Immobilization and Imaging of Live Bacteria in Liquids

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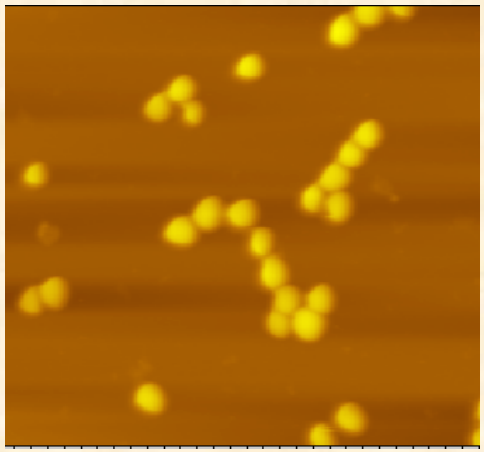
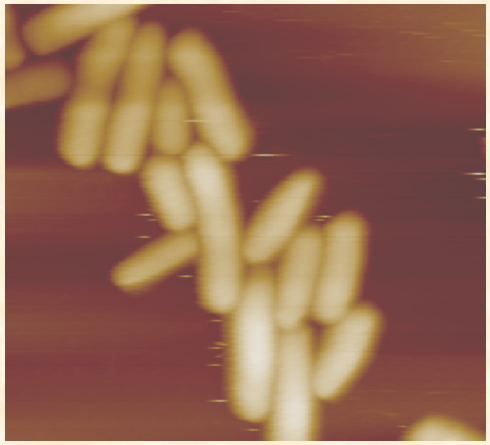
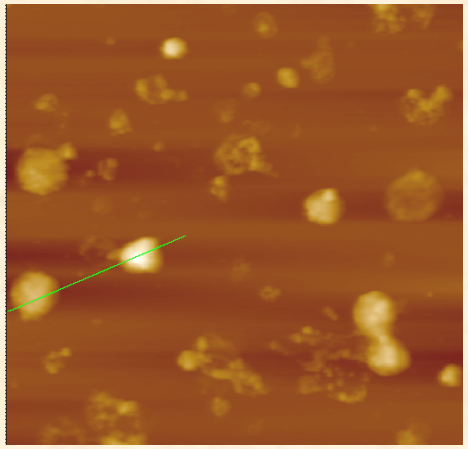


Gram Positive
Staphylococcus aureus



Gram Negative
Escherichia coli



Gram Negative
spheroplast
Escherichia coli

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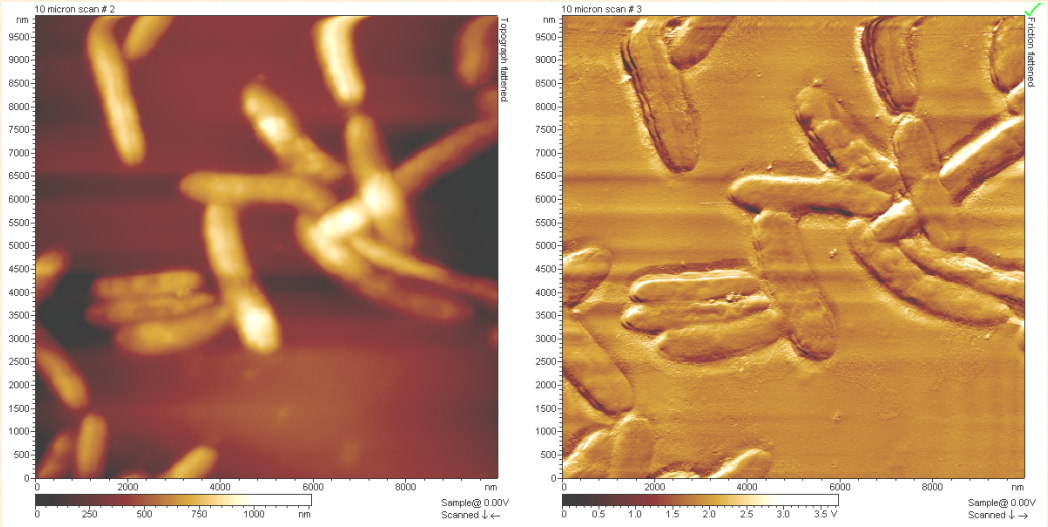
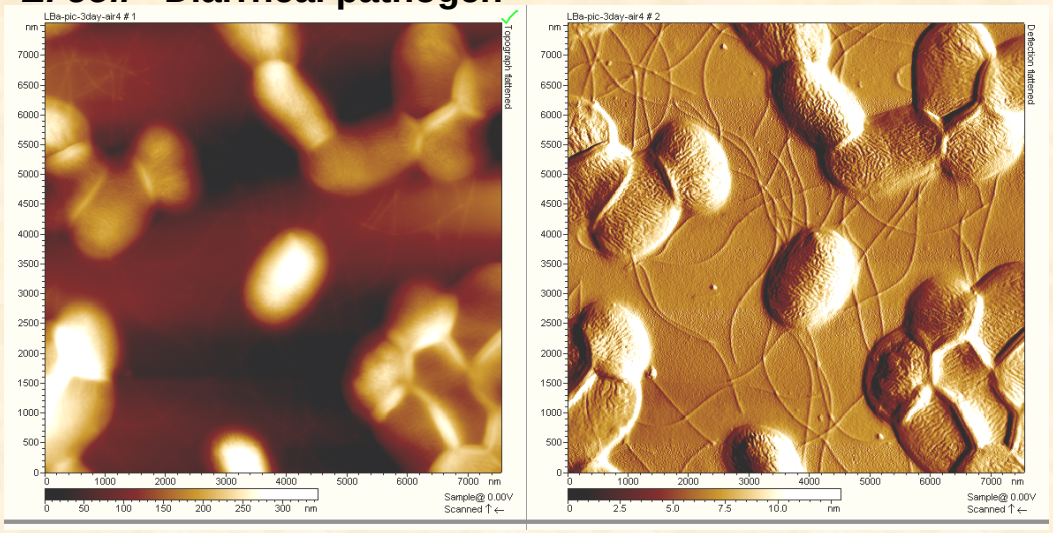
Imaging in air

