

Nematode Epicuticle Visualisation by PeakForce Tapping Atomic Force Microscopy

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[Abstract] The free-living soil nematode *Caenorhabditis elegans* has become an iconic experimental model animal in biology. This transparent animal can be easily imaged using optical microscopy to visualise its organs, tissues, single cells and subcellular events. The epicuticle of *C. elegans* nematodes has been studied at nanoscale using transmission and scanning (SEM) electron microscopies. As a result, imaging artefacts can appear due to embedding the worms into resins or coating the worms with a conductive gold layer. In addition, fixation and contrasting may also damage the cuticle. Conventional tapping mode atomic force microscopy (AFM) can be applied to image the cuticle of the dried nematodes in air, however this approach also suffers from imaging defects. Ideally, the nematodes should be imaged under conditions resembling their natural environment. Recently, we reported the use of PeakForce Tapping AFM mode for the successful visualisation and numerical characterisation of *C. elegans* nematode cuticle both in air and in liquid (Fakhrullina *et al.*, 2017). We imaged the principal nematode surface structures and characterised mechanical properties of the cuticle. This protocol provides the detailed description of AFM imaging of liquid-immersed *C. elegans* nematodes using PeakForce Tapping atomic force microscopy.

Keywords: Atomic force microscopy, Nematodes, *Caenorhabditis elegans*, Cuticle, PeakForce Tapping, Layer-by-Layer assembly

[Background] Nematodes, both free-living and parasitic, have been extensively studied due to their remarkable biology and effects on agriculture and human well-being. Apparently, the most studied and famous species among nematodes is a free-living soil nematode *Caenorhabditis elegans* (Sterken *et al.*, 2015). This round worm has been successfully employed as a versatile model organism in a number of investigations (Fire *et al.*, 1998; Kenyon, 2010; Swierczek *et al.*, 2011; O'Reilly *et al.*, 2014; Stroustrup *et al.*, 2016), securing eventually a Nobel prize for Sidney Brenner, John Sulston and Robert Horvitz in 2002. *C. elegans* nematode is a tiny (~1 mm long) transparent animal which can be easily visualised using an optical microscope. Its cuticle, though thin and transparent, serves as a principal protective barrier between the worm and its habitat. Cuticle is an important marker of disease, when pathogenic bacteria colonize it, leading to the death of the infected animal. In addition, the structure of the cuticle may indicate the ageing in the nematodes. From the human health care and industrial point of view, monitoring of the cuticle of nematodes might be helpful in elucidation of novel antinematode chemicals affecting the cuticle. As a result, nanoscale imaging of the nematode epicuticle in its natural liquid environments may open new avenues in biomedical research.

Previously, the cuticle of nematodes (mostly employing *C. elegans* as a model organism) has been studied using electron microscopy, both transmission and scanning. Unfortunately, electron microscopy does not allow imaging the worms in liquid, which is their native environment. Sample preparation for electron microscopy requires fixation, dehydration, contrast staining or surface sputtering, and also resin embedding for ultrathin slicing. As a result, nematode cuticle may exhibit secondary artifacts preventing from evaluation of native surface structure and mechanical characterisation. Atomic force microscopy has been established as a potent tool in imaging biological samples in situ, including imaging live cells in liquid media (Beaussart *et al.*, 2015). Recently, the application of tapping mode AFM to visualise *C. elegans* nematodes in air has been reported (Allen *et al.*, 2015). Imaging in air suffered from the same drawback as scanning electron microscopy, for example the images demonstrated the typical shrunk and collapsed cuticle surfaces apparently caused by dehydration and air imaging. We envisaged a different technique, based on PeakForce Tapping AFM mode (Alsteens *et al.*, 2012) for successful visualisation of *C. elegans* nematode cuticle in water (Fakhrullina *et al.*, 2017). Here we report a detailed protocol for this technique.

Materials and Reagents

Note: All chemicals were purchased from Sigma-Aldrich unless noted otherwise.

