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Short Communication

In-vivo high resolution AFM topographic imaging of *Caenorhabditis elegans* reveals previously unreported surface structures of cuticle mutants

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Abstract

Atomic force microscopy (AFM) is a powerful method for topographic imaging of surfaces with nanometer resolution. AFM offers significant advantages over scanning electron microscopy (SEM) including the acquisition of quantitative 3D-images and biomechanical information. More importantly, for in-vivo biological imaging, AFM does not require sample dehydration/labeling. We show for the first time high-resolution topographical images of the cuticle of the model organism *C. elegans* under physiological conditions using AFM. *C. elegans* is used extensively for drug screening and to study pathogen adherence in innate immunity; both applications highly depend on the integrity of the nematode's cuticle. Mutations affecting both drug adsorption and pathogen clearance have been proposed to relate to changes in the cuticle structure, but never visually examined in high resolution. In this study we use AFM to visualize the topography of wild-type adult *C. elegans* as well as several cuticle collagen mutants and describe previously unseen anatomical differences. Crown Copyright © 2016 Published by Elsevier Inc. All rights reserved.

Key words: Atomic force microscopy; Nano-scale topography; *C. elegans*; Cuticle mutants; Collagens

AFM achieves nanometer level resolution under ambient conditions, offering exciting possibilities for imaging biological samples.^{1–3} Although SEM can achieve similar resolution, it requires fixative treatment of the sample including harsh dehydration steps.⁴ In this study we characterize the topographical and biomechanical properties of the cuticle of the model

organism *C. elegans*. *C. elegans* is used in medical research for high-throughput drug screening and pathogen host interaction studies, and its cuticle is layered similarly to human skin.^{5–8} Both research applications are critically dependent on the animal's cuticle, and mutations in cuticle proteins can influence both efficiency of drug uptake and resistance to pathogens or biofilm formation.^{9–11} Several SEM studies have described the gross surface structure of the cuticle, whilst TEM sections reveal the structure of the cuticle sub-layers.^{8,12,13} A published AFM study was limited to fixed, partially dry larval stage animals.¹⁴ Therefore, the nano-scale topography of adult *C. elegans* cuticle under physiological conditions and its biomechanical properties, including the differences between wild-type strains and relevant cuticle mutants, remain undescribed. Our study shows for the first time AFM topography images of live adult wild-type worms and reveals new surface structures in collagen mutants.

Conflict of interest: The authors declare that there are no conflicts of interest.

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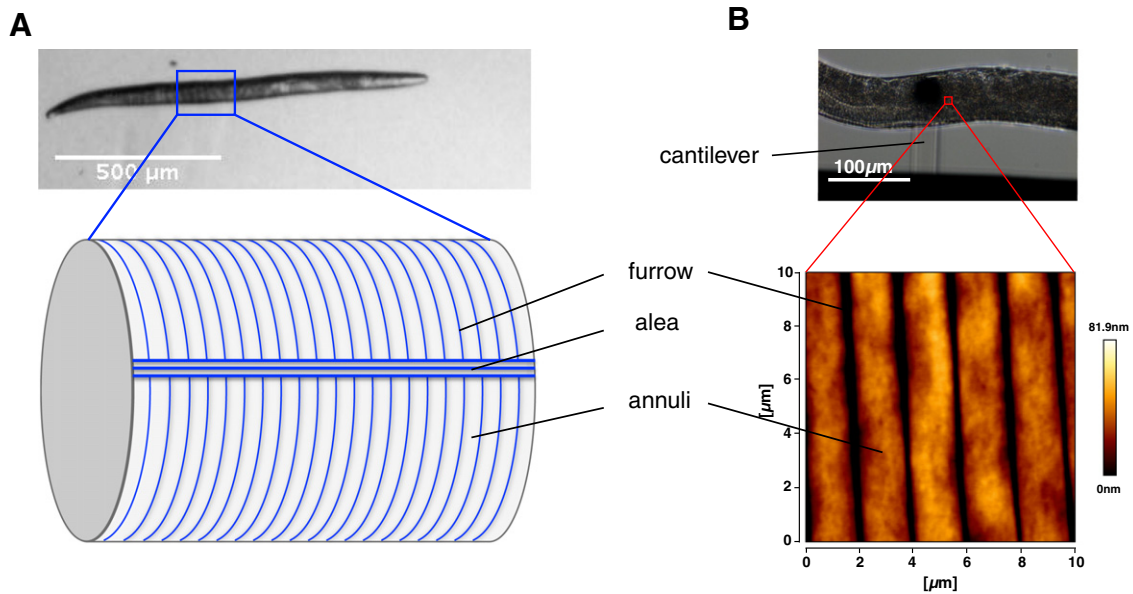


