle from the scan direction) can be calculated from θ , Δ , β , and R (Figure 1) according to

$$\tan \alpha = \frac{y_t}{x_t} \quad (1)$$

$$D = \sqrt{x_t^2 + y_t^2} \quad (2)$$

where x_t and y_t can be calculated (Figure 1c and d) as

$$\tan(\theta + \Delta) = \frac{x_t}{z_t} \quad (3)$$

$$\tan \theta = \frac{y_t}{z_t} \quad (4)$$

and where z_t is defined as the height on the tip where it

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interacts with the bacterium (Figure 1c)

$$z_t = z_b + R \quad (5)$$

If the bacterium is rotated β degrees about the z -axis, the projected cylinder on the x - z plane is an ellipse where (Figure 1c)

$$R_1 = \frac{R}{\cos \beta} \quad (6)$$

The horizontal (x_b) and vertical (z_b) distances from the center of the bacterium to the point of contact with the side of the tip can be related by

$$\frac{x_b^2}{R_1^2} + \frac{z_b^2}{R^2} = 1 \quad (7)$$

At the point of contact between the bacterium and the tip, the slope of the bacterium is equal to the slope of the tip, or

$$\frac{dx_t}{dz_t} = \frac{dx_b}{dz_b} \quad (8)$$

Combining the differentiation of eqs 3 and 7 with eq 8 gives

$$\frac{-z_b}{x_b \cos^2 \beta} = \tan(\theta + \Delta) \quad (9)$$

Equations 3–5 can be combined into eq 2 to give

$$D = (z_b + R)\sqrt{\tan^2(\theta + \Delta) + \tan^2 \theta} \quad (10)$$

Equations 9 and 2 are combined together to solve for z_b :

$$z_b^2 \left(\frac{1}{\cos^2 \beta \tan^2(\theta + \Delta)} + 1 \right) = R^2$$

Finally, combining eqs 9 and 10, D is obtained directly as a function of known geometrical values of θ , Δ , β , and R as

$$D = R \left(1 + \sqrt{\frac{\cos^2 \beta \tan^2(\theta + \Delta)}{1 + \cos^2 \beta \tan^2(\theta + \Delta)}} \right) \sqrt{\tan^2(\theta + \Delta) + \tan^2 \theta} \quad (11)$$

while combining eqs 1, 3, and 4 provides α as a function of θ and Δ .

$$\alpha = \tan^{-1} \left(\frac{\tan \theta}{\tan(\theta + \Delta)} \right) \quad (12)$$

Results

AFM Image Artifacts on Bacteria. AFM topographic images of individual *E. coli* cells taken in tapping mode consistently showed the expected rod-shape cells, as well as the appearance of a sort of “material” alongside the bacterium (Figure 2). This “material” formed large parallel lines that were not in the direction of the scan. An image analysis of the length and orientation of the material, as shown by the thick black lines in Figure 2, indicated that the lines to the right of the cell were $1.1 \pm 0.2 \mu\text{m}$ in length and $27 \pm 3^\circ$ from the horizontal ($n = 13$).

