

# High-speed Atomic Force Microscopy Observation of Internal Structure Movements in Living *Mycoplasma*

Kohei Kobayashi<sup>1, #</sup>, Noriyuki Kodera<sup>2, #</sup> and Makoto Miyata<sup>1, 3, \*</sup>

<sup>1</sup>Graduate School of Science, Osaka City University, 3-3-138 Sugimoto, Sumiyoshi-ku, Osaka 558-8585, Japan

<sup>2</sup>Nano Life Science Institute (WPI-NanoLSI), Kanazawa University, Kakuma-machi, Kanazawa, Ishikawa 920-1192, Japan

<sup>3</sup>The OCU Advanced Research Institute for Natural Science and Technology (OCARINA), Osaka City University, 3-3-138 Sugimoto, Sumiyoshi-ku, Osaka 558-8585, Japan

\*For correspondence: [miyata@osaka-cu.ac.jp](mailto:miyata@osaka-cu.ac.jp)

#Contributed equally to this work

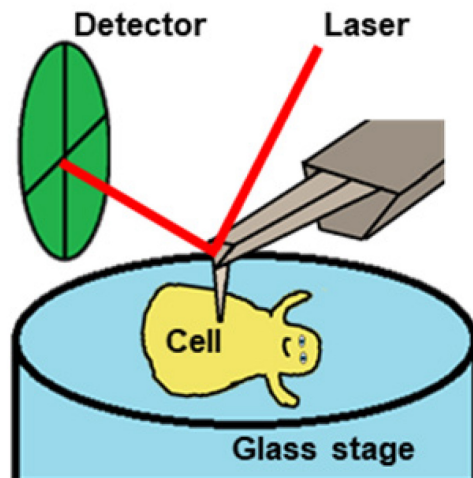
## Abstract

Dozens of *Mycoplasma* species belonging to the class *Mollicutes* bind to solid surfaces through the organelle formed at a cell pole and glide in its direction by a unique mechanism. In *Mycoplasma mobile*, the fastest gliding species in *Mycoplasma*, the force for gliding is generated by ATP hydrolysis on an internal structure. However, the spatial and temporal behaviors of the internal structures in living cells were unclear. High-speed atomic force microscopy (HS-AFM) is a powerful method to monitor the dynamic behaviors of biomolecules and cells that can be captured while maintaining their active state in aqueous solution. In this protocol, we describe a method to detect their movements using HS-AFM. This protocol should be useful for the studies of many kinds of microorganisms.

**Keywords:** AFM, Probing, Pathogenic bacteria, ATPase, Class *Mollicutes*, Internal structure

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## Graphic abstract:



Scanning *Mycoplasma* cell

## Background

Class *Mollicutes*, a small group of bacteria featuring a single-layered cell membrane, has as many as three unique motility mechanisms different from other organisms. Recently, the isolated internal structure of *M. mobile* was shown to hydrolyze ATP with conformational changes, suggesting that the internal structure functions as a force generator for gliding (Miyata and Hamaguchi, 2016; Nishikawa *et al.*, 2019). However, the spatial and temporal behaviors and movements of internal motors in living cells have not been examined.

High-speed atomic force microscopy (HS-AFM) is a powerful method to monitor the dynamic behaviors of biomolecules and cells that can be captured while maintaining their active state in aqueous solution (Ando, 2018). In recent years, this approach has been dramatically improved, and the behaviors of more and more proteins have been elucidated (Kodera *et al.*, 2010 and 2021; Ando, 2018; Kodera and Ando, 2020). In addition, HS-AFM has been applied to understanding the structures in the cell wall surface (Yamashita *et al.*, 2012) and below the cell membrane (Zhang *et al.*, 2017).

Here, we provide a protocol detailing how to detect the movements of the internal structure in a living *M. mobile* cell, based on our recent study (Kobayashi *et al.*, 2021).

## Materials and Reagents

1. 1.5 mL microtube (Greiner Bio-One, catalog number: 616201)
2. Glass substrate stage for HS-AFM (custom-made glass rod with 2 mm in diameter and 2 mm in height, Japan Cell)
3. Cantilever (Olympus, BLAC10DS-A2)
4. #3000 sand paper (Riken Corundum, Precision Polishing Film Sheet #3000, catalog number: 3-9013-06)
5. 100 mL beaker
6. Kimwipes (Nippon Paper Crecia, catalog number: 62015)
7. Filter paper for liquid replacement on glass stage (ADVANTEC, catalog number: 01531090) (Cut into triangles as shown in Figure 2)
8. 25 cm<sup>2</sup> tissue culture flask (AS ONE, catalog number: 2-8589-01)
9. Micro pipette tip (Greiner Bio-One, catalog numbers: 740290 and 739285)

