Best practices in Imaging Biological samples

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migrating c2c12 stem cell
Sample preparation guideline

• Careful consideration of sample preparations is the ‘key’ to achieve high quality, reliable AFM results

• Factors to consider:
  o Imaging conditions (air vs. fluid)
    ➢ Sample concentration
    ➢ Buffering conditions, concentration
    ➢ Appropriate pH, salinity, temperature etc
  o Imaging modes (contact, tapping, force curve, peakforce tapping)
  o Substrate
    ➢ Flat and easy to modify
    ➢ Compatible with sample
  o AFM probes
    ➢ Imaging mode dependent
    ➢ Imaging or force curve measurement, spring constant and coating
    ➢ Sharpened/blunt/particle attached/tipless
Outline

- Substrate selection and modification
- Cantilever and tip selection and modification
- Biomolecule preparation – DNA, Protein, Lipids
- Tip functionalization for molecular recognition
- Cells preparation – Bacteria, Mammalian cells
Commonly used substrate

- **Requirements:**
  - Atomically flat surface (exception: cell imaging)
  - Appropriate surface chemistry (hydrophilic, hydrophobic, charged)

- **Substrates:**
  - mica (negatively-charged) – DNA, proteins
  - AP-Mica or APS-Mica (positively-charged): more rough surface – DNA, proteins
  - Graphite: hydrophobic – proteins
  - Gold – pulling experiments
  - Glass coverslips/slides – cells
  - Polystyrene petri dishes – cells
  - Glass-bottom petri dishes – cells (AFM + light microscopy)
## Probe selection

www.brukerafmprobes.com

<table>
<thead>
<tr>
<th>Probe Model</th>
<th>Force Constant (N/m)</th>
<th>Resonant Frequency (kHz)</th>
<th>Radius of Curvature (nm)</th>
<th>Probe Attributes</th>
<th>AFM Mode</th>
<th>Life Sciences Sample Type</th>
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<tr>
<td></td>
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<td>Reflective Coating Tip Side Coating Catalog Page</td>
<td>Fast Coating ScanAsyst Tapping Contact Force Curves Electrical Magnetic</td>
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Sample preparation: DNA

- **DNA concentration:** 1-10μg/ml

- **Substrate:**
  - Mica (negative charges)
  - AP-Mica (positive charges)

- **Imaging in air:**
  - Remove buffer salt with rinsing in DI water
  - Tapping in air using TESP series probe
  - Peakforce tapping in air using Scanasyst air probe

- **Imaging in fluid:**
  - Buffer containing divalent cations (50mM HEPES (Tris), 10mM NiCl₂ or MgCl₂)
  - Tapping in fluid using DNP, MSCT, SNL probes, driving frequency at 8-12kHz
  - Peakforce tapping in fluid using ScanAsyst-Fluid+ probes
Example of DNA imaging

• Lambda digest DNA on mica: 500nm x 500nm x 2nm

• Top Left: 0.5 fps
  – 92 Hz; 256 x 192 pixels
  – shown at 20x

• Top Right: 1.0 fps
  – 92 Hz; 128 x 92 pixels
  – shown at 10x

• Bottom Left: 2.0 fps
  – 148 Hz; 160 x 74 pixels
  – shown at 5x

• Bottom Right: 3.0 fps
  – 184 Hz; 128 x 62 pixels
  – shown at 5x
Sample preparation: Protein

- **Protein concentration:** 1-100ug/ml
- **Substrate:** Mica (Hydrophilic, negative charges)  
  Graphite (HOPG, hydrophobic)
- **Incubation:** 15min to 24hours
- **Imaging in air:**
  - Remove buffer salt with rinsing in DI water
  - Tapping in air using TESP probe
  - Peakforce tapping in air using ScanAsyst air probe
- **Imaging in fluid:**
  - Buffer pH<pL (mica substrate)
  - Tapping in fluid using DNP, MSCT, SNL probes, driving frequency at 8-12kHz
  - Peakforce tapping in fluid using ScanAsyst-Fluid+ probes
Sample preparation: Protein membrane (cell membrane)
Modulus mapping across individual bR monomers revealed:

- Areas of high stiffness (low flexibility) = \( \alpha \)-helices.
- Areas of low stiffness (high flexibility) = intrahelical loops.
- E-F loops are most rigid structures at low forces, collapse at \( F \sim 125\text{pN} \)

Sample preparation: Lipid bilayer

- **Two methods of lipid bilayer formation:**
  - Langmuir – blogget deposition
  - In situ fusion (pre-formed liposome)

- **Liposome concentration:** 1mg/ml

- **Substrate:** Mica (Hydrophilic, negative charges)

- **Incubation:**
  - 30min to several hours
  - Incubation with CaCl₂
  - heating above Tₘ

- **Imaging in fluid:**
  - Tapping in fluid using DNP, MSCT, SNL probes, driving frequency at 8-12kHz
  - Peakforce tapping in fluid using ScanAsyst-Fluid+ probes
• Molecular mechanism of lipid bilayer formation.
  • Liposomes adsorb onto mica surface.
  • Increased curvature causes liposomes to rupture.
  • Liposomes fuse to form lipid islands.
  • Lipid islands grow to form continuous lipid bilayer.

Images obtained at 6 sec/frame with 256 x 160 pixels. Height images shown.
FastScan Bio AFM
Dynamic Studies of Model Membrane Systems

(1:1) DOPC/DPPC Lipid Bilayers
Left: 0.16fps, 512 x 256 pixels.
Right: 0.5 fps, 256 x 88 pixels.
Schematics for applications with tip functionalization
Tip functionalization protocol

The first step of tip functionalization is generally to introduce amine groups to the tip surface.