Figure 1. Cuticle structures in *C. elegans*. (A) Optical image of wild-type *C. elegans* (top) and schematic diagram showing annuli, furrows and alae (bottom). (B) Optical image from our AFM setup showing the cantilever and body of the worm (top) and typical topography image of the cuticle (bottom).

Methods

Strains

All strains were cultivated at 20 °C and maintained as described previously.¹⁵ N2 (wild-type), CB61 (*dpy-5*), CB88 (*dpy-7*), CB128 (*dpy-10*), CB458 (*dpy-13*) strains were obtained from the Caenorhabditis Genetics Center (University of Minnesota).

Worm Preparation for AFM

Staged young adult worms were paralysed (10 mg/ml BDM), fixed on head and tail with tissue glue (Dermabond) onto an agarose pad in a dish and immersed in 2.5 ml M9-buffer to prevent dehydration. For SEM-fixation, staged young adults were fixed in 3% glutaraldehyde o/n.

AFM

AFM images of worms were obtained using a NanoWizard3 (JPK). Type qp-CONT-10 (nanosensors) cantilevers were used for imaging in contact-mode and NSC18/no AL (MikroMasch) for quantitative imaging (QI) mode. The set point for imaging was 0.3 V (~350pN) at 0.5 Hz scanning speed and for QI 0.3 V (35nN) at 100 μm/s. Cantilever sensitivity and spring constant were calibrated using the JPK calibration tool.¹⁶

Optical Imaging

Staged young adults were stained with the fluorescent lipophilic dye DiI,¹⁷ paralyzed and mounted on a 2% agarose pad in M9 buffer. Focal image series were captured using an upright epifluorescence microscope (BX51WI, Olympus) with a 60×/1.0 objective lens.

Image Analysis

AFM data was analysed using JPK analysis software. Topography images are flattened (256 × 256 pixels) and the elastic modulus was calculated using the Hertz/Sneddon contact mechanics model (image 128 × 128 pixels). Optical images were processed and analysed using custom scripts written in Matlab and ImageJ.

Results

An AFM was used to obtain topographic images of the cuticle of wild-type *C. elegans* under physiological conditions (methods). The major surface structures include the alae, three parallel ridges along the length of the worm on either side of the body, and a series of periodically separated annuli and furrows running perpendicular to the alae (Figure 1, A). Using soft cantilevers, optimized for contact-mode imaging in solution, an area of 10 × 10 μm was scanned at the worm surface (Figure 1, B). The analyzed image shows the ring-like segments of the worm including annuli and furrows (Figure 1, B).

Next we imaged the surface of two different cuticle collagen mutants that are longer (*lon-2*) or shorter (*dpy-5*) than wild-type worms (Figure 2, A) and compared their 3D-structure to that of wild-type animals. Quantifications of annuli width and furrow depth show correlations to the body size of the animals and suggest that the total number of annuli is the same for wild-type, *dpy-5* and *lon-2*. Measured annuli depth and width may depend on scanning force applied, but the line profiles did not change at higher scan forces (Figure 2, B-F).