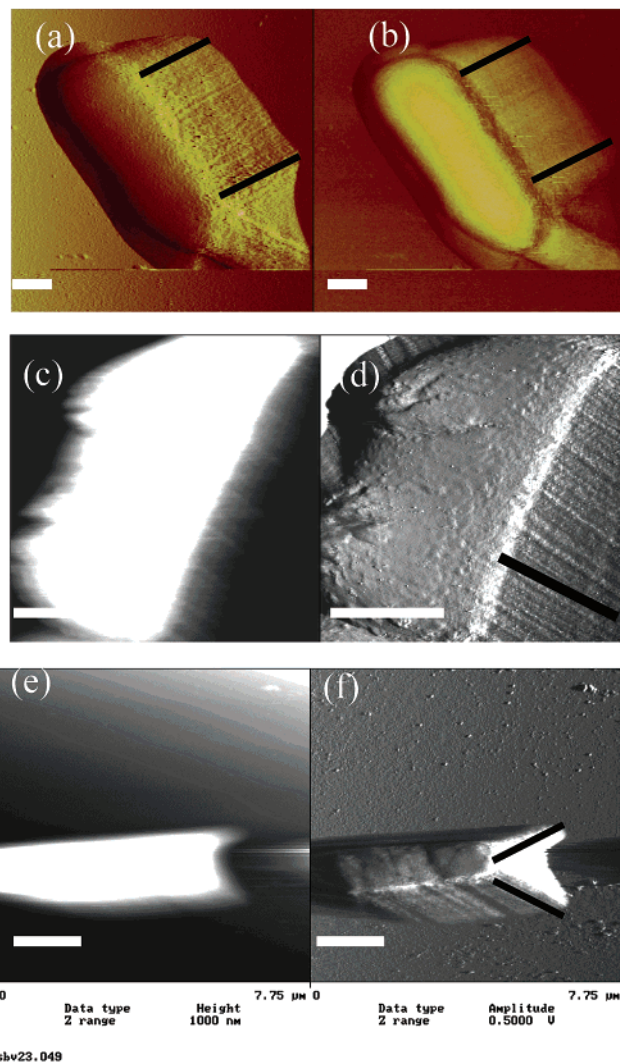


Figure 2. AFM tapping mode images of *E. coli* K12 (D21) showing line artifacts. Parts a and b are amplitude and phase images of D21 with 2.5% glutaraldehyde in 100 mM NaCl + 1 mM Tris. Parts c and d are height and amplitude images of D21 with 2.5% glutaraldehyde in 100 mM NaCl. Parts e and f are height and amplitude images of D21 in 1 mM Tris, respectively. The white bar represents $1 \mu\text{m}$. The vertical scale for the height images is $1 \mu\text{m}$. The scan direction is from right to left with a 0° scan angle. The lines highlighted in black are $\pm 27^\circ$ to the scan direction.

To test whether these image artifacts might be due to the deformation of the cell or to electrostatic interactions between the tip and the surface, we stiffened the cells and changed the solution ionic strength. Glutaraldehyde cross-links proteins in the cell membrane and increases cell stiffness.¹⁹ Force curves taken with AFM have shown that *E. coli* strains become about 3 to 5 times stiffer when fixed in a 2.5% glutaraldehyde solution.⁷ However, glutaraldehyde treatment did not remove the image line artifacts surrounding the cells (Figure 2a and b). Decreasing the solution ionic strength from 100 mM NaCl to 1 mM NaCl solution, which should have increased the Debye length (repulsive layer) surrounding the cell from 1 to 10 nm, also did not remove the lines offset from the cell area (Figure 2e and f). This lack of an effect of ionic strength indicated that the lines were not produced by electrostatic repulsion of the tip by the bacterium.

If the lines were caused by the AFM tip pulling material from the cell onto the surface, then changing the direction