1. Pipette tips
2. Petri dishes
3. Dust-free Nexterion glass slides (Schott)
4. Wild type *C. elegans* (N2 Bristol) nematodes
5. *Escherichia coli* OP50 bacteria
6. Poly(allylamine hydrochloride) (PAH, molecular weight ~17,5 kDa) (Sigma-Aldrich, catalog number: 283215)
7. Poly(sodium 4-styrenesulfonate) (PSS, molecular weight ~70 kDa) (Sigma-Aldrich, catalog number: 243051)
8. Nematode growth media (NGM) (bioWORLD, catalog number: 30620040-1)
9. Nematode lysis solution (aqueous 2% NaOCl/0.45 M NaOH)
10. Sodium hypochlorite (NaClO) (Sigma-Aldrich, catalog number: 425044)
11. Sodium hydroxide (NaOH) (Sigma-Aldrich, catalog number: S8045)
12. Levamisole hydrochloride (Sigma-Aldrich, catalog number: L9756)
13. Glutaraldehyde (25% aqueous) (Sigma-Aldrich, catalog number: G5882)
14. Ultrapure (type 1) water purified by Simplicity (Millipore) water purification system
15. Potassium phosphate monobasic (KH₂PO₄) (Sigma-Aldrich, catalog number: NIST200B)
16. Sodium phosphate dibasic (Na₂HPO₄) (Sigma-Aldrich, catalog number: 71649)
17. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S7653)
18. Magnesium sulfate (MgSO₄) (Sigma-Aldrich, catalog number: 793612)
19. Aqueous M9 buffer (see Recipes)

Equipment

1. Pipettes (mechanical Eppendorf or Gilson pipettes)
2. Eppendorf Scientific Excella E24 temperature-controlled benchtop shaker (Eppendorf, New Brunswick™, model: Excella® E24)
3. Biosan V-1 plus vortex (Biosan, model: V-1 plus)
4. Nikon SMZ 745 T stereomicroscope (Nikon, model: SMZ745T)
5. Dimension FastScan Bio atomic force microscope (Bruker, model: Dimension FastScan) equipped with a liquid submersible scanner

Note: Do not use air scanners for scanning in liquid, this may damage the instrument.

6. Biosan Microspin 12 centrifuge (Biosan, model: Microspin 12)
7. ScanAsyst-Fluid probes (nominal length 70 μm , tip radius 20 nm, spring constant of 0.7 N m^{-1}) (Bruker, model: SCANASYST-FLUID)

Note: Other AFM probes from Bruker or other producers approved for using in PeakForce Tapping mode having characteristics similar to ScanAsyst-Fluid probes can also be used.

Software

1. NanoScope AFM operating software (Bruker Corporation)
2. NanoScope Analysis v.1.7. software (Bruker Corporation)

Procedure

A. Fabrication of layer-by-layer (LbL) polyelectrolyte coated glass substrates

1. Prepare 5 mg ml^{-1} aqueous solutions of PAH and PSS polymers (5 ml each).
2. Drop 50 μl of PAH solution onto the central part of a dust-free glass slide, incubate for 20 min in a humidified chamber (any tightly closed glass or plastic container with moist cotton pad might be used to prevent the premature evaporation), wash several times with water.
3. Repeat the same using PSS solution.
4. Repeat steps A2 and A3 until the resulting coating architecture is (PAH/PSS)₁₂PAH (12 consecutive PAH/PSS bilayers followed by a final PAH layer). The outmost polyelectrolyte should be PAH to ensure the positive charge of the deposited film. More information on fabrication of LbL films can be found elsewhere (Lvov *et al.*, 1995).

B. *Caenorhabditis elegans* nematodes cultivation

1. Cultivate wild type *C. elegans* (N2 Bristol) nematodes on NGM-supplemented agar plates at 20 °C using a thermostated shaker according to a standard protocol (Brenner, 1974). Use *Escherichia coli* OP50 bacteria to feed worms. Observe the animals using a stereomicroscope after 48 h to detect gravid nematodes.