Next we analyzed additional mutants, one with annuli (*dpy-13*) and two in which the cuticle has been reported to lack annuli and furrows (*dpy-7*, *dpy-10*).¹³ *Dpy-13* animals

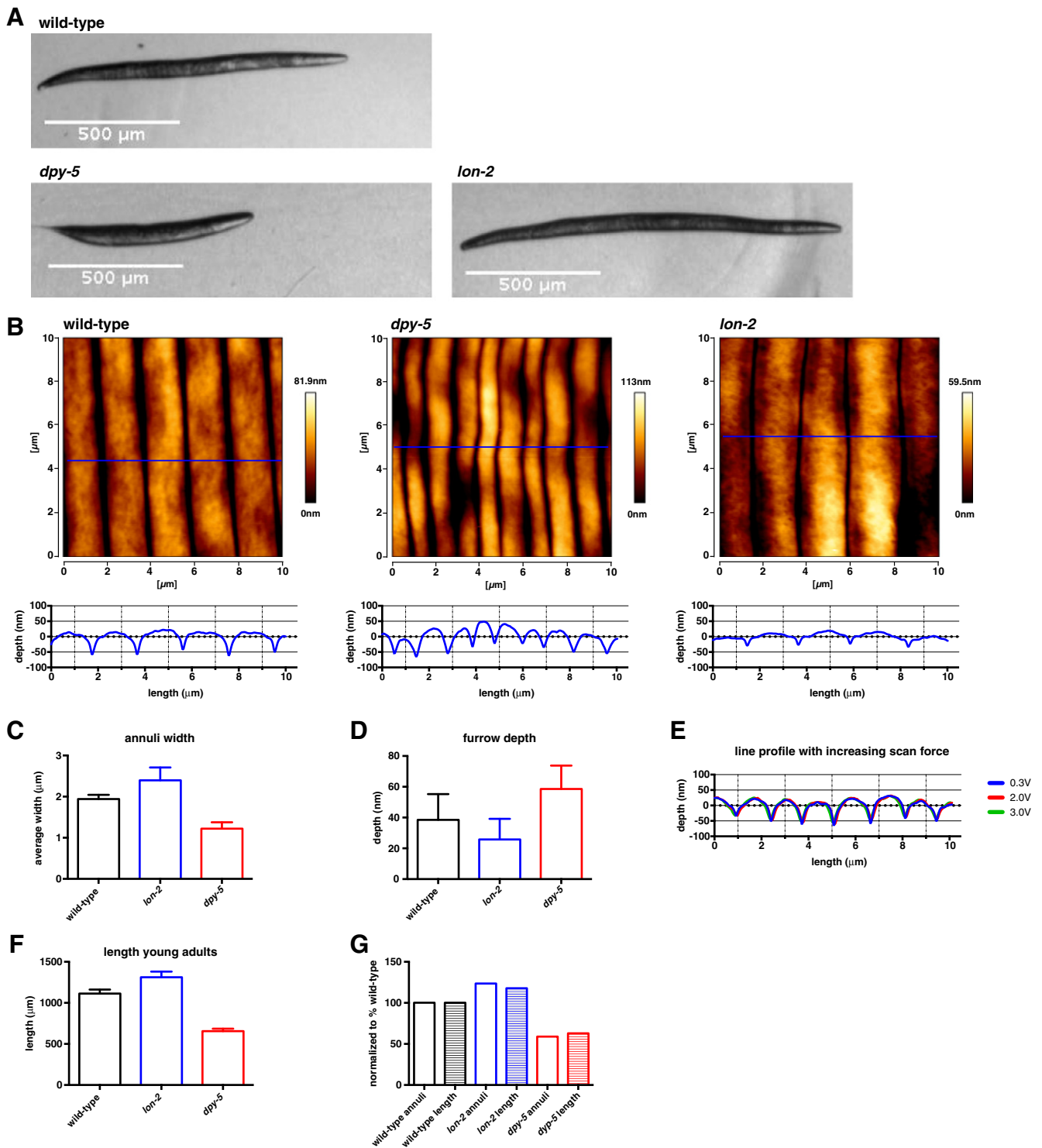


Figure 2. Analysis of wild-type *C. elegans* and cuticle mutant surface structures. **(A)** Brightfield images of wild-type and mutants (*dpy-5*, *lon-2*). **(B)** AFM topography images (top) and line histograms (bottom) of wild-type, *dpy-5* and *lon-2* animals. **(C + D)** Comparison of annuli/furrow width for wild-type and mutants represented as mean \pm SEM, $n > 30$. **(E)** Line profiles for topographic images with increasing scan force. **(F)** Comparison of body size of wild-type and mutants represented as mean \pm SEM, $n > 19$. **(G)** Comparison of body size and annuli width of mutants represented as percentage of wild-type.

show similar size/annuli ratios as *dpy-5* and wild-type animals, confirming a common total number of annuli for each organism (Figure 3, B). Surprisingly, and in contrast to their smooth

appearance in SEM images, AFM images of mutants without annuli reveal irregular surface patterns of similar height and depth as *dpy-5* animals (Figure 3, A).

