Counting and imaging bacteria using fluorescent microscopy & Electron Microscopy and Atomic Force Microscopy (AFM)

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Viewing bacteria using a microscope

- Bacteria ~1 um in size
- Invisible using brightfield microscopy
- Use phase-contrast to see bacteria (wet mount)
- Staining bacteria can help differentiate them (gram stain) based on cell structure
Fluorescent staining

- Fluorescence increases light sensitivity
- Can stain cells for specific materials
- General stains: Acridine orange, DAPI
- Viability/Respiration: CTC
- FISH- fluorescent in-situ hybridization (allows staining of specific types of bacteria)
Phase contrast image (isolate PDX)
Natural assemblage of bacteria- AO stain
Natural assemblage of bacteria-AO stain
Natural assemblage of bacteria- AO stain
Water from Lake Constance (Germany): DAPI
Soil bacteria: SYBR Green II stain

Fluorescent redox probe (CTC) for actively respiring bacteria (*P. putida*)

Viewing particles in seawater on filters using cytoclear slides
Closeup of *Chaetoceros* (brightfield image, AO, AB)
Closeup of *Chaetoceros*
(blue light, AO, AB)
Viruses in Seawater
(stained with Yo-Pro-1, a cyanine-based nucleic stain)

From: Hennes and Suttle, 1995, Limnol. Oceanogr. 40, 1050
Material specific stains

- Other stains can be used to view materials in cells
- Alcian blue (AB) stains only negatively charged polysaccharides
- Used to identify material responsible for large particle aggregation in the ocean (TEP- transparent exopolymer particles)
Alcian Blue stained phytoplankton culture
Alcian Blue stained phytoplankton culture- phase contrast
Using fluorescent in-situ hybridization (FISH) with 16s rRNA-targed oligonucleotide probes
FISH Analysis of Nitrifying Biofilms

Nitrosomonas (ammonia oxidizing)  Nitrospira (nitrite oxidizing)

From: Okabe et al. 1999, Appl. Environ. Microbiol. 65, 3182
FISH Analysis of Toluene-degrading Biofilms

*Acinetobacter* sp  *Pseudomonas putida*

Electron Microscopy

- Scanning Electron Microscopy (SEM)
- Transmission Electron Microscopy (TEM)
- Environmental SEM (ESEM)
SEM Images

*Burkholderia cepacia G4*  
*Pseudomonas fluorescens P17*
TEM Images

*Pseudomonas fluorescens* P17
ESEM Images
Atomic Force Microscopy (AFM)
Imaging with the Bioscope Atomic Force Microscope

Bacteria are attached to glass slides and once attached, AFM experiments can be performed.

- Generate 3-D images of surfaces (topographic imaging)
- Provide information about surface properties such as adhesion properties and chemical composition (phase imaging)
Configuration of the AFM

Sensor to measure cantilever position

Laser

Cantilever with silicon nitride tip

Adapted from image on Digital Instruments' web page
AFM imaging: use a silicon nitride tip mounted on a cantilever

100 $\mu$m = width of human hair!

400 nm

Made of silicon nitride

Spring constant of cantilever $\sim$ 0.1 N/m

Radius of tip = 5 – 50 nm
BIOSCOPE:
Atomic Force Microscope (AFM) is integrated with an inverted microscope.
AFM Head on microscope stage
AFM Cantilever & Tip
The Atomic Force Microscope (AFM) can be used to provide data on:

- surface topography
- surface heterogeneity
- adhesion forces between tip and surface

Data is obtained in different ways, that include:

- Contact mode
- Tapping mode
- Phase (in tapping mode)
- Approach/Retraction curves

Samples can be imaged in water or in air
The **topography** of a surface is measured by monitoring the deflection of the tip (using a laser) as it is pulled across a surface.
AFM-Tapping Mode

The **topography** of a surface is also measured but the tip oscillates during scanning.
Height image

Height signal: $\Delta h(x)$

Deflection image

Deflection signal: $\Delta d(x)$
...$\Delta h_{\text{piezo}}$ decreases
“Height” images not as clear as “Deflection” images
“Residuals” on Surfaces

AFM images of bacteria in air often show some sort of “material” adjacent to cells.
Bacteria imaged with AFM show a “residual”

The side of the AFM tip makes contact with cell giving the appearance of a “Shadow”
Bacteria imaged in air do not have show artifacts (they have less height)

Water drops

Dried bacterium

No residuals when dr
AFM studies of cell morphology

Chemicals can be used to alter cell adhesion properties, but their effects on bacterial morphology are not well known.

Objective:

Use the AFM to probe morphological changes in response to chemical treatments.
Sodium Pyrophosphate

Low IS water

MOPS Buffer
(Control)

Topographic Images of Pseudomonas stutzeri KC

Lysozyme and EDTA

Disodium Tetraborate
Tapping Mode

Free Amplitude

Fluid Layer

Tapping

Amplitude Reduced
Tapping Mode Phase Imaging
AFM Images (in air): Burkholderia cepacia G4 exposed to Tween 20

Tapping mode image

Phase image
Tapping Mode Phase Imaging *Pseudomonas stutzeri* KC

Disodium Tetraborate

Tween 20
Bacterial interaction forces

Objectives:

• Use the AFM to measure forces between bacteria and surfaces.
What is the interaction force between a bacterium and a surface?
A. Glass bead on a tipless cantilever

B. Glass bead in front of the pyramid shape tip

C. Glass bead behind the pyramid shape tip

D. Too much glue on the bead (done intentionally)
Approach

Distance from surface

AFM - Force Measurement

Force (mN)

Attractive Force

Distance from surface
Approach

AFM- Force Measurement

Distance from surface

Force (mN)

Repulsive Force
Anatomy of a deflection curve
Anatomy of a deflection curve
Anatomy of a deflection curve
Anatomy of a deflection curve
Anatomy of a deflection curve
EXAMPLE: Show that force curves must be done on the top of the bacterium.
First, Zoom in on a single bacterium
Now you are ready for deflection curve analysis
Deflection curve on *E. coli* D21f2
Deflection curve on *E. coli D21f2*
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Deflection curve on *E. coli* D21f2
Deflection curve on *E. coli* D21f2
Deflection curve analysis

Must be on the very top of a bacterium to obtain a good force curve
Understanding Force Curves

Force, nN = $k_{\text{cantilever}} \Delta d_{\text{cantilever}}$

Challenge: Where is zero distance?
AFM Force Measurements
(Non-interacting Sample and Tip, “Hard” Sample)

Deflection of Cantilever (nm)

spring constant, $k = \text{N/m}$

Tip-to-Sample Distance (nm)

constant compliance region
AFM Force Measurements
(Non-interacting Sample and Tip, “Soft” Sample)

Deflection of Cantilever (nm)

spring constant, \( k = \text{N/m} \)

constant compliance region?

Tip-to-Sample Distance (nm)
Approach Curves

KT2442 in 1 mM MOPS Buffer

- pH=2.2
- pH=4.75
- pH=7.00
- pH=8.67

Tip-to-Sample Distance (nm)

Force (nN)
Surface roughness is important
AFM vs Electron Microscopy

- AFM does not require the use of formaldehyde or other fixative chemicals
- AFM does not require ultrahigh vacuum, or even any vacuum
- Morphology more clearly observed using AFM
- TEM is best for observing flagella
- In ESEM, samples need not be dried, but we found it very difficult to observe bacteria