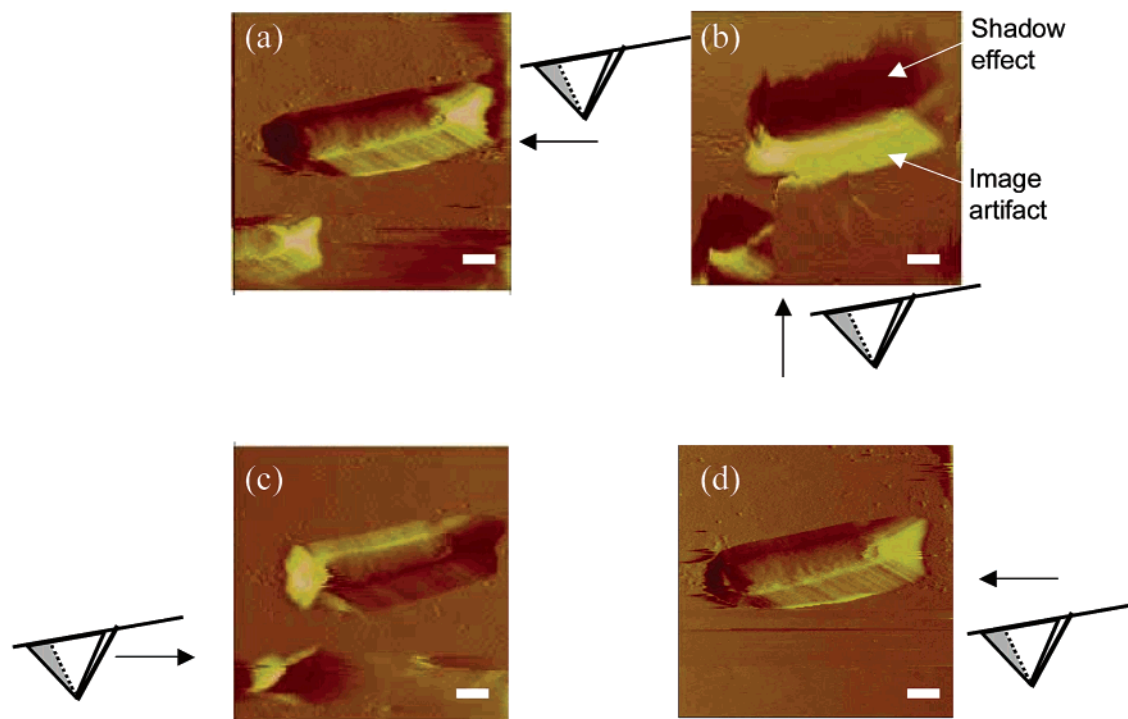


Figure 3. AFM tapping mode images of D21 with a scan angle of 0° (a), 180° (b), or 90° (c). In part d, the scan angle is 180° and the tip direction is from left to right. The lines are always approximately $\pm 27^\circ$ to the tilt of the tip. The dark region (or the “shadow” effect) in each image is due to the scanning direction. The white bars represent 1 μm .

of the scan should have altered the location of the lines and “material” relative to the bacterium. However, changing the direction of the scan did not alter the location of the lines. The lines remained at an angle of approximately 27° relative to the 10° tip tilt, as shown in Figure 3. When the AFM scanning direction is changed, the bacterium remains fixed in the x - y plane, and the AFM tip remains in a fixed orientation in the x - z plane while the direction of the movement of the tip changes in the x - y plane. As a result, changing the scan direction does not alter the relative orientation of the pyramid-shaped tip to the cell, just the direction the tip moves during a scan. The geometry of the artifact in the image of the bacterium shown in Figure 3 looks the same independent of the scanning directions shown in Figure 3, indicating that no material is being pulled off the cell. This indicated that the interaction between the tip and surface must be related to the tip–bacterium geometry and the orientation of the tip relative to the bacterial surface. We therefore hypothesized that the image artifact was due to the height of the cell and that the lines were produced by the interaction of the edge of the tip with the bacterium when the end of the tip was actually on the surface.

Examining the Effect of Cell Height on Image Artifacts. When bacteria were imaged in air, they had a smaller overall height (200–300 nm) relative to that of bacteria imaged in water (800–1000 nm). We observed that when *E. coli* were imaged in air, the line artifacts disappeared (Figure 4), a finding that we attributed to the reduction in cell height. To further investigate the effect of height on the appearance of image artifacts, we imaged bacteria in water that appeared to become damaged or “deflated” during imaging, resulting in a smaller height than that for normal, healthy cells. The healthy bacterium imaged under water showed the image artifact lines (Figure 5a and b), but as the height decreased, the artifact lines started to disappear (Figure 5c and d) until finally there were no longer any lines (Figure 5e and