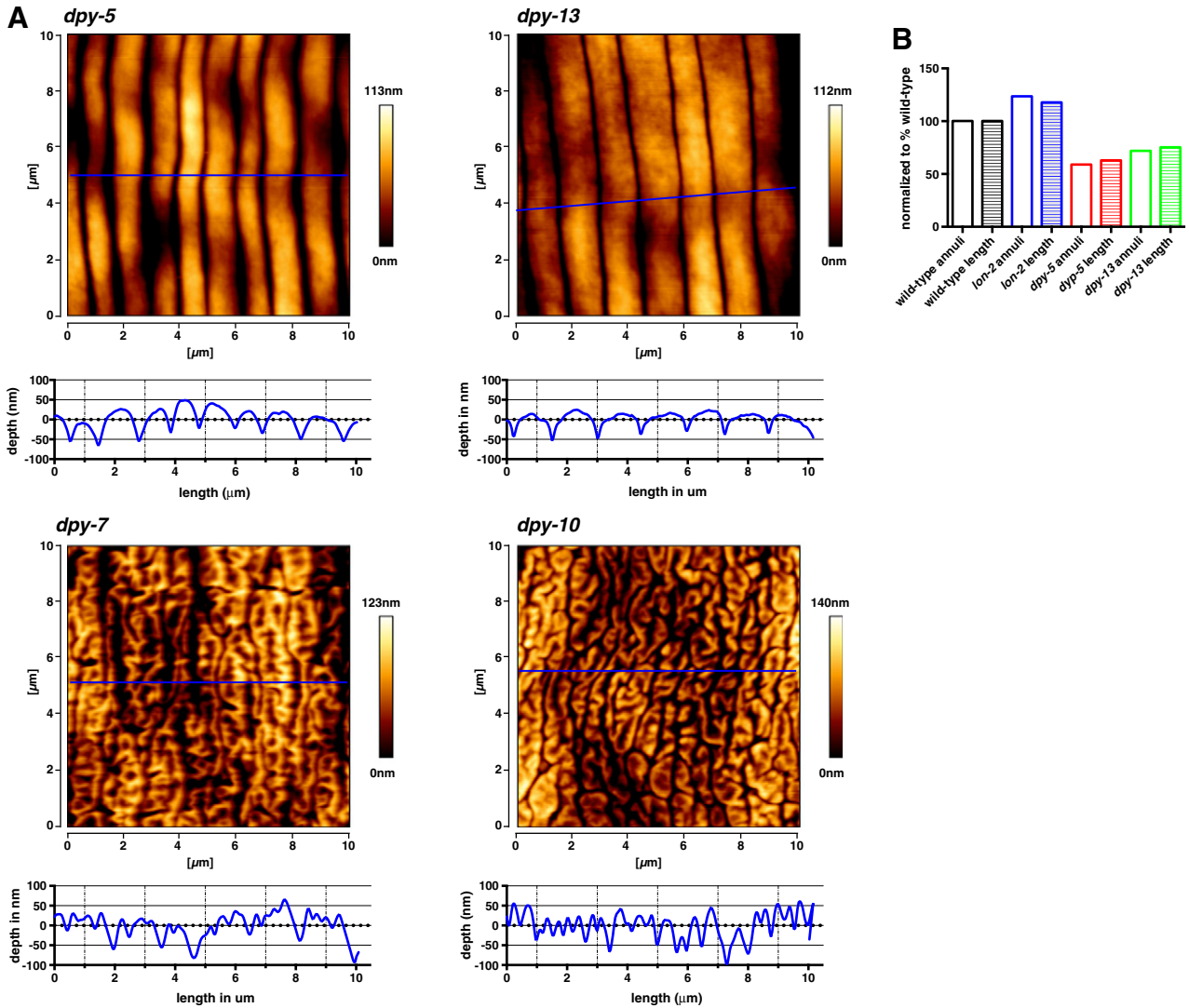


Figure 3. Comparison of mutants with and without annuli. (A) AFM topography images and corresponding line profiles. (B) Measured body size and annuli width of mutants represented as percentage of wild-type.

