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Whole-Animal Mounts of *Caenorhabditis elegans* for 3D Imaging Using Atomic Force Microscopy

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Abstract

The 3D surface of *Caenorhabditis elegans* was imaged at nanometer resolution using atomic force microscopy (AFM). Oscillation of a medium stiffness silicon AFM cantilever at the upper second amplitude peak, typically 6 times above the fundamental frequency, vastly improved image quality on the moist, sticky, and soft worms. Whole-animal mounts of normal and double-headed mutants of the nematode worm were prepared and scanned. Well-preserved anatomical features including annuli, furrows, alae, and rows of never before seen nanometer-sized pores dotting the molted worm's outermost surface coat were resolved. This AFM method represents a simple and rapid new approach for nanometer-resolved 3D imaging and analysis of whole-animal specimens of *C. elegans*.

Graphical Abstract

C. elegans is a useful model to study genetics as well as genomic and environmental interactions as we have shown. Even though a large amount of data exists on the various 'normal' and variant *C. elegans*, the structure of these organisms still remains a mystery. We have refined the understanding of *C. elegans* morphology by studying images generated by atomic force microscopy. We show that the skin has considerable cuticles and also the variant *vab-1* (EPH mutation) has the notched head phenotype. (Graphical Abstract: Figure 2)

Allen, *et. al.*, Scanning Whole-Animal Mounts of *Caenorhabditis Elegans* Using Atomic Force Microscopy

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Keywords

C. elegans; atomic force microscopy; AFM

Introduction

The nematode worm, *C. elegans*, is well-characterized regarding its genetic mutations, cellular development, behavioral aspects, and as a model of human diseases¹⁻⁵. While high resolution imaging of tangential and oblique views of the worm ultrastructure can be achieved with transmission electron microscopy (TEM), preparation of the worm's outermost surface for TEM is extremely difficult^{6,7}. Scanning electron microscopy (SEM) has been used for characterizing the worm's surface but at resolutions substantially lower than by TEM⁸. Atomic force microscopy (AFM) applied to embedded sections of *C. elegans*⁹, its isolated proteins¹⁰, and DNA¹¹ have shown resolutions comparable to TEM. While AFM-based quantitative assessments of the mechanical properties of whole worms were attainable¹² and affinity-based AFM had been applied to single cells^{13,14}, a method that allowed 3D nanoscale imaging of the surface of whole-animal mounts had not been developed. Here we report the first such images of *C. elegans*.

Methods

AFM instrumentation

We used the Nanoscope IIIa controller with extender module operated with version 6 software (Bruker AXS, Santa Barbara, California, USA). This controller was coupled to a Multimode AFM equipped with a low noise optical head, calibrated E- and J- piezoelectric scanners and PicoForce hardware (Bruker AXS). The imaging system is suspended on bungee cords with a vertical resonance of ~ 0.5 Hz.

AFM sample preparation

Individual worms were picked from agar plates and fixed in 0.5% glutaraldehyde (Sigma-Aldrich, St. Louis, Missouri, USA) for 30 minutes in PBS (Sigma) pH 7.4. Pooled worms were spin-washed 3× in pure water (ACS grade, Sigma) at $500 \times g$ for 2 min and the supernatant discarded leaving 10-20 worms in a 20 µl volume at the bottom of the tube. Worms were deposited and physisorbed to a small area 3-5 mm in diameter at the center of a 10 mm glass coverslip from a 10-20 µl droplet over a 15 min incubation period or until the worms were still moistened by liquid accumulated at their body perimeters and then immediately scanned. Specimens were aligned beneath the AFM probe using a manual x,y micrometer AFM stage and top-view video microscope (10×) integrated with the AFM. While still moist when imaged, the worms were exposed to drying forces during their attachment to the AFM substrate and might be damaged during spin-washing. Therefore, we first fixed the worms in glutaraldehyde. While this would help retain structural integrity, fixatives can also induce small structural alterations.

AFM image acquisition

The fundamental resonance frequency of the cantilever-probe used in this study is approximately 70 kHz (probe model AC240TS, Olympus Microcantilevers, Tokyo, Japan). This rectangular aluminum-coated silicon probe is 240 µm, 40 µm, 2.3 µm in length, width, and thickness, respectively and has a nominal spring constant of 2 nN/nm. The imaging-tip used has a radius of curvature of 9 nm and a tip length of 14 µm. The second resonance peak in the frequency sweep spectrum between 0-500 kHz in air is ~6-fold higher than the fundamental resonance, i.e., at ~435 kHz. Tuning to this higher resonance peak allows high quality scans of the worms. 'Light tapping' conditions of ~100 pN were maintained by using low drive voltages (50 mV) coupled with a setpoint voltage just below probe pull-off, enough to allow good tracking (0.30-0.50 V). Line-scan frequencies were 0.3-1.0 Hz at 512 data points/line. Images were limited to 256 lines/scan to minimize sample damage in the slow scan axis.