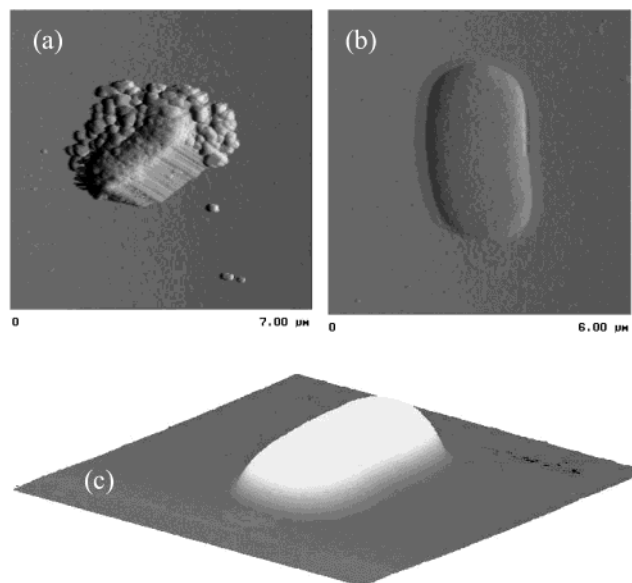


Figure 4. AFM images (contact mode) of *E. coli* in air. (a) Image taken immediately after adhesion to glass. The cell height is 830 nm, and lines are present in the lower right side of the image. The spherical protrusions surrounding the cell are likely water droplets that have not yet evaporated. (b) Image after water has evaporated and the cell height is reduced to 240 nm. (c) Surface plot of the same image in part b. Notice that image artifacts are no longer present due to the reduced height of the cell in parts b and c.

f). Another deflated bacterium shown in Figure 5g and h also does not show any image line artifacts due to the reduced height of the bacterium.

Image Artifacts with a Polystyrene Microsphere. If the line artifacts are produced solely by the interaction of the side of the AFM tip with the bacterium, it was reasoned that images of a microsphere on a surface would produce line artifacts exactly like those observed for a

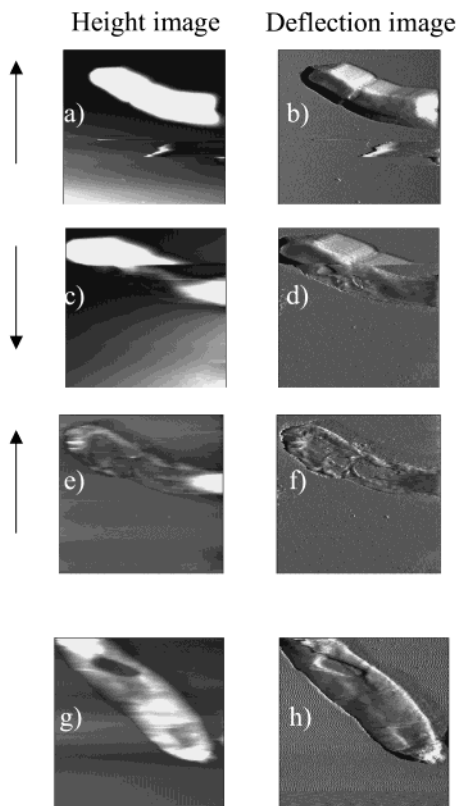


Figure 5. Creation of a deflated bacterium and disappearance of artifacts. Parts a, c, e, and g are height images while parts b, d, f, and h are deflection images. The bacterium is deflated in parts c and d at the dotted line. When the height of the bacterium decreases due to deflation, the imaging artifacts disappear and are not present in parts e and f. A similar deflated bacterium is shown in parts g and h.

bacterium. It was found that polystyrene latex microspheres having about the same height ($1\ \mu\text{m}$) as that of a normal bacterium did produce image artifacts (Figure 6) on the right side of the sphere, where Δ was $+10^\circ$, $D = 1.4\ \mu\text{m}$, and $\alpha = 27^\circ$, as well as to the left of the sphere, where Δ was -10° , $D = 0.33\ \mu\text{m}$, and $\alpha = 58^\circ$.

Force Curve Artifacts on Bacteria. Taking force curves at different locations on a single bacterium provided a mechanism to determine the height of a bacterium and to further study the interactions of the tip with the side of the bacterium. When obtaining a force image, the rastering of the cantilever is stopped and the tip is moved only in the vertical direction. The relative piezo position is recorded for each force curve and used to determine the height.