We confirmed our AFM surface analysis results by performing fluorescence imaging of DiI stained animals (Figure 4, A). Deconvolved focal series of images were analyzed for annuli width and matched the AFM results (Figure 4, B). When viewed as a maximum intensity projection, the cuticles of both non-annuli mutants appeared smooth in the optical images, similar to published SEM results. However, single viewing image planes reveal irregular net-like structures consistent with the AFM results (Figure 4, C).

To investigate whether sample preparation for SEM accounts for differences in the appearance of the cuticle, non-annuli mutants were treated with 3% glutaraldehyde, a fixative used for SEM.^{13,18} Comparing AFM images of fixed and non-fixed mutants reveals a slight reduction in surface details, however the cuticle still contained significant topographic structures (Figure 5, A).

To characterize biomechanical properties of the *C. elegans* cuticle we utilized the QI mode of the AFM, in which force-distance curves are recorded to determine topography and local mechanical

properties.¹⁹ We applied the Hertz/Sneddon contact mechanics model for small indentations considering the tip-shape of the indenter to estimate the elastic modulus at each location (Figure 5, B, Suppl Figure 2), RMS is in Figure 5, D.^{20–22} A 3D-overlay of topography and elastic modulus of the worm cuticle indicates that minimum values occur in annuli regions and maximal values in the furrows, the indentation depth is approximately 500 nm (Figure 5, C). The overall values are within the range of previously described data for *C. elegans* cuticle.^{23,24}

Discussion

We developed an experimental protocol to acquire high-resolution 3D surface topography data of paralyzed young adult *C. elegans* using AFM. In contrast to SEM, which requires dehydrated samples held under vacuum, our methodology enabled organisms to be imaged in-vivo under physiological conditions. Our results revealed

