

Force Measurement on *Mycoplasma mobile* Gliding Using Optical Tweezers

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[Abstract] Dozens of *Mycoplasma* species, belonging to class *Mollicutes* form a protrusion at a pole as an organelle. They bind to solid surfaces through the organelle and glide in the direction by a unique mechanism including repeated cycles of bind, pull, and release with sialylated oligosaccharides on host animal cells. The mechanical characters are critical information to understand this unique mechanism involved in their infectious process. In this protocol, we describe a method to measure the force generated by *Mycoplasma mobile*, the fastest gliding species in *Mycoplasma*. This protocol should be useful for the studies of many kinds of gliding microorganisms.

Keywords: *Mycoplasma*, Optical tweezers, Force, Avidin-biotin, Bead

[Background] Surface motility systems are spread over many bacterial species and they are not well elucidated compared to bacterial flagella and eukaryotic motor proteins (Jarrell and McBride, 2008), although potentially they can give us critical information to understand the survival strategy of bacteria. To elucidate a motility mechanism, we need information about the structure of machinery, the flow of energy, and the mechanical characters including speed and force. Optical tweezers are a special method used for micromanipulations or force measurements in the piconewton range under microscopy, by which an object with a diffractive index different from the medium is trapped at the center of focused laser beam (Ashkin *et al.*, 1986). This method has greatly contributed to clarifying the features of motility systems including myosin, dynein, and kinesin, and now an established method in the field of biophysics. Here, we provide a protocol on how to measure force generated by surface moving microorganisms, based on our studies (Miyata *et al.*, 2002; Tanaka *et al.*, 2016) for gliding mechanism of *M. mobile* the fastest gliding species in class *Mollicutes*. This is the first protocol for force measurement made using optical tweezers in bio-protocol.

Materials and Reagents

1. 18 x 18 mm Square microscope cover slip (Matsunami Glass, catalog number: C218181)
2. 22 x 40 mm Square microscope cover slip (Matsunami Glass, catalog number: C022401)
3. Double-sided tape (NICHIBAN, NICETACK™, catalog number: NW-5)
4. Mending tape (3M, Scotch®, catalog number: 810-3-15)
5. Filter paper $\phi 70$ (ADVANTEC, catalog number: 00021070)

6. 1.5 ml microtube
7. *M. mobile* 163K strain (ATCC, catalog number: 43663)
8. Polystyrene beads 1.0 μm in diameter (Polybead[®] Carboxylate Microspheres 1.00 μm) (Polysciences, catalog number: 08226-15)
9. PolyLink Protein Coupling Kit for COOH Microspheres (Polysciences, catalog number: 24350-1)
 - a. PolyLink coupling buffer
 - b. PolyLink EDAC
10. Avidin from egg white (Sigma-Aldrich, catalog number: A9275)
11. PBS with 20 mM glucose (PBS/G)
12. PBS with 40 mM glucose
13. Sulfo-NHS-LC-LC-biotin (Ez-Link[™] Sulfo-NHS-LC-LC-biotin) (Thermo Fisher Scientific, Thermo Scientific[™], catalog number: 21338)
14. Glucose
15. Nail polish
16. Heart infusion broth (BD, catalog number: 238400)
17. Yeast extract (BD, catalog number: 212750)
18. 10 N NaOH
19. Horse serum (Thermo Fisher Scientific, Gibco[™], catalog number: 16050122)
20. Amphotericin B (Sigma-Aldrich, catalog number: A2942)
21. Ampicillin Na (Nacalai Tesque, catalog number: 02739-32)
22. Sodium phosphate (pH 7.3)
23. NaCl
24. Aluotto medium (see Recipes)
25. Phosphate-buffered saline (PBS) (see Recipes)

Equipment

1. Centrifuge (Sigma Laborzentrifugen, model: Sigma 1-14)
2. 25 cm² tissue culture flask (AS ONE, catalog number: 2-8589-01)
3. 25 °C incubator (Tokyo Rikakikai, model: LTI-400E)
4. Sonicator (Emerson Industrial Automation, BRANSON, model: 2510J-MT)
5. Optical microscope (Olympus, model: IX71)
6. Optical tweezers system (Note 1)
7. High speed charge-coupled device (DigiMo, model: LRH2500XE-1)
8. Power meter (Laser Power/Energy Meter) (Coherent, model: FieldMaxII-TOP, catalog number: 1098580)
9. Autoclave