2. Wash off gravid adult hermaphrodites with M9 buffer (see Recipes) into a tube and harvest by centrifugation for 2 min (1,100 x g, applicable to eggs, larvae and adult hermaphrodites). Dissolve the animals using aqueous 2% NaOCl/0.45 M NaOH solution (4.5 ml) to obtain nematode eggs. Wash the eggs with M9 buffer several times, then collect by centrifugation as described above and place onto the sterile NGM agar plates.
3. Cultivate the nematodes at 20 °C to obtain synchronised adult hermaphrodites. Alternatively, cultivate the worms for shorter time to obtain larvae animals (Brenner, 1974).
4. Collect the nematodes as described in step B2. Euthanise the collected worms with 45 mM aqueous levamisole hydrochloride. Wash consequently with water.
5. Fixate the animals using 2 ml of buffered glutaraldehyde (in M9 buffer) for 2 h at 25 °C, then wash with water.

C. Atomic force microscope setup

1. It is assumed that the AFM operator is familiar with the AFM working principles and has previous experience with Dimension FastScan Bio AFM. For introduction into PeakForce Tapping AFM mode refer to microscope manual or to the following publication (Durkovic *et al.*, 2014)
2. AFM should be initiated before the sample is ready for imaging (see below). Ideally, these steps should be performed at the same time.
3. Switch on the microscope and the operating software.
4. Install and calibrate the mechanical properties of the probe (ScanAsyst-Fluid) according to the manufacturer's manual. Start calibrating in air, then repeat in water.

D. Atomic force microscopy imaging

1. Place a droplet (100 µl) of water-suspended fixated nematodes onto the LbL-coated glass slides.
2. Wait 30 min for settling and attaching the animals to the LbL polyelectrolyte film.
3. Remove the excess water from the glass surface by pipetting, then wash the slide with water 3 times to remove any loosely immobilised worms.
4. Add 200 µl of water on the top of the LbL-deposited film retaining the worms. The adhesion of the animals to the glass slide can be checked using a stereomicroscope prior to AFM imaging.
5. Secure the glass slide on the microscope stage using vacuum suction. This should be done with due care to avoid removing the liquid from the glass slide.
6. Locate a worm using the internal AFM optical camera. The worms will appear on the computer screen connected to the AFM internal camera as they would be seen under a stereomicroscope (Figure 1).

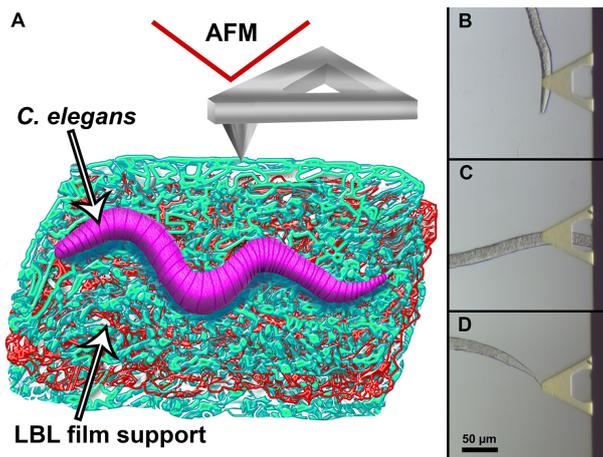


Figure 1. Immobilisation of nematodes on the support film. A sketch demonstrating immobilisation of an adult *C. elegans* nematode on (PAH/PSS)₁₂PAH LbL-deposited film and subsequent AFM imaging in liquid (A). Optical microscopy camera snapshot demonstrating the approach of the AFM probe to head (B), body (C) and tail (D) region of an immobilised *C. elegans* adult hermaphrodite.

7. Position the probe above a certain area of the worm (*i.e.*, head, tail or body regions).
8. Approach the tip to the surface. Use relatively low scanning resolution (*i.e.*, 64 lines) for locating the cuticle surface.
9. After locating the region of interest (ROI), reduce the scanning frequency (0.8-0.9 Hz) and peak force setpoint (1 nN); increase the scanning resolution (up to 512 lines). The typical AFM images of an adult *C. elegans* nematode are shown in Figure 2.