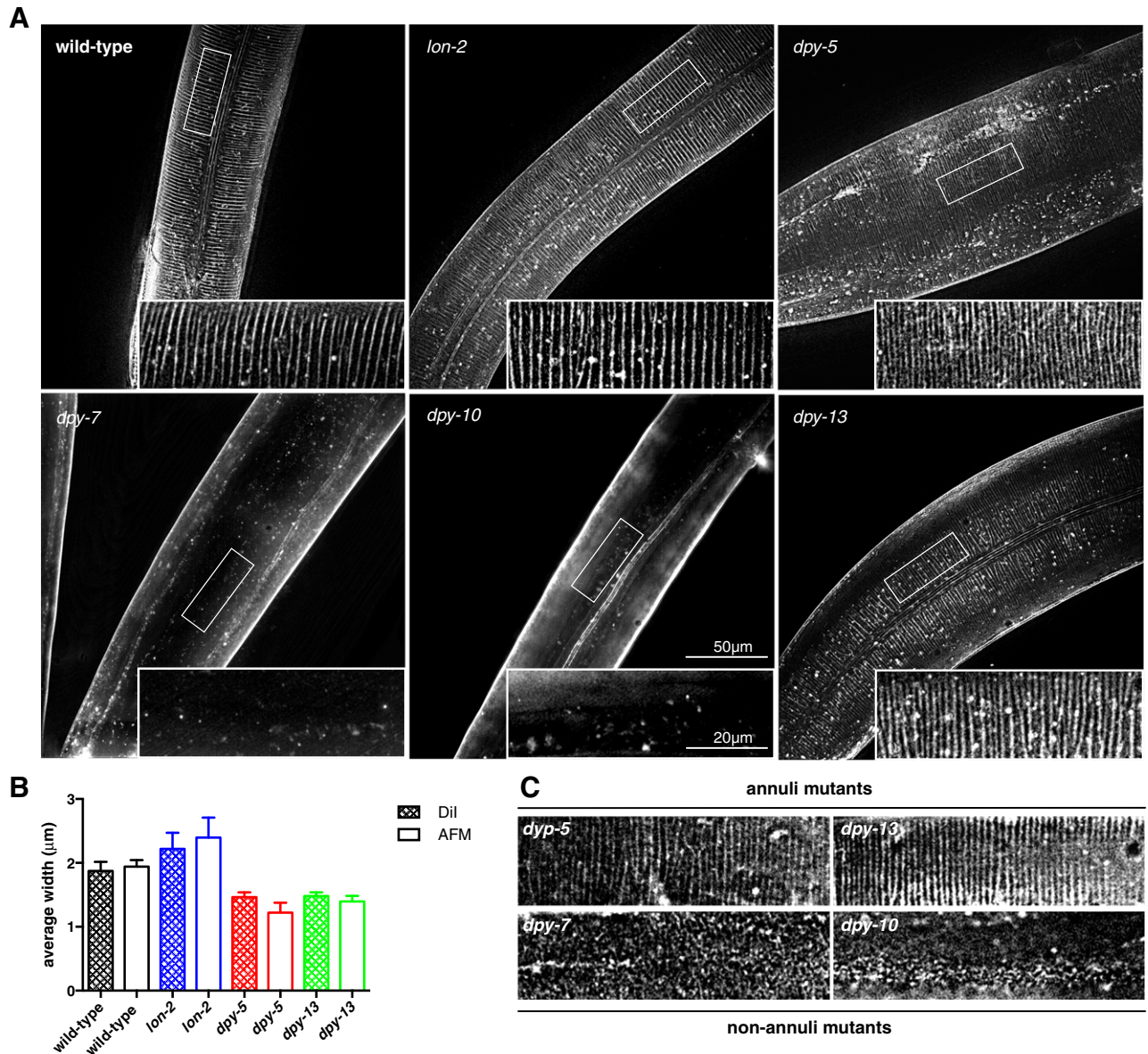


Figure 4. Fluorescence imaging of surface structures. **(A)** Representative images of deconvolved axial image projections of wild-type and mutants stained with DiI (see methods). Insets showing surface structures (annuli, furrows) highlighted by DiI staining. **(B)** Comparison of annuli width from AFM and fluorescence images of wild-type and mutants represented as mean \pm SEM, $n > 30$ (AFM), $n > 8$ (fluorescence). **(C)** Transverse image section showing DiI-highlighted structures in mutants.

previously unreported surface structures in *C. elegans* cuticle mutants which appeared smooth using SEM. Treating the worms with SEM fixative did not significantly remove the surface structure suggesting that subsequent dehydration steps, gold-particle coating, or data analysis might explain these differences. In particular, ethanol dehydration is likely to affect cuticle proteins, which are ethanol-soluble.²⁵ *C. elegans* is widely used in clinical research for drug screening and pathogen adherence studies. Mutations in cuticle proteins (e.g. *bus-5*, *srf-3*) affect the integrity of the cuticle and therefore pathogen adherence^{10,11} and drug uptake efficiency. In most cases the changes are attributed to the surface topography of these mutants but evidence is limited. Our AFM imaging technique offers an exciting new possibility to visualize and characterize these cuticle

mutants. In addition to the acquisition of detailed surface images we were able to measure force-displacement relationship and estimate cuticle elasticity. We have shown that QI imaging allows insights into spatial variations of stiffness (furrows stiffer than annuli) providing more detailed information about the cuticle's biomechanics. One possible explanation is that the collagen layer above the hypodermis and muscles is thinner in furrows, and that furrows are supported by collagen struts.⁸ Moreover, actin bundles at the apical layer of the hypodermis assisting to structure the furrows might contribute to the higher stiffness.

Together with topographic images, local biomechanical properties of cuticle mutants could shed light on the function of the mutated cuticle proteins like collagens, which remain largely unknown.