AFM data rendering

AFM data-images were imported into the Nanoscope Analysis software program (version 1.40, Bruker AXS) for analysis and rendering using continuous color scale and illumination display graphics. Boxes, bars and arrows were drawn onto the images using Powerpoint (Microsoft, Redmond, Washington, USA).

Results

The fundamental resonance frequency of the rectangular cantilever-probe used for imaging *C. elegans* is approximately 70 kHz while the second resonance peak in the spectrum is ~6-fold higher at ~435 kHz (Figure 1). By driving and feedback-monitoring at this second resonance, we were able to obtain high quality scans of still moist, whole nematode worms. Attempts to scan the worms at 70 kHz failed to produce images due to the weak cantilever spring causing the probe to become stuck on the moist specimen by adhesive forces. Use of contact-mode AFM, where the probe is gently rubbed back-and-forth without oscillation, resulted in the scanning probe displacing and damaging the specimen.

Individual worms were picked from agar plates, fixed in 0.5% glutaraldehyde and spin-washed in pure water. The worms were then deposited and physisorbed to coverslip glasses and scanned as the cantilever/probe was driven at its second amplitude peak, ~435 kHz. An AFM scan of a normal early larval stage worm showed characteristic anatomical features such as the annuli, furrows, and alae (Figures 2A-C). Annuli spacing, as measured by AFM, averaged 660.2 nm (n=37, sd=76.9). These morphological/structural features observed by AFM are consistent with EM micrographs, as depicted in a drawing of the worm's outer surface¹⁵ (Figure 2D), confirming our AFM sample prep has preserved the specimens.

The double-headed *vab-1*¹⁶ larval stage mutant was observed by AFM with evident molting of the outer cuticle (Figure 3A). AFM scans along the mid-body surface worm revealed micron-sized scale-like fractures in the epicuticle layer formed during ecdysis (Figure 3B). Intermediate resolution AFM scans of the underlying fiber layer revealed a series of parallel rows of numerous nanometer-sized pores (Figure 3C). Higher resolution AFM images show the highly globular topography of the outer surface coat as well as the 3D landscapes of individual pores, which measure 25-30 nm in diameter and 10 nm in depth (Figures 3D-E). These small 10 nm globules and 25-30 nm nanopores, each the size of individual higher-ordered macromolecular protein complexes, covering the fiber layer surface demonstrate the nano-scale resolution of the method described.

Discussion

Here we describe a new AFM sample method, quicker and simpler than those used for EM, and resulting in nanometer-resolved 3D images of whole-animal worms. No extensive preparative methods are required, including coatings, stains or freeze fractures, and the AFM scanning probe can be located quickly to individual worms using integrated optics and a micrometer stage. In the past, use of a soft cantilever-probe with a low spring constant for contact- or AC-mode AFM on moist, soft, and sticky biological specimens such as *C. elegans* was extremely problematic due to the cantilever-probe spring being too weak to overcome the adhesion forces encountered by the AFM probe. Although not in the context of application to biological imaging, use of cantilever-probe resonances driven above the fundamental frequency (notably at the third eigenmode) had been reported to enhance AFM image contrast formation appearing to highlight materials properties information of the specimen in addition to topography¹⁷. Here, the cantilever-probe driven at its second resonance, also near the third eigenmode (approximately 6-times the fundamental frequency), has allowed the successful application of a moderately soft, non-damaging AFM cantilever to an important yet previously problematic biological specimen for conventional AFM. And we have demonstrated the feasibility of nanoscale analyses on whole worms of differing genetic backgrounds and developmental and molting stages.