10. Acetone (FUJIFILM Wako Pure Chemical Corporation, catalog number: 012-00343)
11. Potassium hydroxide (KOH) (Nacalai Tesque, catalog number: 28616-45)
12. Ethanol (FUJIFILM Wako Pure Chemical Corporation, catalog number: 057-00456)
13. Fluorosurf (FluoroTechnology, catalog number: FG-3020C-20)
14. Colorless nail polish (Shiseido, MAQuillage (Top base coat))
15. Deionized water
16. 3-aminopropyltriethoxymethylsilane (APTES) (Shin-Etsu Chemical, catalog number: KBE-903)
17. Glutaraldehyde (Sigma-Aldrich, catalog number: G5882)
18. Heart infusion broth (BD, catalog number: 238400)
19. Yeast extract (BD, catalog number: 212750)
20. 10 N NaOH (Nacalai Tesque, catalog number: 31511-05)
21. Horse serum (Thermo Fisher Scientific, Gibco™, catalog number: 16050122)
22. Amphotericin B (Sigma-Aldrich, catalog number: A2942)
23. Ampicillin Na (Nacalai Tesque, catalog number: 02739-32)
24. di-Sodium hydrogenphosphate (Nacalai Tesque, catalog number: 31801-05)
25. Sodium dihydrogenphosphate dihydrate (FUJIFILM Wako Pure Chemical Corporation, catalog number: 192-02815)
26. NaCl (Nacalai Tesque, catalog number: 31320-05)
27. Glucose (Nacalai Tesque, catalog number: 16806-25)
28. Phosphate-buffered saline containing glucose (PBS/G) (see Recipes)
29. Aluotto medium (see Recipes)

## Biological materials

1. A mutant strain (*gli521*[P476R]) isolated from *M. mobile* 163K strain (ATCC, catalog number: 43663) (Uenoyama and Miyata, 2005)

## Equipment

1. Micro pipette (Gilson, catalog numbers: F123600, F123601, and F123602)
2. Centrifuge (Sigma Laborzentrifugen, model: Sigma 1-14)
3. 25°C incubator (Tokyo Rikakikai, model: LTI-400E)
4. Heater (TAITEC CORPORATION, CTU-Neo)
5. Magnetic stirrer (AS ONE, model: CT-1AT)
6. HS-AFM (An apparatus with the same performance is commercially available from Research Institute of Biomolecule Metrology Co., Ltd., model: SS-NEX)
7. Autoclave (TOMY SEIKO, model: LSX-700)

## Procedure

### A. Cell preparation

1. Inoculate 1 mL of frozen *M. mobile* stock into 10 mL of Aluotto medium in a 25 cm<sup>2</sup> tissue culture flask and statically cultivate it at 25°C for a few days, to reach an optical density at 600 nm of 0.06–0.08.
2. Collect 2 mL of cultured cells by centrifugation at 12,000 × g and room temperature (RT) for 4 min, discard the supernatant, resuspend the cell pellet in 400 µL of PBS/G, and centrifuge it again at 12,000 × g and RT for 4 min (Note 1).
3. Repeat this process twice.
4. Resuspend the cells in 100 µL of PBS/G to 20-fold density of the original culture.

## B. Glass stage treatment

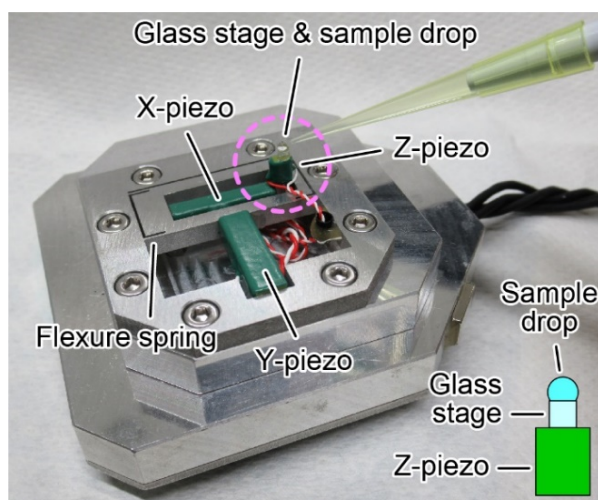
1. Lightly press the substrate surface against the #3000 sandpaper and scrub 200 times back and forth to scratch the surface (The rough surface provides a more stable observation. This step is optional). Note that this process should not be applied to the other side.
2. Put the glass stage into the 1.5 mL microtube filled with acetone and shake it to remove glass debris. (Optional: as mentioned above)
3. To prepare saturated KOH-ethanol solution, add 10 g of KOH into 50 mL of ethanol, stir using a magnetic stirrer for 5 min, and transfer the supernatant of the saturated KOH-ethanol solution into a new beaker.
4. Put several glass stages into the prepared saturated KOH-ethanol solution.
5. Leave for 15 min to hydrophilize the glass surface.
6. Discard the solution, add some deionized water, and shake the beaker gently.
7. Repeat this process ten times to remove the chemicals.
8. Move the glass stages onto Kimwipes and remove the water.
9. Keep the glass stages with some slant in a heater, for 20 min at 40–45°C to dry.