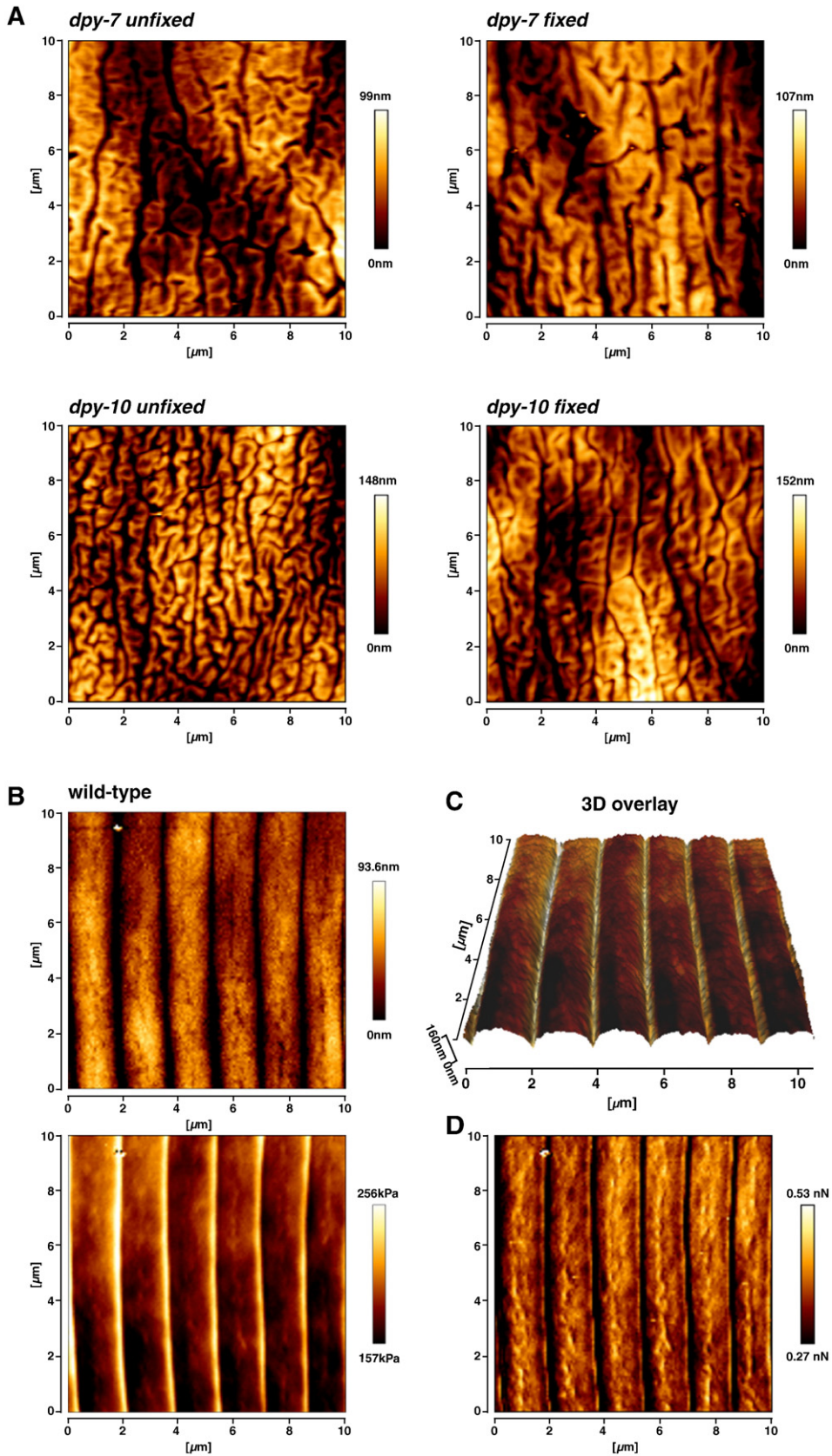


Figure 5. Surface characteristics. **(A)** AFM topography images of non-annuli mutants without and without SEM-fixative treatment. **(B)** AFM images of wild-type animals acquired in QI mode: topography (upper panel), elastic modulus (lower panel). **(C)** 3D-overlay of topography and elastic modulus from B. **(D)** RMS histogram shows accuracy of the fit.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.nano.2016.09.006>.

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