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References

1. Brenner S. The genetics of *Caenorhabditis elegans*. *Genetics*. 1974; 77:71–94. [PubMed: 4366476]
2. Kaletta T, Hengartner MO. Finding function in novel targets: *C. elegans* as a model organism. *Nat Rev Drug Discov*. 2006; 5(5):387–98. [PubMed: 16672925]
3. Markaki M, Tavernarakis N. Modeling human diseases in *Caenorhabditis elegans*. *Biotechnol J*. 2010; 5(12):1261–1276. [PubMed: 21154667]
4. Artal-Sanz M, de Jong L, Tavernarakis N. *Caenorhabditis elegans*: a versatile platform for drug discovery. *Biotechnol J*. 2006; 1(12):1405–1418. [PubMed: 17109493]
5. Wood, WB. *The Nematode Caenorhabditis elegans*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press; 1988. community of *C. elegans* researchers.
6. Blaxter ML, Page AP, Rudin W, Maizels RM. Nematode surface coats: actively evading immunity. *Parasitol Today*. 1992; 8(7):243–246. [PubMed: 15463630]
7. Peixoto CA, Kramer JM, Souza W. *Caenorhabditis elegans* cuticle: a description of new elements of the fibrous layer. *J Parasitol*. 1997; 83(3):368–372.
8. Altun, ZF.; Herndon, LA.; Crocker, C.; Lints, R.; Hall, DH. [Accessed 6/3/2015] WormAtlas:Hermaphrodite:Cuticle. <http://www.wormatlas.org/hermaphrodite/cuticle/Cutframeset.html>
9. Matsko N, Mueller M. AFM of biological material embedded in epoxy resin. *J Struct Biol*. 2006; 34:3057–3066.
10. Foeger N, Wiesel N, Lotsch D, Mücke N, Kreplak L, Aebi U, et al. Solubility properties and specific assembly pathways of the B-type lamin from *Caenorhabditis elegans*. *J Struct Biol*. 2006; 155:340–350.
11. Moreno-Herrero F, Seidel R, Johnson SM, Fire A, Dekker NH. Structural analysis of hyperperiodic DNA from *Caenorhabditis elegans*. *Nucleic Acids Res*. 2006; 34:3057–3066. [PubMed: 16738142]
12. Park S, Goodman MB, Pruitt BL. Analysis of nematode mechanics by piezoresistive displacement clamp. *PNAS*. 2007; 104(44):17376–17381. [PubMed: 17962419]
13. Chtcheglova LA, Waschke J, Wildling L, Drenckhahn D, Hinterdorfer P. Nano-Scale Dynamic Recognition Imaging on Vascular Endothelial Cells. *Biophys J*. 2007; 93(2):L11–L13. [PubMed: 17496017]
14. Ahmad SF, Chtcheglova LA, Mayer, Kuznetsov SA, Hinterdorfer P. Nanosensing of Fcγ receptors on macrophages. *Anal Bioanal Chem*. 2011; 399(7):2359–2367. [PubMed: 20676615]
15. Altun, ZF.; Herndon, LA.; Crocker, C.; Lints, R.; Hall, DH. [Accessed 3/12/2015] WormAtlas. <http://www.wormatlas.org>
16. George SE, Simokat K, Hardin J, Chisholm AD. The VAB-1 Eph Receptor Tyrosine Kinase Functions in Neural and Epithelial Morphogenesis in *C. elegans*. *Cell*. 1998; 92:633–643. [PubMed: 9506518]
17. Stark RW, Drobek T, Heckl WM. Tapping-mode atomic force microscopy and phase-imaging in higher eigenmodes. *Applied Physics Letters*. 1999; 74(22):3296–3298.

List of abbreviations

TEM	transmission electron microscopy
SEM	scanning electron microscopy
AFM	atomic force microscopy

Figure 1.

Frequency sweep of the AFM cantilever used for the scanning of *C. elegans* showing the amplitude response at approximately 70 kHz, its fundamental frequency, and the secondary amplitude peak at roughly 6 times higher frequency (435 kHz). To image the worms, the AFM cantilever was driven and monitored at this second resonance during scanning with its amplitude changes serving as the feedback signal.

Figure 2.

Normal morphology. (A) Amplitude and (B) topographic [from yellow boxed area in (A)] AFM images of the early larval worm head region showing annulus (green arrows), and furrows. Head region z-thickness, $\sim 3.0 \mu\text{m}$. (C) Topographic AFM image of the mid-body region shows alae running along the long axis midline (arrows). Mid-body z-thickness, $\sim 3.0 \mu\text{m}$. (D) Schematic showing alae, annuli, and furrows as determined by SEM (adapted from the Worm Atlas). Full-scale z-axis, (A) 0.3 V; (B) 3.5 μm ; (C) 4.0 μm .

Figure 3.

vab-1 mutant morphology. (A) Topographic AFM image showing entire head region of a double-headed early larval stage *vab-1* mutant with evident molting of the outer cuticle (white arrows). Head region z-thickness, 4.25-4.75 μm (B) Lower resolution topographic AFM image of the cuticle surface in the mid-body region of a molting larval double-headed mutant worm, *vab-1*. Micron-sized scale-like fractures in the epicuticle/cortical layer formed during ecdysis are evident (white arrows). (C) Intermediate resolution topographic AFM image reveals parallel rows of nanometer-sized pores likely related to the struts of the fiber layer which underlies the epicuticle and exposed following molting. (D) Topography of the parallel rows of nanopores (white arrows). (E) High resolution topographic AFM image shows several such nanopores; inset, section profile of 2 nanopores with diameters measuring 29.4 nm (red arrows) and 26.5 nm (green arrows) and approximately 10 nm in depth. The surface surrounding the nanopores consists of a high density of 10 nm globular structures. Full-scale z-height, (A) 5.6 μm ; (B) 250 nm; (C) 100 nm; (D) 50 nm; (E) 30 nm.