- **Additional ultrastructure is often visible**
- **Bacteria often appear dehydrated**

***Clostridium thermocellum* - Possess extracellular structure that degrades cellulose**



***E. coli* - Diarrheal pathogen**



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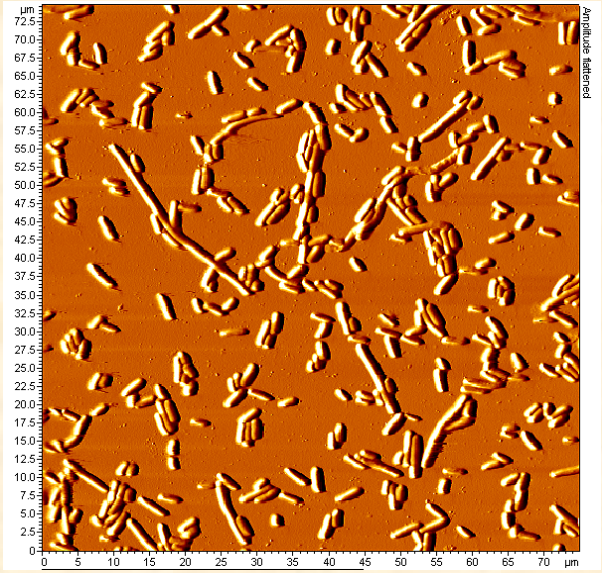
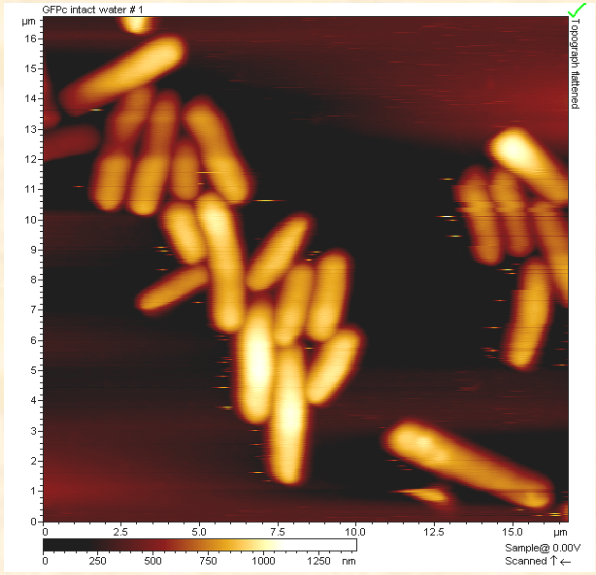
Immobilization of bacteria on gelatin-coated mica

- A thin layer of warm gelatin is added to the mica and allowed to dry
- An aliquot of the bacterial suspension is allowed to incubate on the treated mica





Doktycz et al. Ultramicroscopy (2003) 97(1-4): 209-216

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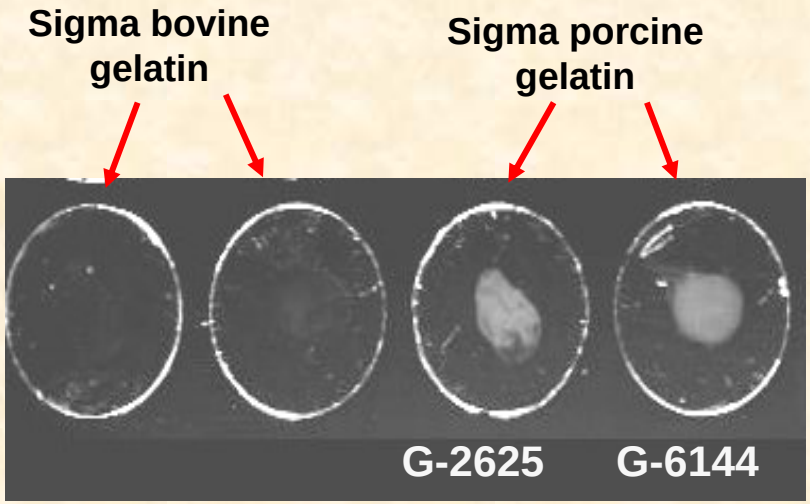
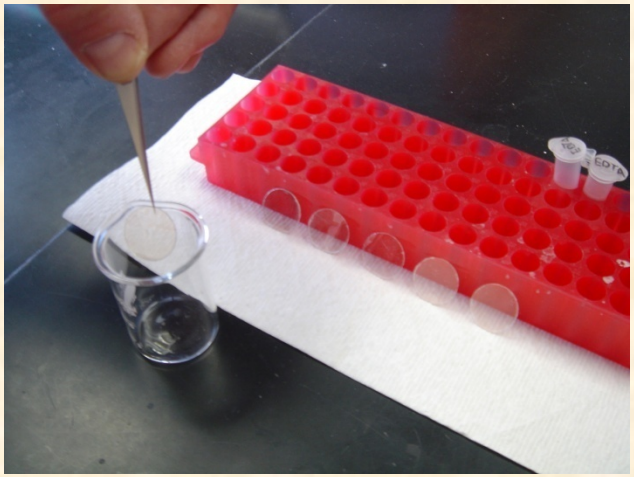
Preparation of the gelatin coated mica surface