Software

1. ImageJ (<http://rsbweb.nih.gov/ij/index.html>)
2. IGOR Pro 6.33J (WaveMetrics, Portland, OR)

Procedure

A. Coating polystyrene beads with avidin

1. Warm polystyrene beads, coupling buffer and PBS to room temperature (RT).
2. Centrifuge 60 μ l polystyrene bead suspension at 2,000 $\times g$ for 6 min at RT.
3. Discard supernatant, resuspend the pellet in 400 μ l of coupling buffer and centrifuge it at 2,000 $\times g$ for 6 min at RT.
4. Discard supernatant, resuspend the pellet in 160 μ l of coupling buffer.
5. Just before use, dissolve 11.7 mg of EDAC in 50 μ l of coupling buffer, add 20 μ l of this solution to the suspension and incubate for 5 min at RT.
6. Dissolve 0.4 mg of avidin in 10 μ l of coupling buffer to be 0.13 mM as the final concentration, add the avidin solution to the suspension and incubate it with gently end-over-end mixing for 3 h at RT.
7. Centrifuge the suspension at 2,000 $\times g$ for 6 min at RT, discard the supernatant and resuspend the pellet in 450 μ l of PBS. Repeat this step five times.
8. Transfer the suspension to new microtube and storage it at 4 °C until use (it can be stored for 6 months).

B. Biotinylation of *M. mobile* cell surface

1. Inoculate 1 ml of frozen stock of *M. mobile* into 10 ml of Aluotto medium in a 25 cm² tissue culture flask and statically cultivate it at 25 °C to reach an optical density at 600 nm of 0.06-0.08.
2. Collect 9 ml of cultured cells by centrifugation at 12,000 $\times g$ for 4 min at RT, discard supernatant, resuspend the cell pellet in 900 μ l of PBS/G and centrifuge it at 12,000 $\times g$ for 4 min at RT (Note 2).
3. Just before use, prepare a 0.5 mM of Sulfo-NHS-LC-LC-biotin solution by dissolving 0.3 mg of Sulfo-NHS-LC-LC-biotin in 1 ml of PBS/G.
4. Discard supernatant of step B2, resuspend the cell pellet (Note 2) in 300 μ l of Sulfo-NHS-LC-LC-biotin solution and incubate the cell suspension for 15 min at RT (Hiratsuka *et al.*, 2006).
5. Centrifuge the suspension at 12,000 $\times g$ for 4 min at RT, discard supernatant, and resuspend the cell pellet (Note 2) in 300 μ l of PBS/G. Repeat this step three times.

C. Force measurement by using optical tweezers

1. Prepare a tunnel chamber (5-mm interior width, 18-mm length, 60- μ m wall thickness) composed of two cover glasses from box, and double-sided tapes (Figure 1).

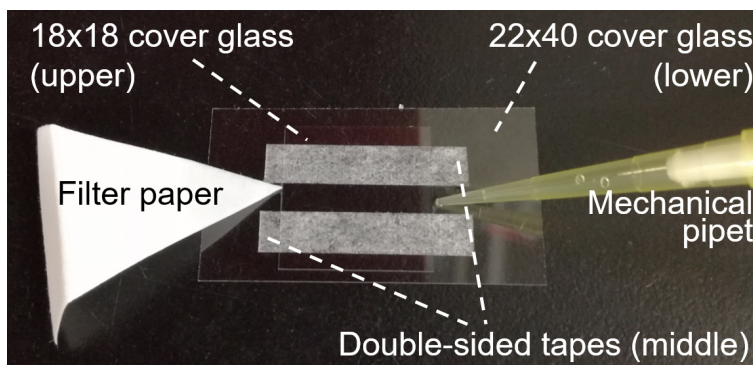


Figure 1. Tunnel chamber (step C1)

2. To coat the glass surface with glycoproteins, inject 20 μ l of 10% horse serum in PBS/G into the tunnel chamber and incubate it for 15 min at RT (Note 3).
3. Dilute the suspension of biotinylated cell with a 10 to 20-fold volume of PBS/G.
4. Wash the tunnel chamber by flowing 40 μ l of PBS/G, inject 20 μ l of the diluted cell suspension into the tunnel chamber and incubate it for 15 min at RT.
5. To remove floating cells, insert 20 μ l of PBS/G into the tunnel chamber.
6. Add the avidin-coated beads in PBS to the same volume of PBS containing 40 mM glucose, for adjusting glucose concentration to 20 mM.
7. Dilute the bead suspension with PBS/G to get an appropriate bead density (10-20 beads per a 125.4 x 70.6 μ m field of view) for the force measurements.
8. Transfer 40 μ l of bead suspension to a new microtube and sonicate it for 20 sec.
9. Inject sonicated bead suspension into the tunnel chamber and seal both ends with nail polish.
10. Set and fix the tunnel chamber by mending tapes onto the stage of inverted microscope equipped with optical tweezers (Figure 2).

