# Do Immobilization Methods Affect Force Spectroscopy Measurements of Single Bacteria?

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**ABSTRACT**

Increasing antibiotic resistance in bacteria is a critical issue that often leads to infections or other morbidities. Mechanical properties of the bacterial cell wall, such as thickness or elastic modulus, may contribute to the ability of a bacterial cell to resist antibiotics. Techniques like Atomic Force Microscopy (AFM) are used to quantify bacterial cell mechanical properties and image cell structures at nanoscale resolutions. An additional benefit of AFM is the ability to probe samples submerged in liquids, meaning that live bacteria can be imaged or evaluated in environments that more accurately simulate in vivo conditions as compared to other methods like electron microscopy.

However, because AFM measurements are highly sensitive to small perturbations in the deflection of the tip of a sensor probe brought into contact with the specimen, immobilization of bacteria prior to measurement is essential for accurate measurements. Traditional chemical fixatives crosslink the molecules within the bacterial cell wall, which prevents the bacteria from locomotion. While effective for imaging, chemical crosslinkers are known to affect the measured stiffness of eukaryotic cells, and also may affect the measured stiffness of the bacterial cell wall. Alternative immobilization methods include Cell-Tak™, an adhesive derived from marine mussels that does not interact with the bacterial wall, and filters with known pore sizes which entrap bacteria. Previous studies have examined the effect of these immobilization methods on successful imaging of bacteria, but have not addressed differences in measured modulus. This study compares the effects of immobilization methods including chemical fixatives, mechanical entrapment in filters, and Cell-Tak™ on the stiffness of the bacterial cell wall as measured by force spectroscopy.

Keywords: atomic force microscopy, mechanobiology, live cell immobilization

## INTRODUCTION

Physical structures of bacteria are critical to the viability of cells, and can affect how they move, grow, and reproduce[1-4]. Previous studies show that the elastic modulus, or stiffness, of the bacterial cell wall, may be related to the antibiotic resistance of bacteria [5, 6], which is of interest due to increasing antibiotic resistance in bacteria often leading to infections or other morbidities [7-10]. Understanding the relationship between the mechanical behavior of a cell and its antibiotic resistance may help in the development of strategies for treatment or prevention of bacterial infections.

Atomic force microscopy (AFM) is a technique used to image cell structures and quantify bacterial cell mechanical properties at high resolutions [11, 12]. One advantage to using AFM to characterize biological samples is that imaging can be performed in liquid at atmospheric pressures, simulating in vivo conditions for bacteria. Additionally, because AFM measures samples through a contact based process, mechanical properties such as stiffness and surface roughness can also be measured. To determine the stiffness of a cell, a probe is brought into contact with the sample. The force applied to the sample and the distance the probe travels into the sample are recorded. Mathematical models, such as the Hertz fit model, are then applied to calculate properties such as elastic modulus.

However, one challenge in obtaining high quality AFM measurements of bacterial cells is cell motility. Because AFM relies on physical contact to measure cell stiffness, the cells must be held stationary and adhered to a surface during measurement. A common method of immobilizing cells for imaging is to use a chemical crosslinker such as paraformaldehyde, which crosslinks molecules in the bacterial cell wall, "fixing" the cells. Though chemical fixatives are effective for imaging, previous studies show that these fixatives can affect the measured Young's modulus of eukaryotic cells [13], and may affect bacterial cells as well. Previous studies which compare the effect of fixatives on bacteria focus on preserving the morphology of cell features [14, 15]. This study compares common immobilization methods to determine if the fixation method affects the measured Young's modulus of *Staphylococcus aureus* cells.