- Weigh out 0.5 gm of gelatin --- Then In a 150 ml Wheaton or Gibco glass bottle add 100 ml of nanopure distilled water, place in microwave and bring to a boil, add the gelatin and shake to dissolve. Note, chromium potassium sulfate 10 mg is sometimes added to cross link gelatin but is not necessary.
- When the gelatin is about 60-70 ^{o}C, pour into a 20 ml beaker as shown below, dip freshly cleaved mica surfaces, let dry overnight standing on edge.
- The gelatin solution can be stored in the refrigerator and used for 60-90 days. For reuse heat in the microwave 60-70 ^{o}C.
- For best results, a drop of bacteria suspended in water is placed on the gelatin and spread with a pipette tip. After 10 min rinse the surface in a stream of water or buffer,

Very important not all gelatin works!!





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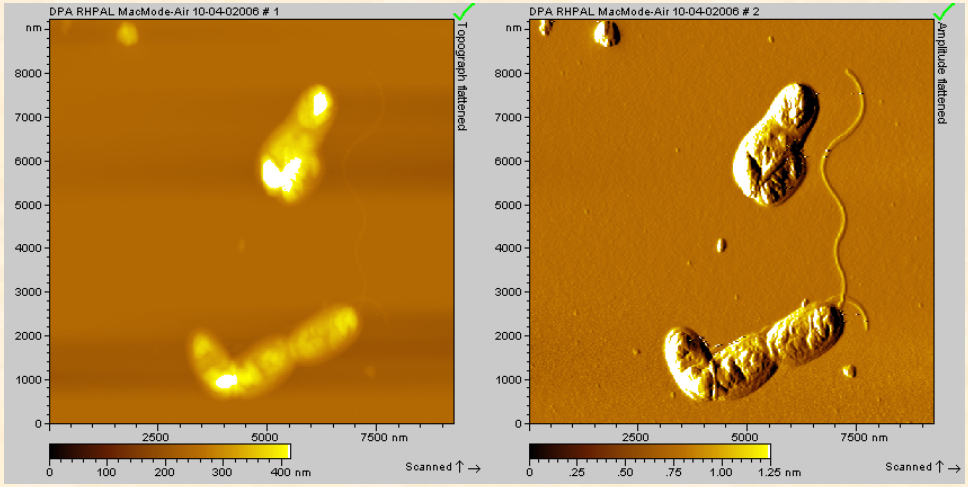
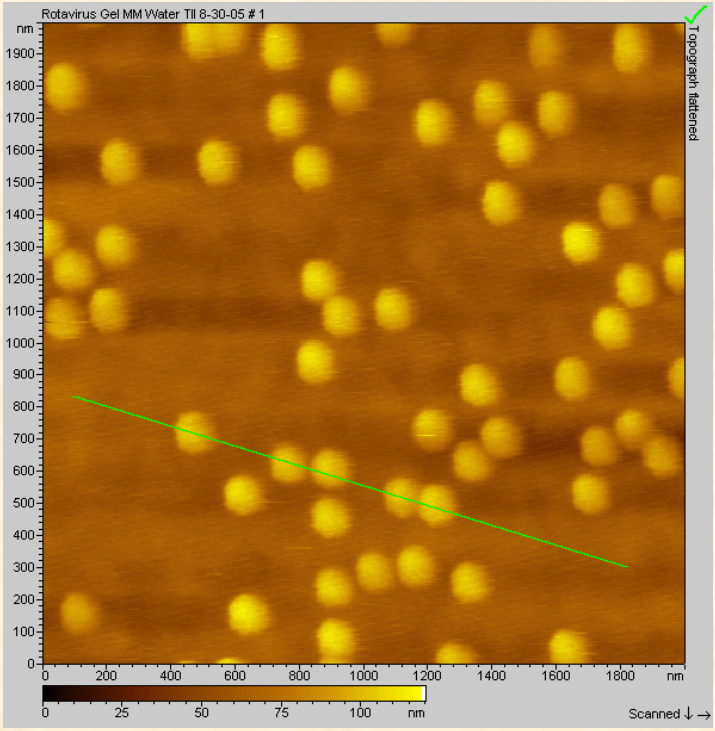
Imaging in solution

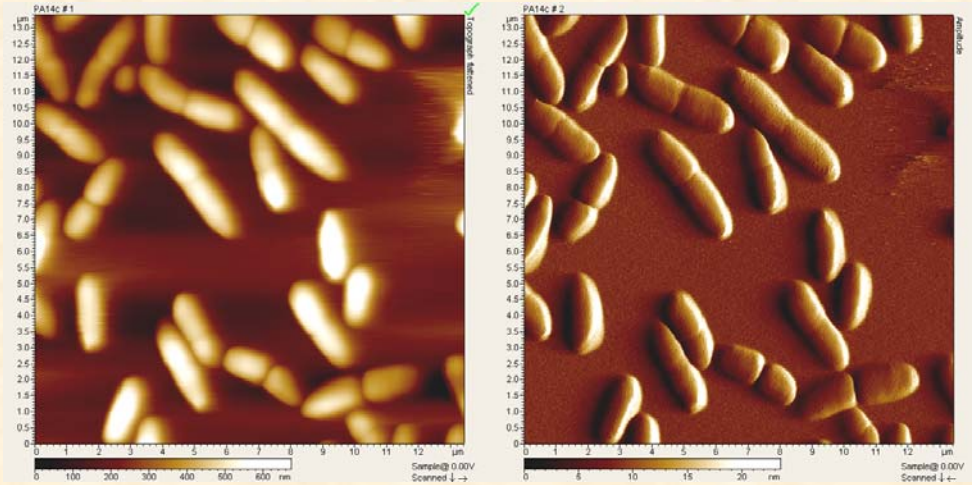
***Rhodopseudomonas palustris* (diverse energy metabolizer)**