## C. Cell immobilization on the glass surface

1. Treat the sides of the glass stage with Fluorosurf for hydrophobization.

*Note: If not treated, the liquid placed on the substrate surface will spill over to the sides.*

2. Fix the glass stage onto the Z-piezo of the HS-AFM scanner with nail polish (Figure 1).



**Figure 1. HS-AFM scanner with glass stage and sample drop.**

Piezoelectric elements are used to drive each axis in the XYZ directions. Z piezo, glass stage, and sample solution are seen in a pink dashed circle, as illustrated in the lower right. The glass stage is fixed onto the Z-piezo using nail polish.

3. Just before use, dilute 1  $\mu$ L of APTES with 999  $\mu$ L of water.
4. For silanization on the glass stage surface, load 5  $\mu$ L of APTES solution onto the treated glass substrate immediately after the dilution and leave it for 10 min.
5. Rinse the substrate surface with 20  $\mu$ L of water dropwise. Repeat this four times (Figure 2).
6. Replace the water on the glass substrate with 0.1% glutaraldehyde solution and leave for 5 min.
7. Rinse the substrate surface with 20  $\mu$ L of water dropwise. Repeat this four times.

8. Replace the glutaraldehyde solution on the substrate with PBS/G.
9. Replace the PBS/G on the substrate to cell suspension and leave for 10 min at 25–28°C.
10. Rinse the substrate surface with 20  $\mu$ L of PBS/G dropwise. Repeat this four times.

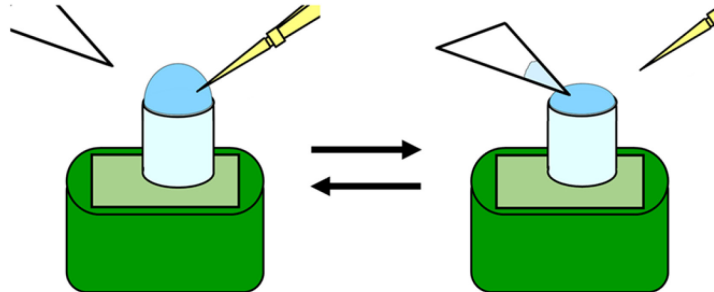


Figure 2. Procedure for liquid replacement on the glass stage.

## D. Observation by HS-AFM

1. Set the HS-AFM scanner equipped with the glass stage and sample solution upside down to the HS-AFM mechanical unit and start imaging (Figure 3).

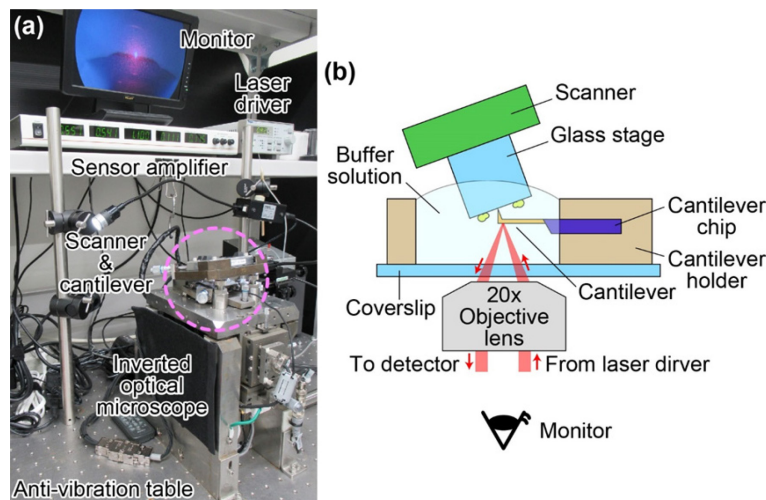


Figure 3. HS-AFM.

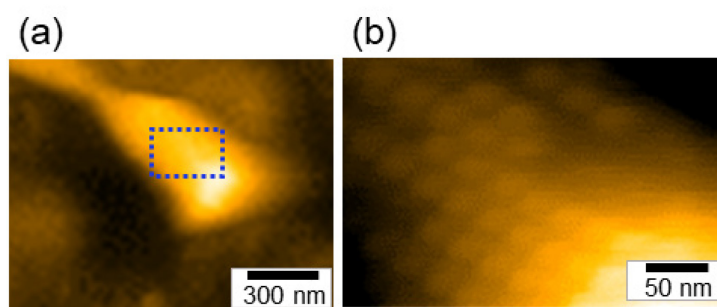
(a) Picture showing the mechanical parts and some of the electrical devices of HS-AFM. The AFM unit (scanner and cantilever) is mounted on an inverted optical microscope which is placed on an anti-vibration table. (b) Schematic of experimental setup. Cantilever and *M. mobile* cells are not in scale. The optical view around the cantilever and the glass substrate can be observed through the monitor.

