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Local Stiffness Measurements of *C. elegans* by Buckling Nanoprobes inside an Environmental SEM

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***Abstract*— This paper presents the local stiffness measurements of *Caenorhabditis Elegans* (*C. elegans*) by buckling nanoprobes through the nanorobotic manipulation system inside an Environmental-Scanning Electron Microscope (E-SEM). *C. elegans* has complex outer and inner structures constructed by approximately one thousand cells. For example, the lateral alae are the surface fine structure by seam cell body. In this paper, their fine structures were observed by E-SEM directly; without any drying or dyeing processes. The observation environments are controlled under different E-SEM chamber pressures for clear observation of *C. elegans*. The local stiffness of *C. elegans* was measured by buckling measurement method of the nanoprobe fabricated by Focus Ion Beam (FIB) etching at the tip of Atomic Force Microscope (AFM) cantilever. The measurement position can arbitrarily be controlled by the nanorobotic manipulator inside the E-SEM. In this work, From experimental results, the measured elasticity on lateral alae was 1.6 times larger than it around lateral alae. This local stiffness measurement technique can readily be applied to reveal unknown biological local stiffness, cell health conditions and novel cell diagnosis.**

I. Introduction

Single cells analysis has been much more attentions based on the recent progress of the micro/nano scale techniques on the local environmental measurements and controls [1]. The local environmental control/manipulation/measurement techniques are needed to realize an effective cell analysis as shown in Fig. 1.

Stiffness evaluation of biological cells is one of important technique to reveal the unknown properties of biological cells, cell health condition, novel cell diagnosis, and so on. The micromanipulation techniques are readily used for biological mechanical evaluations under optical microscope, for example, by the micro-glass plate [2], the optic fiber probe [3], the mechanical manipulation of micro-pipette [4].

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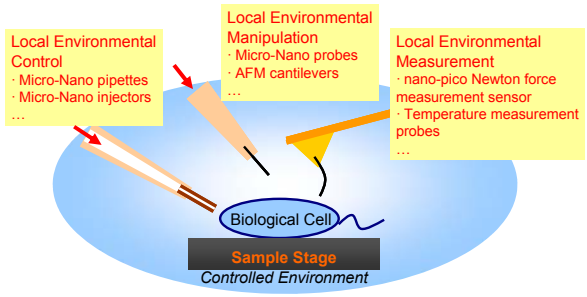


Fig. 1 Biological nanomanipulation for single cell analysis based on local environmental control/manipulation/measurement.

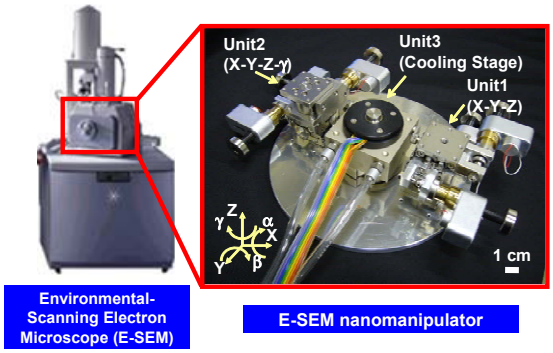


Fig. 2 Overview of E-SEM nanorobotic manipulation system.

Optical trapping technique is also investigated under optical microscope [5]. By optical microscope, the limitation of its imaging resolution prevent for nano-scale evaluation, caused by the diffraction limit of optical wavelength [6]. By the optical trapping technique, the applying forces are limited pico-Newton order, and the irradiation of laser beam might be affecting damage for the biological samples.

To realize nano-scale biological evaluation, an AFM is an effective microscope under various environments, such as vacuum, air and water [7-8]. The imagining probe can be also used as manipulations [9]. However, their observation and manipulation area is basically limited in the 2-D plane [10]. Real time observation is normally difficult caused by the mechanical scanning motion of the cantilever probes. On the other hand, the electron microscopes; scanning electron microscope (SEM) and transmission electron microscope (TEM), have superior on the 3 dimensional and real-time manipulations [11]. The sample chambers of these electron

Table 1. Specifications of Nanorobotic Manipulation System inside an Environmental-Scanning Electron Microscope (E-SEM)

Items	Specifications
DOFs	Unit1: 4 DOFs (X-Y-Z-γ), Unit2: 3 DOFs (X-Y-Z) Total: 7 DOFs
Actuators	7 Picomotors™, (Unit1, Unit2)
Work. Space	~ 16 mm × ~ 16 mm × ~12 mm × ±5°
Positioning Resolution	~ 30 nm (Unit 1, Unit2)
Cooling Stage	Unit3 (Cooling water temp. ± 20 °C)
Environmental Scanning Electron Microscope (E-SEM, FEI, Quanta 600)	
Vacuum Mode	E-SEM Mode (10–2600 Pa) Low Vacuum Mode (10–130 Pa) High Vacuum Mode (~10^{-4} Pa)
Acc. Voltage	0.2 ~ 30 kV
Resolution	3.5 nm (E-SEM Mode) 15 nm (Low Vacuum Mode) 3.5 nm (High Vacuum Mode)
Obs. Space	150 mm × 150 mm × 65 mm
Max. Obs. Area	ϕ0.5 mm (E-SEM Mode) ϕ18 mm (Low and High Vacuum Mode)
Detectors	SED, RED

microscopes are set under the high vacuum (HV) to reduce the disturbance of electron beam for observation. To observe water-containing samples, for example bio-cells, drying treatment processes are additionally needed. Hence, direct observations of water-containing samples are normally quite difficult through these electron microscopes.