Rotavirus particles



Pseudomonas aeruginosa



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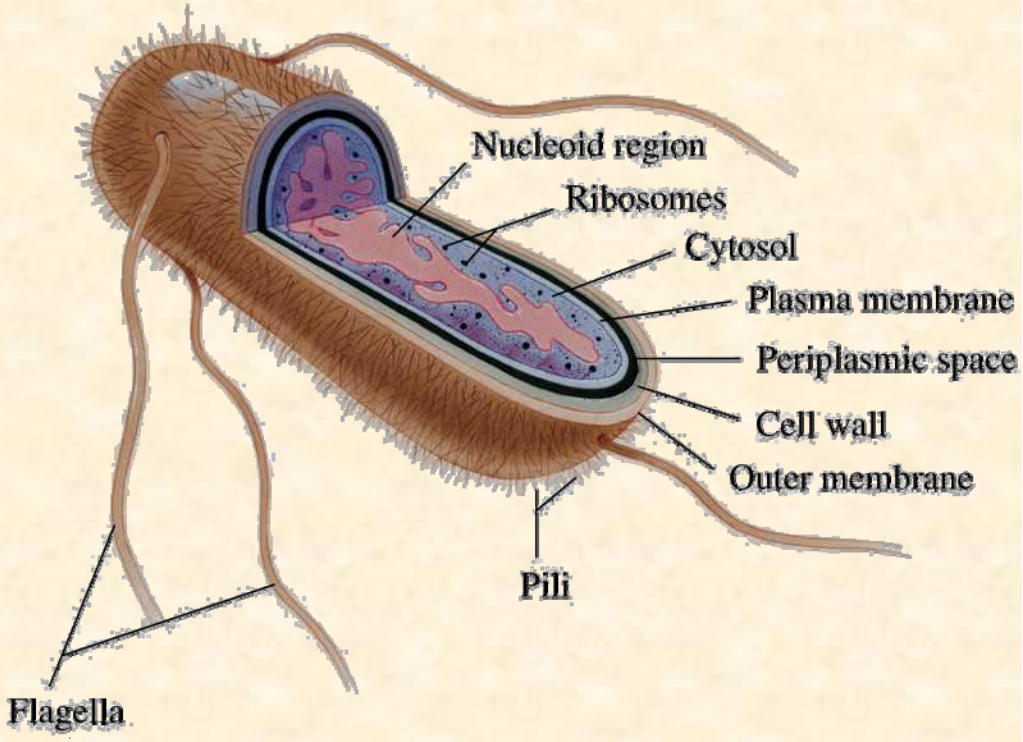
Locating membrane bound proteins

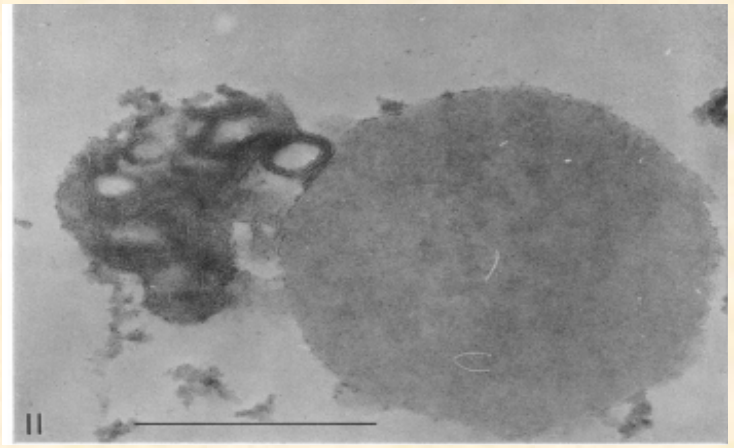
- Selective transport occurs via transport proteins located on the cytoplasmic membrane
- Requires removal of the outer membrane and peptidoglycan
  - The peptidoglycan gives the cell its shape and rigidity
  - Immobilization procedures need to change
- Need to verify that the cells are alive

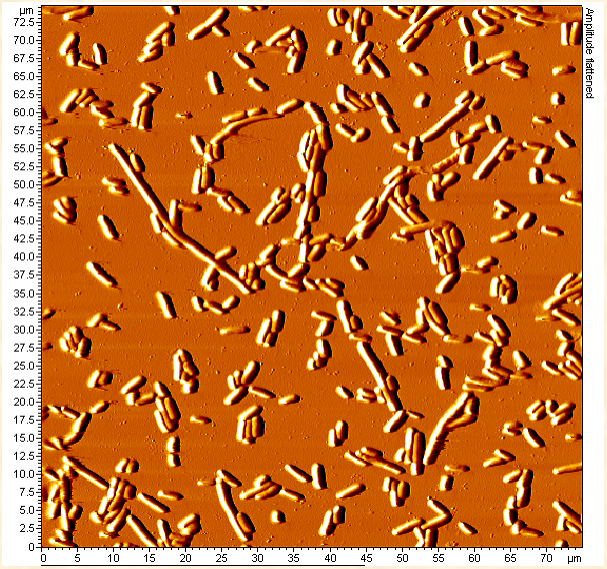


From: Principles of Biochemistry, Horton, Moran, Ochs, Rawn, Scrimegour Prentice Hall