2. Set the cantilever's free oscillation amplitude ( $A_0$ ) and set-point amplitude ( $A_{sp}$ ) at  $\sim 2.5$  nm and  $\sim 0.8 \times A_0$ , respectively. Under these conditions, the average tapping force  $\langle F \rangle$  can be approximated as  $\sim 40$  pN using the following equation:

$$\langle F \rangle = \frac{k_c}{2Q_c} \sqrt{A_0^2 - A_{sp}^2}$$

where  $k_c$  and  $Q_c$  are the spring constant and the quality factor of the cantilever, respectively. The nominal values of  $k_c$  and  $Q_c$  in liquid are  $\sim 0.08$  N/m and 1.5, respectively. The detail conditions were described previously (Uchihashi *et al.*, 2012).

3. For searching cells (Figure 4a), scan the sample at  $150 \times 150$  pixels with an imaging rate of 1,000 ms per frame in an area of  $3,000 \times 3,000$  nm<sup>2</sup>.  
(<https://journals.asm.org/doi/10.1128/mBio.00040-21#movS1>).
4. To observe particle structure like Figure 4b, scan the cell surface with an imaging rate of 330 or 200 ms per frame in an area of  $200 \times 200$  nm<sup>2</sup>, with  $100 \times 100$  pixels.  
(<https://journals.asm.org/doi/10.1128/mBio.00040-21#movS3>).
5. Other details are common with general observation of samples in solution using HS-AFM (Uchihashi *et al.*, 2012).



**Figure 4. Representative images taken by HS-AFM.**

(a) Whole cell image (b) Particle structure image [Magnified image of the boxed area of the panel (a)]. This figure was modified a previous paper (Kobayashi *et al.*, 2021).

## Data analysis

All procedures were published in our previous paper (Kobayashi *et al.*, 2021, (Kobayashi *et al.*, 2021, <https://journals.asm.org/doi/10.1128/mBio.00040-21>). Briefly, videos were processed by three steps (i) to (iii) and analyzed for particle movements. (i) The image contrast was improved by a bandpass filter. (ii) Image drifts were corrected by a plugin for ImageJ, “align slices in stack” (Tseng *et al.*, 2012). (iii) Image noises were reduced by averaging three consecutive slices. Then, each particle image was cropped, binarized, and traced for the mass center. All analyses were performed with ImageJ version 1.52A.

## Notes

1. Suspension of cell pellet  
More than 100 up-and-down movements with the pipette are recommended to separate cells completely.
2. Heat treatment of horse serum  
For *M. mobile* growth and gliding, the horse serum should be inactivated by heat treatment at 56°C for 30 min.

## Troubleshooting

1. Unable to obtain even a flat substrate image.  
Cells or other suspended particles may be sticking to the cantilever. To shake them off, keep the cantilever away from the substrate surface, set the cantilever's amplitude at free oscillation for 1 min, and try imaging again. If

this is not improved after several attempts, remove the scanner and wash the cantilever, using a pipetting water stream in the chamber.

2. No cells are found on the field.  
Small molecules, including amino groups, may be bound to the glass stage and block cell binding. Prepare the cell suspension and the glass stage again carefully. For the cell preparation, increase the number of pipetting times to remove small molecules. Note that too many washes may also reduce cell number. Check the cell conditions by optical density at 600 nm and an optical microscope, because old cultures tend to include particles other than cells.
3. Cells easily detach from the substrate during observation.  
The glass stage with a rough surface may be helpful [refer to Glass stage treatment option (Procedure B)].
4. The cell images look strange.  
The cells may have dried out. Replace the cell suspension and the glass stage. Place a wet tissue at the edge, to humidify the area around the chamber.
5. The particle structures are not found on the cells.  
This may be caused by damage on the cantilever tip. Replace the cantilever.

## Recipes

### 1. Aluotto medium

1% heart infusion broth  
0.56% yeast extract  
0.035% 10 N NaOH

*Note: Dissolve the above three reagents as a mixture, autoclave, cool to lower than 37°C, and add below three reagents in clean bench.*

10% horse serum (Note 2)  
0.025% amphotericin B  
0.005% ampicillin Na

### 2. Phosphate-buffered saline containing glucose (PBS/G)

75 mM sodium phosphate (pH 7.3)  
68 mM NaCl  
20mM Glucose  
Sterilize by using filtering or autoclaving

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This protocol was adapted from a previously published paper (Kobayashi *et al.*, 2021).

## Competing interests

There are no conflicts of interest or competing interests.

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