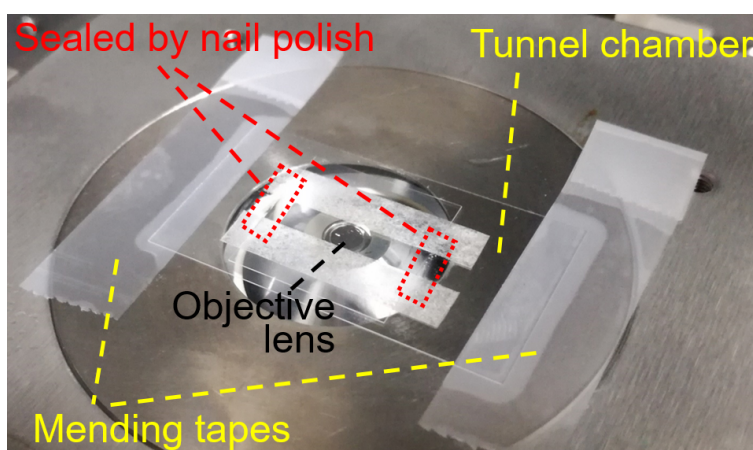


Figure 2. Setting tunnel chamber on microscope stage (step C10)

11. Trap a floating bead near the bottom of glass surface by focused laser beam, start to record the bead movements at 200 frames per second and immediately attached the bead to the back end

of the gliding cell through the avidin-biotin interaction. The cell pulls the bead from trap center of optical tweezers and finally stalls (Video 1). The recording should be continued at least 30 sec after the start of stall (Note 4). After recording, turn off the laser beam.

12. Remove the tunnel chamber and objective lens, turn on the laser beam to the same power as the step C11 and measure the applied laser power of the parallel light, which was achieved by the removal of objective lens, with using a power meter.
13. Trace the bead movement from the trap center of optical tweezers and calculate the force from the distance between centers of the bead and the laser beam, by using ImageJ and IGOR Pro, as previously described (Tanaka *et al.*, 2016).

Video 1. Trapping of bead and attachment to gliding cell (step C11). Focused point of laser beam is indicated by a red circle, marked 'Trap center'. At 5 sec, a flowing bead came from the right side and trapped at the beam. At 18 sec, a gliding cell was moved into the field by stage handling. At 27 sec, the bead was attached to the gliding cell. The cell pulled the trapped bead and stalled from 33 sec to the end.



Data analysis

The calculation method for bead positioning is described in Thompson *et al.* (2002).

Notes

1. Construction of optical tweezers was described in Tanaka *et al.* (2016). Similar systems are also commercially available (Laser Optical tweezers – Mini type Basic model/without shutter, SIGMAKOKI, catalog number: LMS-M1064-2000. OTM200 Optical Tweezer Add-On, THORLABS, catalog number: OTM200).
2. Suspension of cell pellet
More than 100 ups and downs with the pipette is recommended to separate cells completely.
3. 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.

4. Available data size for ImageJ v1.43u is limited around 1 GB. The data size of movie depends on the recording time, the field size, and the frame rate. One example is 180 sec, 4 x 4 µm square, and 200 frames per second.

Recipes

1. Aluotto medium
2.1% 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
0.025% amphotericin B
0.005% ampicillin Na
2. Phosphate-buffered saline (PBS)
75 mM sodium phosphate (pH 7.3)
68 mM NaCl
Sterilize by using filter or autoclave

Acknowledgments

This work was supported by Grants-in-Aid for Scientific Research on Innovative Area, 'Harmonized Supramolecular Motility Machinery and Its Diversity' (grant 24117002 to M. Miyata) and by a grant-in-aid for scientific research (B) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (grant 24390107 to M. Miyata).

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