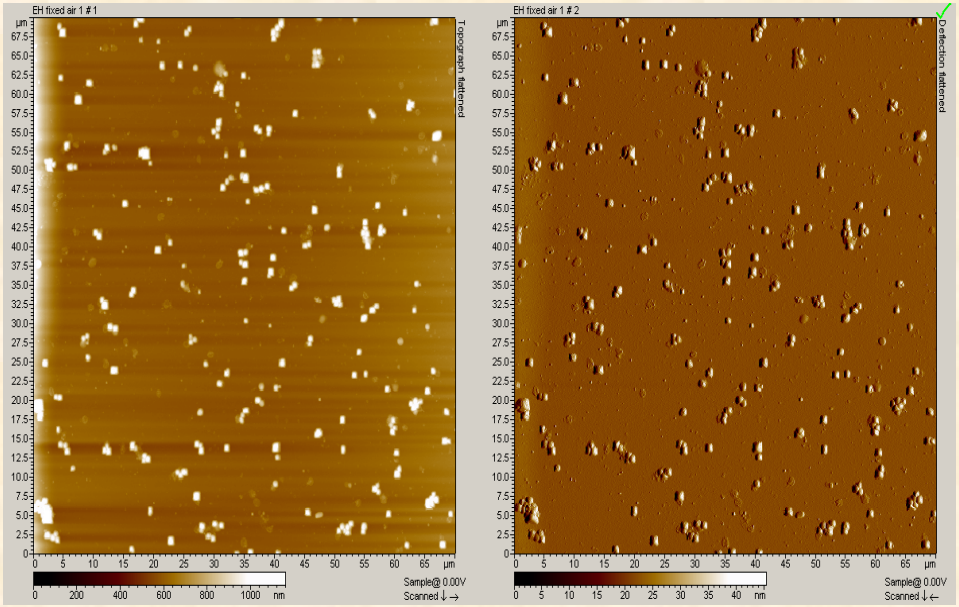
Spheroplasting

Spheroplasting involves removal of the cell wall through treatment with lysozyme and EDTA





Intact *E. coli*



Spheroplast

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Spheroplasting procedure

Sucrose solution

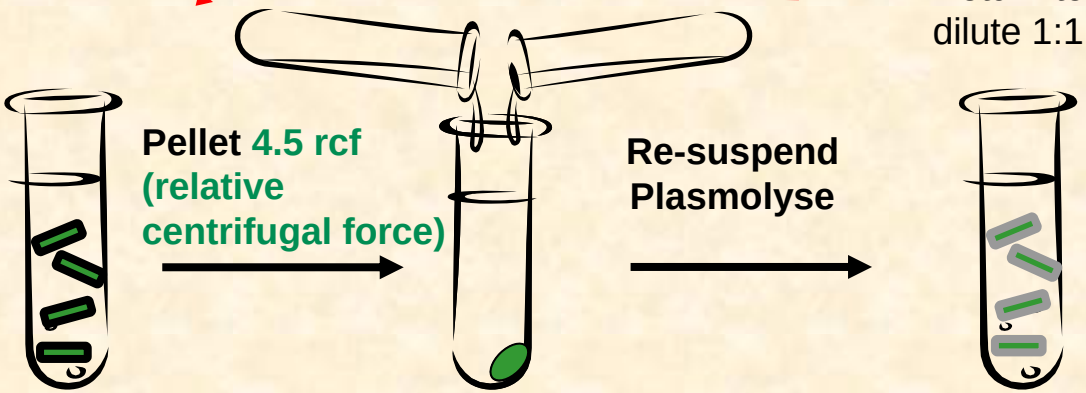
Step (1) Add 500 µl (TB)/ 0.5M sucrose(TBS1) and place on incubator shaker 20 min 37 ^{0}C

Lysozyme solution

Step (2) after 20 min on incubator shaker add lysozyme to cell suspension final concentration 50µg/ml. Return to shaker 20 min, dilute 1:1 with TB.

Begin with

1 ml overnight LB *E. coli* pelleted, suspend in 1ml Tris-HCl pH 8.0 (TB)



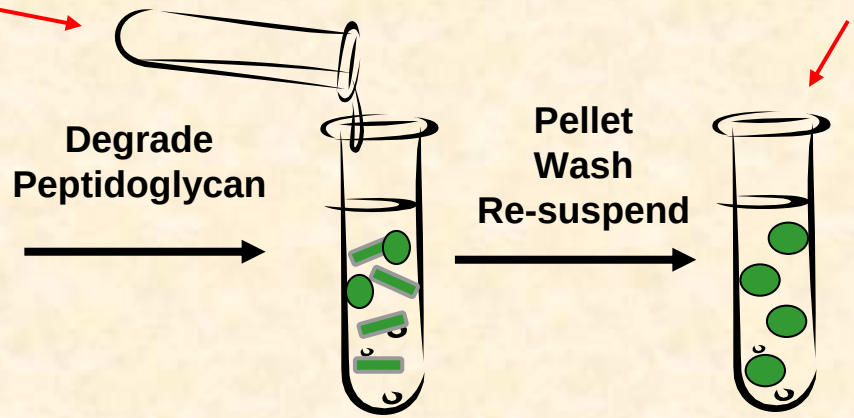
Pellet 4.5 rcf (relative centrifugal force)

Re-suspend Plasmolyse

EDTA

Step (3) EDTA was added to final conc. of 10mM and sample returned to shaker 20-30 min.