Previous studies, which use a variety of fixation methods and bacteria species, have reported modulus values between 60 kPa and 9 MPa [16-18]. However, AFM measurements are sensitive to experimental parameters such as probe tip size and shape, probe approach speed, and indention force into the sample [1, 19, 20], so a direct comparison must be made to determine the effect of a particular sample preparation method. In this study, cells will be fixed with two chemical crosslinkers, glutaraldehyde and paraformaldehyde, which are both commonly used for chemical immobilization of cells and are known to affect the viability of cells. Adhesion methods which do not kill cells include Cell-Tak™, an adhesive derived from a protein from marine mussel shells [15], and poly-L-lysine, which has a positive ionic charge and provides an attachment point for negatively charged bacterial cells [21]. However, bacteria attached with these methods can release from the surface during contact measurements. Another method used to immobilize cells for imaging is mechanical entrapment in filters with pores that correspond to cell size. *S. aureus*, which has a spherical shape, is compatible with this method, but rod-shaped bacterial species such as *Escherichia coli* cannot be immobilized in filters [15]. Future studies will compare mechanical entrapment to the chemical fixation methods as well.

## EXPERIMENTAL

*Bacteria Culture: Staphylococcus aureus* is a spherical, gram-positive bacterium. *S. aureus* stock was added to filter sterilized Todd Hewitt Yeast broth with an inoculation loop and cultured for 24 hours at 37°C.

*Specimen Preparation:* Dish preparation was performed according to established procedure [12]. Poly-L-lysine (0.1% solution in water, Sigma-Aldrich) was added to sterile glass-bottomed 35mm dishes to cover the bottom of the dish and left to sit for one hour. Poly-L-lysine was aspirated from the dishes and the dishes were rinsed three times with phosphate buffered saline (PBS, VWR). 1 mL of *S. aureus* solution was added to each dish. After one hour, bacterial solution was aspirated and the dishes were rinsed three times with PBS to remove planktonic bacteria. For dishes with chemical fixatives: paraformaldehyde (4% solution in PBS, Sigma-Aldrich) or glutaraldehyde/formaldehyde (2.5% solution, Electron Microscopy Sciences) was added to the dish to cover the glass bottom and allowed to sit for an hour until bacteria was fixed. Fixative was aspirated then dishes were rinsed three times with PBS. Sample dishes were transported dry. Bacteria-coated surfaces were covered with PBS prior to imaging to simulate in vivo conditions for bacteria.

*Atomic Force Microscopy Procedure:* Atomic force microscopy was performed with a JPK Nanowizard 4 in Quantitative Imaging mode combined with a Nikon confocal microscope. HQ:CSC17/Cr-AU BS cantilevers (MikroMasch) with tip radius of 8 nm and stiffness between 0.06-0.4 N/m were used for imaging single bacteria. Cells were imaged with a tip extend speed between 25-75 μm/s and a setpoint between 1.5-2 nN. Brightfield microscopy images at a magnification of 60x were taken of an area on the dish with bacterial cells prior to imaging with AFM. If the cells in the area were successfully imaged with AFM, then a second image, intended for modulus measurement, was recorded on top of one of the cells. A region from the center of this image was cropped to dimensions of 100 pixels x 50 pixels to remove issues with measurements caused by curvature at the edges of the cell. For individual force curves, JPK data analysis software calculated Young's modulus of the cell using the Hertzian model for stress between contact of two bodies. These modulus values were averaged to provide a modulus value for an individual cell.

## RESULTS AND DISCUSSION

Representative AFM imaging and modulus results for *S. aureus* cells fixed with glutaraldehyde are shown in Figure 1. Figure 1(a) shows a brightfield microscopy image of *S. aureus* cells with a 60x magnification. At this magnification, individual cells and cells that were not adhered or still moving could be visually identified. Dishes that had been treated with only poly-L-lysine had sparse cells and cells were frequently still moving, increasing the difficulty of obtaining an accurate AFM measurement. After optical microscopy, the AFM was used to record a height map of individual bacterial cells or small clusters of cells, as seen in Figure 1(b) and Figure 1(c). No difference in cell size

was noted for different fixative methods. The cells shown in these images are noted in the optical image in Figure 1(a).