Nine force curves were taken along a straight path that crossed the surface of a bacterium (Figure 7). The effect of side interactions of the tip with the bacterium can be seen by changes in the shape of the force curves taken at several locations along a straight path across the bacterium. An example of a series of such force curves is shown in Figure 7a, where the square symbols indicate force curves obtained on a PEI-coated glass slide on either side of the bacterium and the other symbols indicate locations where the tip was influenced by the bacterium surface. Force curves taken as the tip was moved down across the top of the bacterium surface are shown with filled symbols, while open symbols are used to indicate locations as the tip moved past the cell; the + sign indicates a location on the very top or crest of the bacterium. The piezo displacement of the force curve taken on the top of the cell (+) compared with the glass-only force curve (\square) indicates

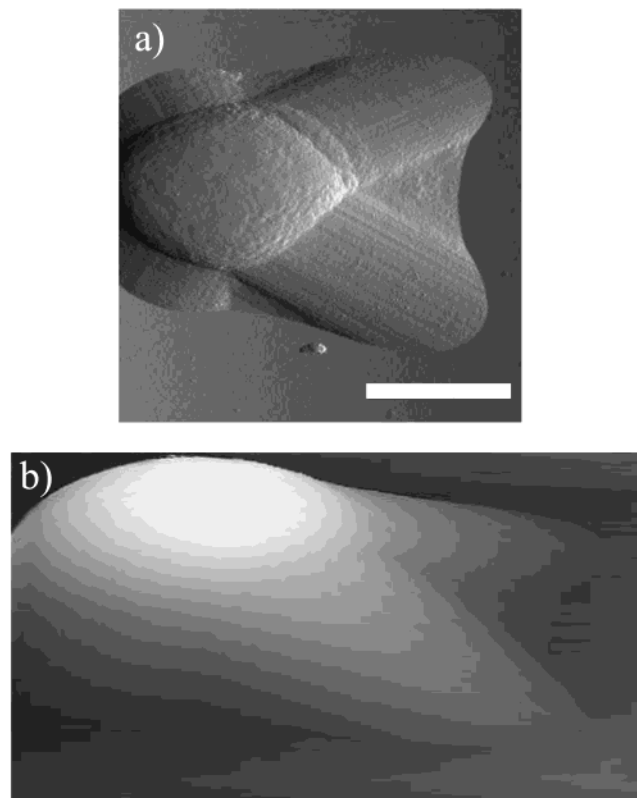


Figure 6. AFM tapping amplitude mode images of a $1\ \mu\text{m}$ polystyrene sphere. The white bar represents $1\ \mu\text{m}$. Part a is a top view, and part b is a surface plot.

that the surface is $950\ \text{nm}$ high (Figure 7a). The decreased slope of the top of the force curve on the bacterium indicates deformation of the bacterium. Notice that some force curves centered over a point not on the bacterium showed a highly nonlinear response, indicating that the tip was experiencing an interaction with the side of the bacterium. The size of the linear image artifact region to the right of the cell is $950\ \text{nm}$; there also appears to be a shadowed area on the bottom left of the bacterium that is much smaller than the artifact area to the top right of the bacterium (Figure 7b). It can be seen from examining these nine force curves that there are tip–bacterium interactions at relatively large distances from locations where the AFM tip is located directly over the cell surface.

Discussion

The image artifacts, consisting of shadow regions and lines angled $27 \pm 3^\circ$ away from the scan direction, indicate interactions between the edge of the pyramidal tip and the side of the bacterium during tapping and contact mode imaging. If the flat face of the tip were to be positioned directly normal to the bacterium ($\beta = 0$), the entire face of the side of the tip would interact with the side of the bacterium. However, the tip is usually positioned at an angle to the side of the bacterium so that the *edge* of the pyramidal tip interacts with the bacterium during imaging. The shadow or artifact zone is generated by the edge of the tip bumping into the high side of the bacterium. The size and location of the image artifact, and the direction of the lines relative to the scan direction and cell orientation, are consistent with the geometry of the bacterium and the tip.