Light microscopy used to monitor spheroplast formation



Degrade Peptidoglycan

Pellet Wash Re-suspend

Step (4) After 90 % of rod shaped bacteria are spherical, centrifuge 25 min 0.5 rcp, remove supernatant with pipette, suspend pellet In 1 ml 0.25M sucrose/0.01M Tris-Hcl/10mM $MgSO_4$(TBS2). repeat centrifugation step and suspend pellet in200 µl of TBS2.

Birdsell, D. C., and Cota-Robles, E. H. (1967)
***Journal of Bacteriology* 93(1), 427-437**

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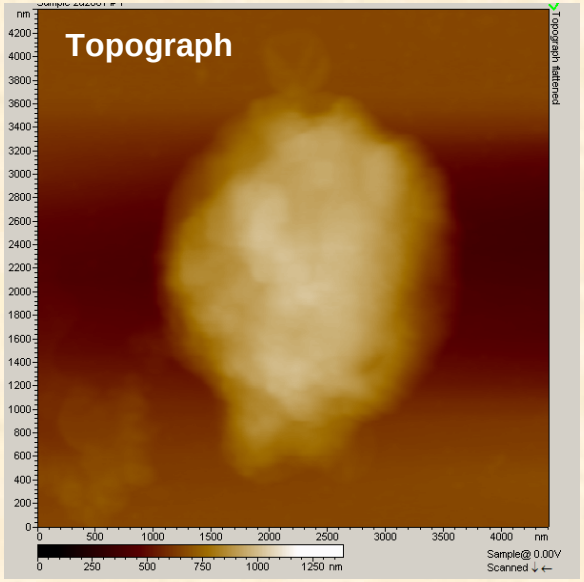
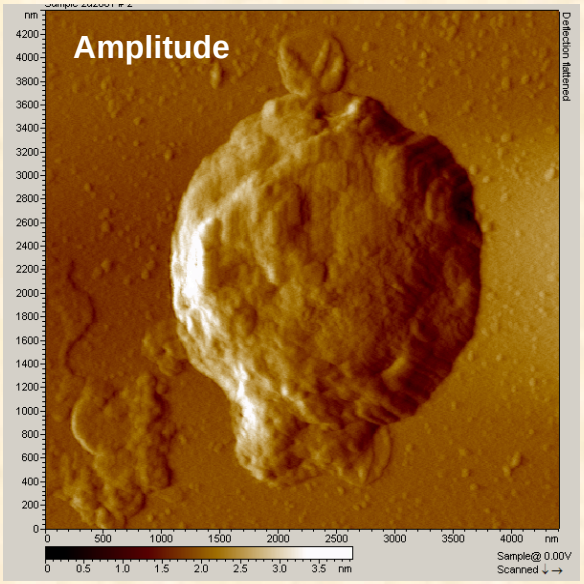
Immobilizing Spheroplasts

- Pretreat mica with aminopropyltriethoxysilane and glutaraldehyde (APTES/glut)
  - Previously shown to successfully immobilize chromatin on mica (Wang, H.D., et al., Biophysical Journal, 2002. 83(6): 3619-3625)
- Incubate the spheroplast suspension on the treated mica
  - Immobilization results from interactions between the proteins and the substrate
  - Conceivably, only the surface in contact with the substrate is affected, leaving the exposed surface in its native state and accessible to the tip
- Rinse and image in sucrose buffer