Fig. 12. Formation of amine-terminated surfaces: (a) by silanization with 3-aminopropyltriethoxysilane (APTES); (b) by etherification with ethanolamine; (c) by hydrosilylation of protected-amino-alkene; (d) by chemisorption of amino-alkanethiols on gold-coated surfaces.
Tip functionalization protocol

Fig. 9. Modification of AFM tips by a three step coupling procedure using biotin-avidin binding properties.
Tip functionalization protocol

Common binding chemistries

- OH + HOOC- Asp or HOOC- Glu → -O-CO-
- COOH + OH- Ser or OH- Thr → -CO-O-
- COOH + SH- Cys → -CO-S-
- COOH + H₂N- Lys → -CO-NH-
- NH₂ + HOOC- Asp or HOOC- Glu → -NH-CO-
- SH + HOOC- Asp or HOOC- Glu → -S-CO-
Malaria-infected erythrocytes (IE’s) are misshapen with knob-like structures on surface.

IE’s exhibit cytoadherence via knob like protrusions.

Prevents IE elimination by the spleen and causes vascular blockages.
AFM probes were functionalized with endothelial surface receptor CD36.

Used PeakForce QNM with functionalized probe to obtain 2D map of the distribution of CD36 molecular binding sites on IE.
Three dimensional confocal images of functionalized AFM tips stained with antibody conjugated with fluorescent dyes.

(A) Freshly prepared AFM tip with TSP conjugated. (B) Unmodified AFM tip as a control. (C) Used but still functional tip. (D) Used and ‘dead’ tip.

Images were taken using Nikon N1 confocal microscope equipped with a 100X oil immersion objective. Scale bars represent 5 mm.
• CD36 binding sites (high adhesion) correlate to knob structures (circles).

• Adhesion is a result of specific interactions between CD36 and knobs and not due to crosstalk between data channels (arrow).
**Bacteria sample preparation**

- *E.Coli cells immobilized on glass slides*
- Cells deposited on PLL treated glass surface
- *Lactococcus lactis cells* immobilized in porous polymer membranes
- Cells trapped in porous polycarbonate filter
Cells exposed to 10 µg/mL CM15.

Increased surface roughness with incorporation into LPS-rich OM.

Andra et al. (2008) Biochem. J.

Onset of changes to individual cells occurred at different times.


Images obtained at 18 sec/frame with 1024 x 256 pixels. Phase images shown.
Ordered structures on the surface of the E. coli outer membrane.

Previous studies on excised outer membrane patches showed that structures were closely packed porin molecules.


Images obtained at 34 sec/frame with 1024 x 1024 pixels.

FastScan Bio AFM of Membrane Structures
Live Cell High Resolution Imaging of Outer Membrane

High-Res topography image of E. coli OM.

2D FFT filtering reveals areas of ordered structures.
Cells exposed to 20 µg/mL CM15.

Changes observed after ~96sec.
  • Membrane rippling/corrugation.
  • Disruption of ordered structures.
  • Membrane protrusions.
  • 'Pore-like' lesions (~15nm).

Elapsed time = ~224 sec.
Cell imaging sample preparation
-- fixed cell preparation and imaging

- Sample: mice skin fibroblast cells
- Commonly used fixatives:
  - Glutaraldehyde
  - Formaldehyde/Paraformaldehyde
  - Formaldehyde and Glutaraldehyde mixtures
  - Osmium tetroxide
  - Methanol
  - Acetone
  - Formal saline
- Imaged in air: tapping mode or peakforce tapping mode
- Imaging in liquid: tapping mode or peakforce tapping mode
Sample preparation: live cells

- **Cell concentration:**
  - 50-80% confluency, if necessary, starving the cells with low serum (2%) to get flatter morphology

- **Substrate:**
  - Plastic/glass bottom petridish
  - Glass coverslip/glass slides
  - Adhesives: Poly-L-lysine, Fibril nectin, Collagen, Cell-tak

- **Imaging in fluid:**
  - Buffer or culture media solution
  - Contact mode in fluid using DNP, MLCT or Biolever probes
  - Tapping mode in fluid using DNP, MLCT probes
  - Peakforce tapping in fluid using ScanAsyst-Fluid probes
PF-QNM of Mammalian Cells

The Role of Mechanical Forces in Disease

BioScope Catalyst operated in fluid using ScanAsyst-Fluid probes.


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- PF-QNM allowed researchers to monitor the effects of glyphosate on living human epidermal cells.

- Changes in cell stiffness were directly correlated to the appearance of cellular substructures.
AFM combined with optical microscopy on live cells

Overlay of fluorescence and QNM images of living HUVEC cells

a: overlay of fluorescence (nucleus and actin) and AFM topography images.
b: Topography channel
c: Modulus channel: 0-4 MPa
d: Deformation channel: 0-250 nm

Optically labeled objects can be traced in real time with mechanical properties changes.
Dynamic study of stem cells migration

Mouse embryonic stem cells. Obtained over 12hrs with a 40x phase contrast objective. © The Exploratorium, www.exploratorium.edu

Obtained over ~1hr at 40sec/frame at 512 x 128 pixel resolution. Phase images shown. (~10 μm scan size)

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Summary
Advancing AFM in Biological Research

- AFM provides unique insights into biomolecules, cells and tissues.
  - Structure
  - Mechanical Properties
  - Dynamic Processes
- New advances in Bruker’s AFM Technology provides new avenues of research in life science.
  - PeakForce Tapping™ & ScanAsyst™
  - PeakForce QNM™
  - BioScope™ Catalyst with MIRO™
  - Dimension FastScan Bio™ AFM

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