The calculated values for the distance, D , observed in the AFM images agree fairly well with the calculations from eq 11 (Figure 8a–c). For a bacterium modeled as a

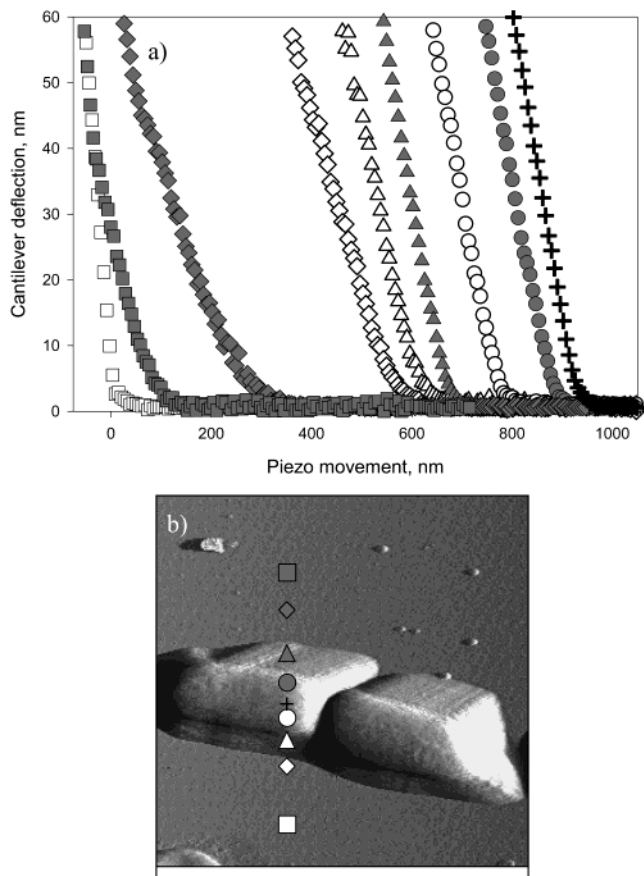


Figure 7. AFM force curves (a) corresponding to the AFM deflection image (b) of *E. coli* K12. The symbols shown on the force curve (a) correspond to the positions of the symbols on the image (b). Note the high position of the piezo at the "artifact" region of the image.

cylinder with a radius of $0.5 \mu\text{m}$, and tip and bacterium angles of $\alpha = 10^\circ$ and $\beta = 35^\circ$ (see Figure 2a for the experimental equivalent), the edge of the AFM tip was calculated to contact the side of the bacterium at a distance $D = 1.0 \mu\text{m}$. The distance D is predicted to decrease linearly with the radius of the object being imaged (Figure 8a), which explains why smaller objects do not display this effect. Rotating the bacterium on the surface (changing β) has a very small effect on the calculated value of D (Figure 8b). A change in the tilt of the cantilever, Δ , is predicted to decrease the value of D (Figure 8c). Qualitatively, the prediction of a decrease in D is consistent with our observations shown in Figure 6a: we observed a decrease in D from 1.4 to $0.35 \mu\text{m}$ with a decrease in Δ from $+10^\circ$ to -10° . However, the model predicts a decrease from 1.0 to $0.8 \mu\text{m}$ for the same change in Δ , but this discrepancy is probably due to the fact that the model is for a cylinder and not a sphere.

The angle α is predicted by eq 12 to be 35° for $\theta = 35^\circ$ and $\Delta = 10^\circ$. This is slightly larger than the experimentally determined value of $27 \pm 3^\circ$ (Figure 8d). This larger value of α may be due to the greater tilt angle of the cantilever than that assumed in our calculations. As seen in eq 12, α is a function of the half-cone angle of the pyramid (θ) and the tilt of the cantilever (Δ). If the tilt angle of the cantilever is assumed to be 19° instead of 10° , α would be calculated as 27° . The tilt angle of the tip when it is in contact with the bacterium may be different than 10° during tapping mode imaging because the cantilever is oscillating in the z -direction. In order for Δ to change from 10° to 19° , the oscillations would have to be 150 nm for a $100 \mu\text{m}$ long cantilever (or 300 nm for a $200 \mu\text{m}$ long