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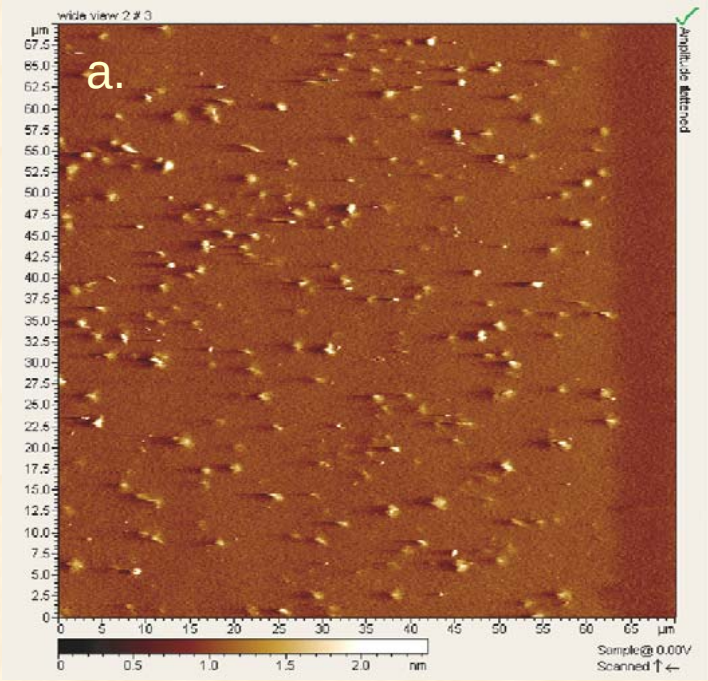
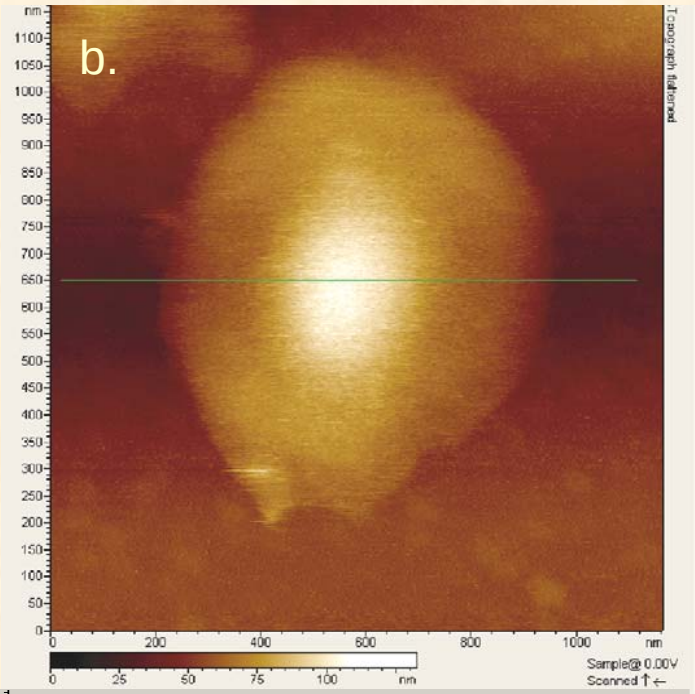
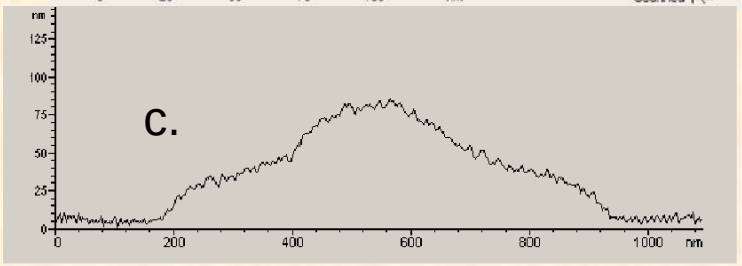
Spheroplasts of *E. coli* Immobilized on APTES/Glut-Treated Mica







- **(a,b) MAC Mode® images generated with a silicon nitride cantilever (0.1 nN/nm). Immobilization is robust.**
- **(c) Cross section of (b) shows lateral diameter ~1µm and height of only ~100nm.**

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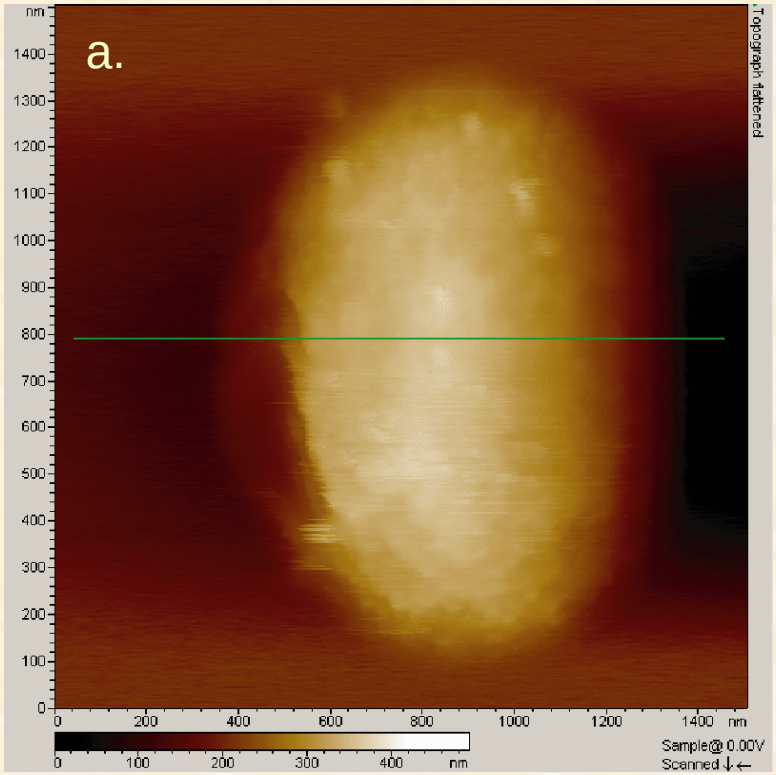
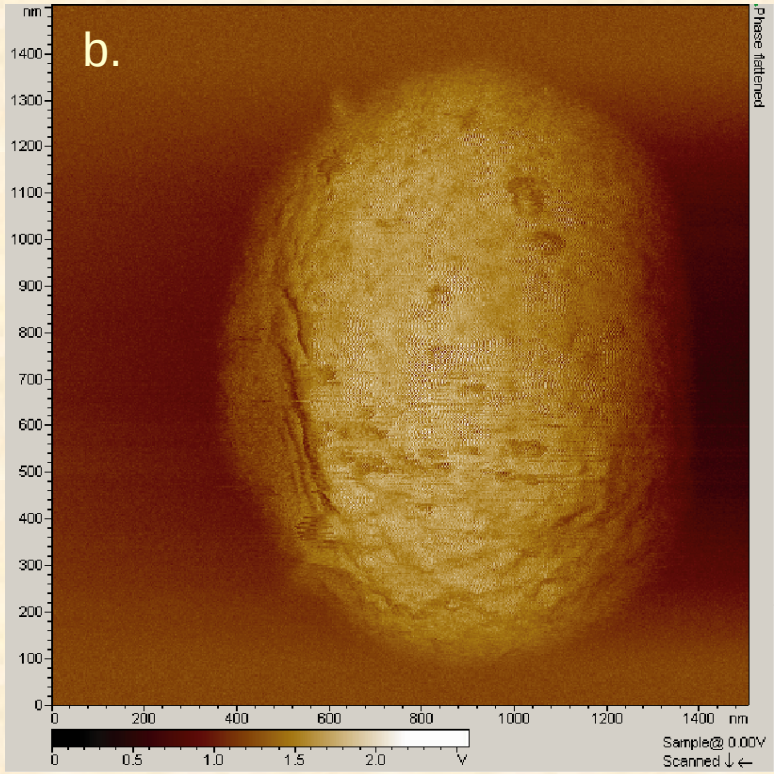
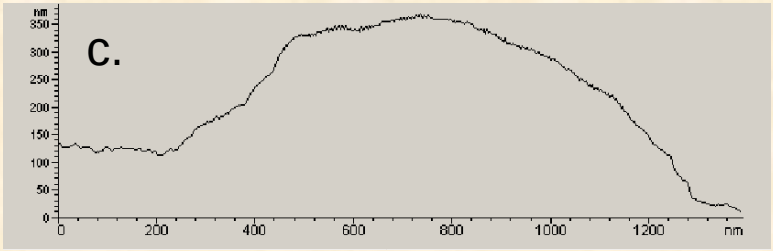
Glutaraldehyde-Fixed Spheroplasts