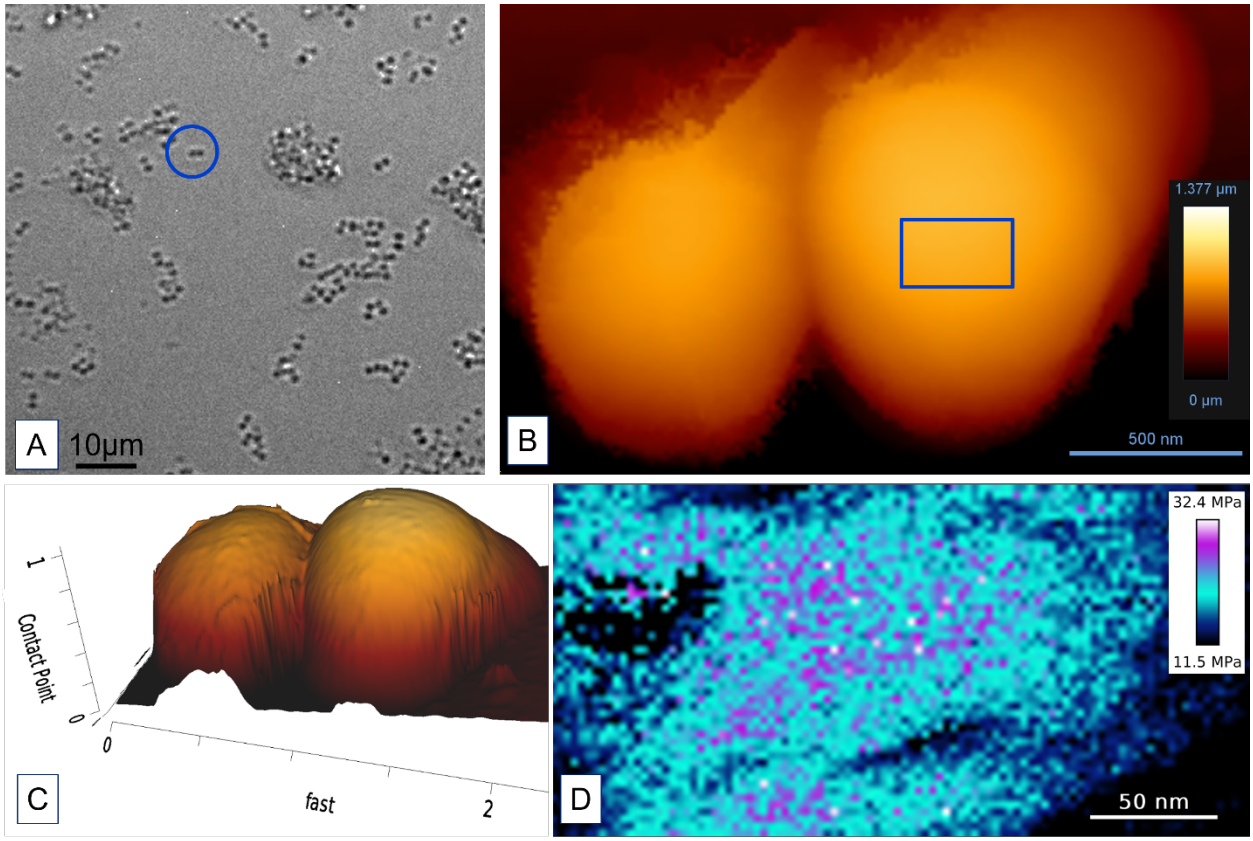


**Figure 1:** AFM imaging and data analysis process. (a) 60x brightfield microscopy image of *Staphylococcus aureus* fixed with glutaraldehyde. (b) Atomic force microscopy map of staphylococcus aureus cluster noted in (a). (c) 3-Dimensional height map of the *S. aureus* cluster shown in (b). (d) Modulus map of area on top of cell highlighted in (b) that has been cropped to an area of 100 pixels x 50 pixels.