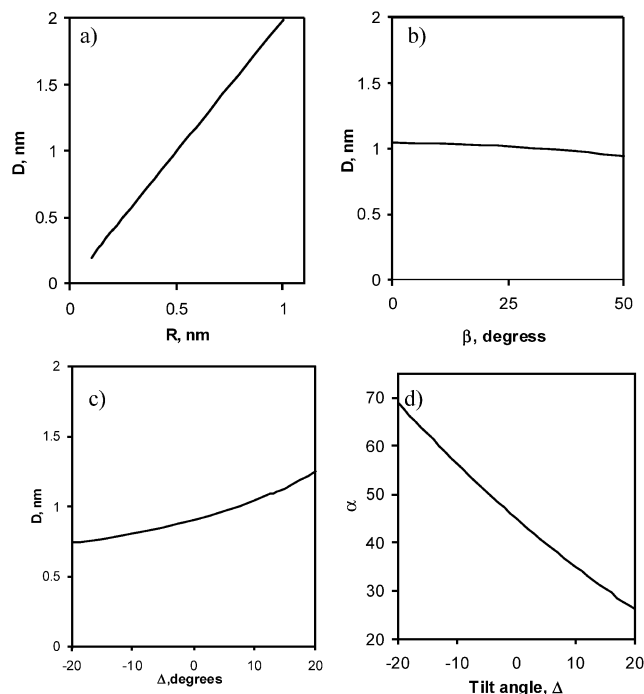


Figure 8. Calculated value of D given by eq 11 as a function of the bacterium radius, R (a), the angle of the bacterium on the surface, β (b), and the tilt angle, Δ (c). Part d shows α as a function of the tilt angle, Δ , using eq 11. The following values were used: half cone angle, $\theta = 35^\circ$; $R = 0.5 \text{ nm}$; $\Delta = 10^\circ$; $\beta = 35^\circ$.

cantilever). An offset of this magnitude is quite large relative to typical tapping mode deflection distances. Another possibility is that the value for the half-cone angle, θ , is not equal to 35° . However, eq 12 is not very sensitive to the value of θ : Changing θ from 35 to 20 and 50 changes α from 35 to 32.2 and 34.5 . A final possibility is that the tip may be sharpened at the point, resulting in a higher value for z_t than that predicted for the model. Nevertheless, the model predicts a reasonably close value for α .

The lines in the artifact image are believed to result from the interaction of the edge of the pyramid with the nonuniform side of the bacterium. This nonuniformity may be due to differences in elasticity of the side of the bacterium or to small protrusions on the side of the bacterium. Because glutaraldehyde did not appear to remove the lines, it is likely that the lines reflect protrusions on the surface of the bacterium. Nonuniform surfaces have been previously reported for bacteria.²⁰

There have been numerous images of bacteria and living cells published in the literature, so why have these image artifacts only been observed in a few cases for bacteria? One reason is that the heights of some bacteria (e.g. *B. cepacia* G4 and *P. stutzeri* KC³) are much less than those of the bacteria (*E. coli*) examined here. Also, many images of bacteria are taken in air. As we showed in Figure 4, bacteria fixed to slides in air are smaller in height than those imaged in water, and therefore the side of the bacterium does not interact over a very large distance with the bacterium. Images of *E. coli* obtained in liquid by others^{6,21} often show a monolayer of bacteria, or bacteria closely packed together. Artifacts are not evident for images of a monolayer of bacteria for two reasons. First,

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when the bacteria are imaged very close together, the AFM tip position is never down close enough to the surface for the side of the tip to interact with the bacterium. Second, if the scan size is so large that many bacteria are imaged together, the edge effects do not become as noticeable due to the resolution of the image, as could be seen in images of *E. coli* by Bolshakova²¹ and by Velegol and Logan.⁷ Thus, the large image artifacts observed here result from the size of the image relative to the scan size as well as the height of the bacterium relative to the tip size.

The aim of this study was to explain and model the prominent lines that are observed on our AFM images of *E. coli* as well as images obtained by others of *P. chrysosporium* spores¹³ and liver endothelial cell (LEC).¹⁰ These artifacts appear when the height of the object being

imaged is on the order of 1 μm and when the image is obtained in an aqueous environment. These artifacts do not change with scan direction (such as the "shadow" effects) and are due primarily to the tilt of the cantilever and the interaction of the object being imaged with the side of the AFM tip.

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