- After immobilization, spheroplasts were fixed with 0.5% glutaraldehyde
- (a,b) MAC Mode® image generated with a silicon nitride cantilever (0.1 nN/nm)
- (c) Cross section of (a) shows lateral diameter ~1µm and height of ~250nm

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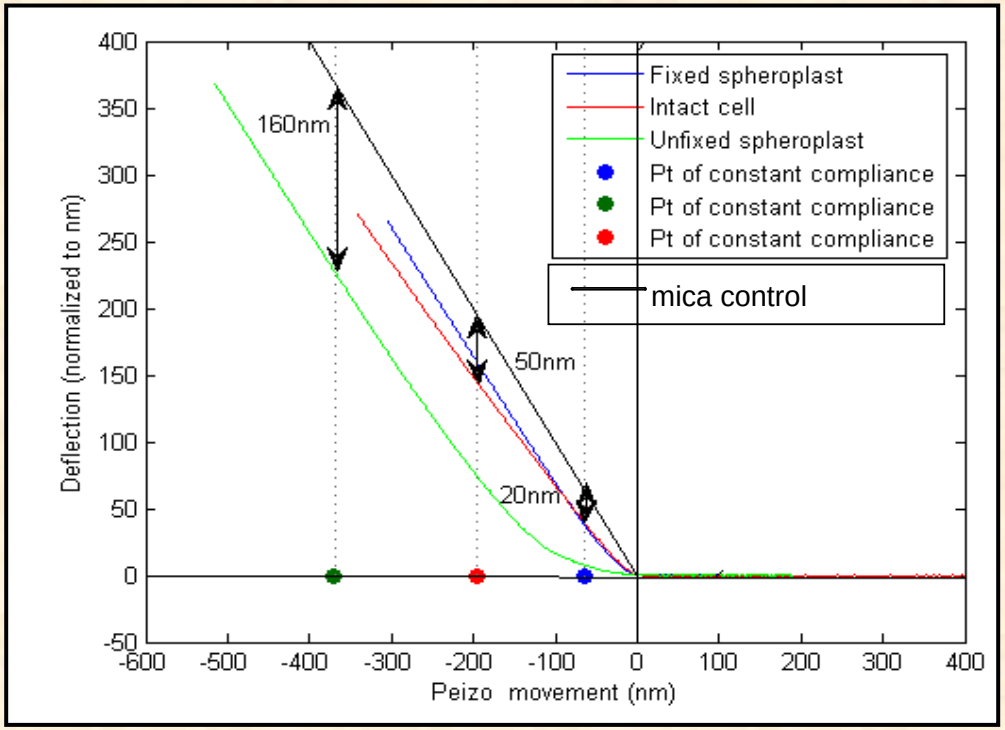
Indentation and Elasticity

- **Spheroplasts are much softer than the cantilever**
- **Crosslinked spheroplasts are stiffer and indent less (more practical for recognition imaging)**



Sample	Cantilever Spring Constant (k_c)	Slope	Indentation	Spring Constant of Bacteria (k_b)
Intact	0.0343 nN/nm (+/- 0.002)	0.85 (+/- 0.02)	50nm (+/- 11.5)	0.194nN/nm
Untreated Spheroplasts	0.0519 nN/nm (+/- 0.016)	0.97 (+/- 0.05)	160nm (+/- 21)	---
Fixed Spheroplasts	0.0364 nN/nm (+/- 0.003)	0.94 (+/- 0.06)	20nm (+/- 5)	0.571nN/nm

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Summary

- Due to forces exerted by the AFM tip imaging samples with scanning probe microscopes require immobilization techniques
- Gelatin coated mica surfaces can be effectively used to immobilize both gram positive and gram negative bacteria for AFM imaging
- Immobilization of spheroplasts cannot be accomplished with gelatin coated mica surfaces
- Spheroplast immobilization can be accomplished by a technique using a mica surface treated with APTES and glutaraldehyde

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