If bacteria were successfully mapped, then a second scan was obtained from a region on top of one of the imaged cells. The approximate region chosen is shown in Figure 1(b). This scan was cropped to a region of 100 pixels x 50 pixels to reduce errors caused by curvature at the edges of the cell. Each pixel represents one contact-based measurement of the cell surface, as shown in the representative force curve in Figure 2(a). Each force curve was fit to the Hertz model for contact stress between two bodies and used to calculate the Young's modulus at that point.

A map of modulus data for the top of one cell is shown in Figure 1(d). For a given map, all 5000 modulus values were averaged to determine an average value for a particular cell. Modulus values for cells adhered with poly-L-lysine and cells fixed with glutaraldehyde and paraformaldehyde are shown in Figure 2(b). In this study, six cells were characterized for each chemical fixative and five cells for only poly-L-lysine. Modulus values for fixed cell with both paraformaldehyde and glutaraldehyde were in a similar range to values reported in literature [16-18]. Modulus values for unfixed cells, however, were much higher than values for fixed cells, and are not commonly reported in literature. Cells fixed with paraformaldehyde had the widest range in measured modulus, with values between 70 kPa and 12 MPa. Future studies will include more cell measurements, as well as additional immobilization methods.

## CONCLUSIONS

Individual *Staphylococcus aureus* cells prepared with a variety of immobilizing methods were imaged with AFM. Cells without chemical fixative often detached from the surface during AFM contact measurements, increasing the difficulty of obtaining accurate measurements. Young's modulus of cells adhered with only poly-L-lysine had higher values than cells prepared with either chemical fixative. Cells prepared with paraformaldehyde had a higher range of

experimental modulus values than cells prepared with glutaraldehyde. Future studies will compare additional immobilizing methods.

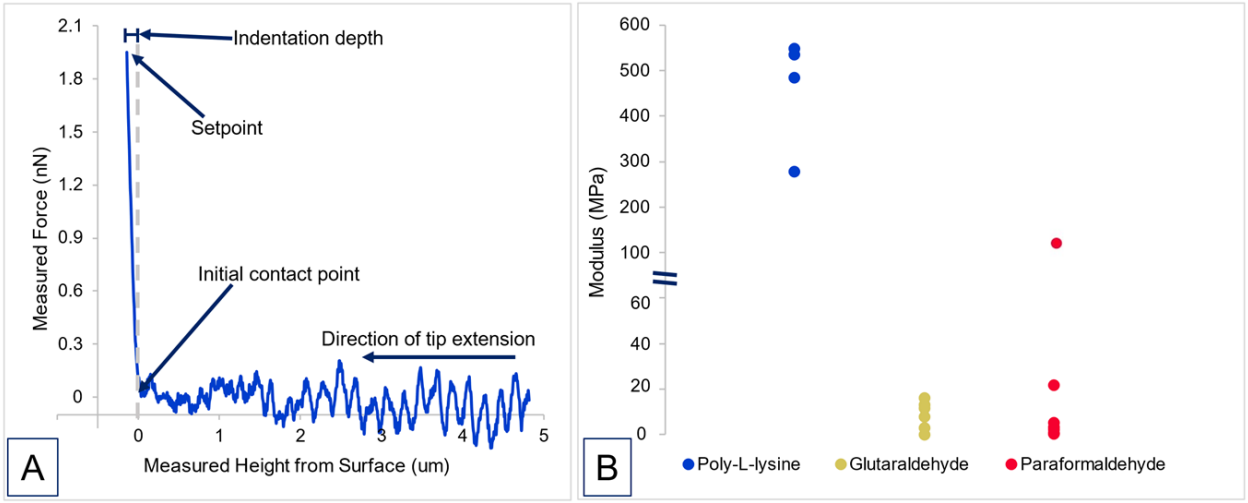


**Figure 2:** (a) Representative force curve with experimental parameters labeled. In this force curve, indentation depth is 141 nm. (b) Experimental Young's modulus for cells with poly-L-lysine, poly-L-lysine + glutaraldehyde, and poly-L-lysine + paraformaldehyde. Note the discontinuous vertical axis.