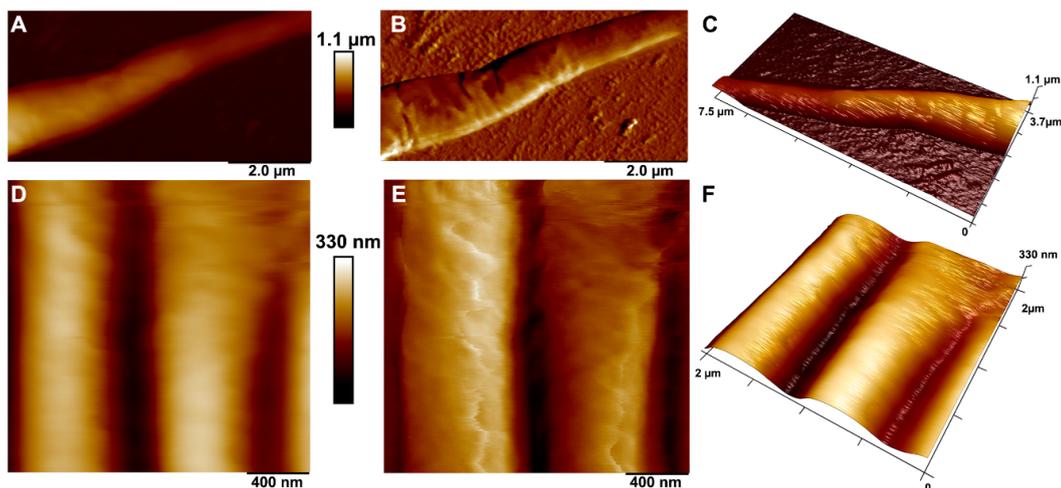


Figure 2. PeakForce Tapping AFM images of an adult *C. elegans* nematode immobilised on (PAH/PSS)₁₂PAH LbL-deposited film: tail region (upper row) and body region (lower row). A and D. Height sensor topography images; B and E. Peak force error images; C and F. 3-D rendering of height sensor data with peak force error data overlaid.

10. Collect images in the following channels: height sensor, peak force error, adhesion and Sneddon modulus mode. Figure 3 demonstrates the images obtained in all four channels.

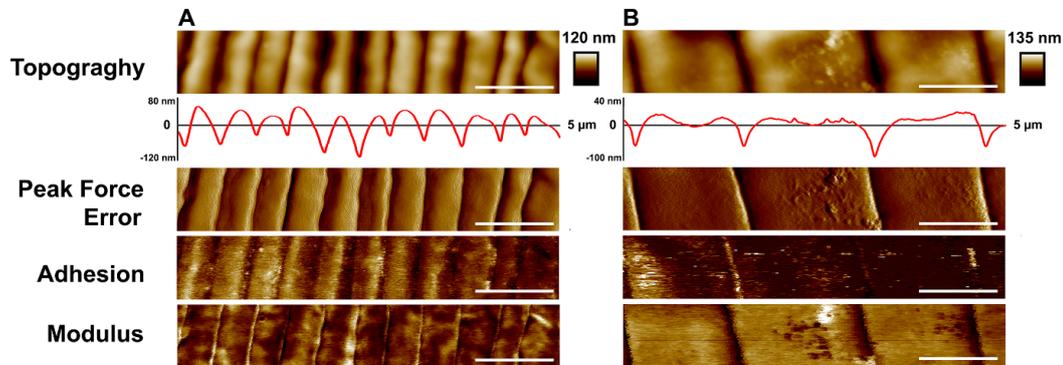


Figure 3. PeakForce Tapping AFM images of L1 larvae (A) and adult *C. elegans* (B) nematode imaged in water in topography (height sensor), peak force error, adhesion and modulus (Sneddon model) channels. Scale bars = 1 μ m.

11. Scans can be obtained using higher aspect ratios (*i.e.*, 5-6), which allows increasing the longitude of the images (compare aspect ratios in Figures 2 and 3).
 12. For statistically-significant quantitative evaluation examine 5-10 individual randomly selected nematodes.
- E. Atomic force microscopy raw data processing
1. Export raw data files to NanoScope Analysis software.
 2. Open a file, then flatten the image using 3^d flattening order.
 3. Adjust colour scale using 'Color Bar Scale Relative to Minimum Data Cursor' function.
 4. Export high-resolution tiff files using Journal Quality Export function.
 5. For advanced image processing and nanomechanical characterisation refer to the NanoScope Analysis software operation manual.

Recipes

1. M9 buffer
22 mM KH_2PO_4
42 mM Na_2HPO_4
85.5 mM NaCl
1 mM MgSO_4

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