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## REFERENCES


1. Ding, Y., et al., *Are elastic moduli of biological cells depth dependent or not? Another explanation using a contact mechanics model with surface tension.* Soft Matter, 2018. **14**(36): p. 7534--7541.
2. Bailey, R.G., et al., *The interplay between cell wall mechanical properties and the cell cycle in staphylococcus aureus.* Biophysical Journal, 2014. **107**(11): p. 2538--2545.
3. Rojas, E.R. and K.C. Huang, *Regulation of microbial growth by turgor pressure.* Current Opinion in Microbiology, 2018. **42**: p. 62--70.
4. Wheeler, R., et al., *Bacterial cell enlargement requires control of cell wall stiffness mediated by peptidoglycan hydrolases.* mBio, 2015. **6**(4): p. 1--10.
5. Matheli-Guinlet, M., et al., *Bacterial Cell Mechanics Beyond Peptidoglycan.* Trends in Microbiology, 2020. **28**(9): p. 706--708.
6. Liu, L., et al., *Mechanical penetration of $\beta$-lactam-resistant Gram-negative bacteria by programmable nanowires.* Science Advances, 2020. **6**(27): p. 1--12.
7. Frieri, M., K. Kumar, and A. Boutin, *Antibiotic resistance.* Journal of Infection and Public Health, 2017. **10**(4): p. 369--378.
8. Garcia-Bustos, J. and A. Tomasz, *A biological price of antibiotic resistance: Major changes in the peptidoglycan structure of penicillin-resistant pneumococci.* Proceedings of the National Academy of Sciences of the United States of America, 1990. **87**(14): p. 5415--5419.
9. Neu, H.C., *The crisis in antibiotic resistance.* Science, 1992. **257**(5073): p. 1064--1073.
10. Longo, G., et al., *Antibiotic-induced modifications of the stiffness of bacterial membranes.* Journal of Microbiological Methods, 2013. **93**(2): p. 80--84.
11. Gaboriaud, F., et al., *Surface structure and nanomechanical properties of Shewanella putrefaciens bacteria at two pH values (4 and 10) determined by atomic force microscopy.* Journal of Bacteriology, 2005. **187**(11): p. 3864--3868.

12. Sandin, J.N., et al., *Near simultaneous laser scanning confocal and atomic force microscopy (Conpokal) on live cells.* Journal of Visualized Experiments, 2020. **2020**(162): p. 1--25.
13. Riethmller, C., et al., *Vacuolar structures can be identified by AFM elasticity mapping.* Ultramicroscopy, 2007. **107**(10-11): p. 895--901.
14. Chao, Y. and T. Zhang, *Optimization of fixation methods for observation of bacterial cell morphology and surface ultrastructures by atomic force microscopy.* Applied Microbiology and Biotechnology, 2011. **92**: p. 381--392.
15. Meyer, R.L., et al., *Immobilisation of living bacteria for AFM imaging under physiological conditions.* Ultramicroscopy, 2010. **110**(11): p. 1349--1357.
16. Cerf, A., et al., *Nanomechanical properties of dead or alive single-patterned bacteria.* Langmuir, 2009. **25**(10): p. 5731--5736.
17. Chen, Y., et al., *Bacterial cell surface deformation under external loading.* mBio, 2012. **3**(6).
18. Mularski, A., et al., *Atomic Force Microscopy Reveals the Mechanobiology of Lytic Peptide Action on Bacteria.* Langmuir, 2015. **31**(22): p. 6164--6171.
19. Kopycinska-Mller, M., R.H. Geiss, and D.C. Hurley, *Contact mechanics and tip shape in AFM-based nanomechanical measurements.* Ultramicroscopy, 2006. **106**(6): p. 466--474.
20. Ozkan, A.D., et al., *Atomic force microscopy for the investigation of molecular and cellular behavior.* Micron, 2016. **89**: p. 60--76.
21. Colville, K., et al., *Effects of poly(L-lysine) substrates on attached escherichia coli bacteria.* Langmuir, 2010. **26**(4): p. 2639--2644.