We have been investigated the manipulation for biological samples using the Environmental-SEM (E-SEM) nanorobotic manipulation system [12]. It can be realized a direct observation of water-containing samples with nanometer high resolution by specially built secondly electron detector in the nanometer scale observation resolution. The evaporation of water is controlled by the low sample temperature and high sample chamber pressure.

In this paper, we focus on the manipulation for the he *Caenorhabditis Elegans* (*C. elegans*). The *C. elegans* were widely investigated as model organism for understanding molecular and cellular mechanism of human genes, because they have many homologues genes with the human disease genes. Some studies have been done on the homologues of human disease genes, for example lrk-1 (a Parkinson's disease gene homolog) [13], and wnk-1 (a familial hypertension gene homolog) [14]. Their complex inner and outer structures are revealed by optical microscopes. For example, the lateral alae are the surface mark of seam cell body [15]. However, the local stiffness depended on their fine structures has not been measured and evaluated.

Firstly, we present the E-SEM nanomanipulation system and observations of *C. elegans* under different chamber pressures. Their fine structures were observed by E-SEM directly; without any drying or dyeing processes. Then, the local mechanical stiff nesses are measured by the nanoprobe

Fig. 3 Optical Microcsope of *C. Elegances*.

fabricated by focused-ion-beam (FIB) at the tip of an AFM cantilever controlled by nanorobotic manipulation system. The local stiffness on or around the lateral alae are measured.

II. Nanorobotic Manipulation System inside Enviromental Scanning Electron Microscope

We have been constructed the nanorobotic manipulators inside an E-SEM [12]. The E-SEM can be realized a direct observation of water-containing samples with nanometer high resolution by specially built secondly electron detector. The evaporation of water is controlled by the sample temperature (0 ~ 40 °C) and sample chamber pressure (10 ~ 2600 Pa).

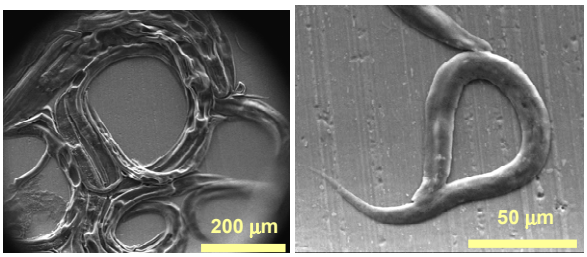
Figure 2 shows the overview of E-SEM nanorobotic manipulation system. This system realizes an effective sample preparation with 3 units and 7 degrees of freedom (DOFs) in total. The temperature of sample is controlled by the cooling stage unit, as Unit3. Table I is a list of their detail specifications of the E-SEM nanorobotic manipulation system.

III. Direct Observation of *C. elegans* by Enviromental-SEM

The *C. elegans* were observed with ESEM under different chamber pressures. One *C. elegans* has complex outer and inner structures by about one thousand cells. For example, the lateral alae are surface mark of seam cell body [15]. Their fine structures are also observed directly by E-SEM under hydroscopic condition.

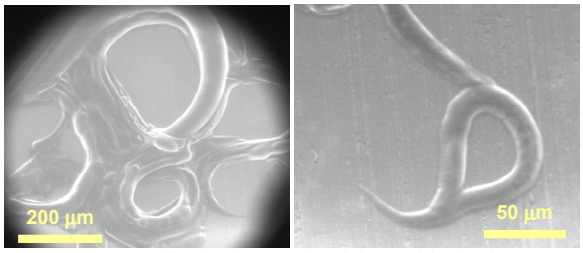
A. E-SEM Observation of C. Elegance under Different Pressures

The wild type *C. elegans* were cultured on the BP plate (0.3% NaCl, 1.5% agarose, 0.25 % peptone) for around 3 days at room temperature. The *C. elegans* are picked up from the cultured plate and dispersed in pure water. Figure 3

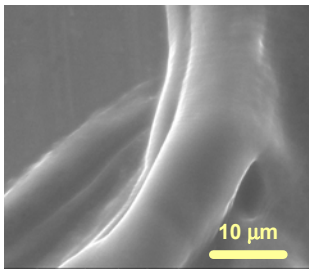


(a) Low magnification image (b) High magnification image

Fig. 4 E-SEM image of *C. elegans* under HV mode.



(a) Low magnification image (b) High magnification image

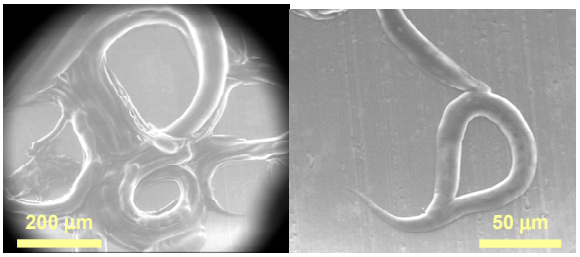


(c) High magnification image

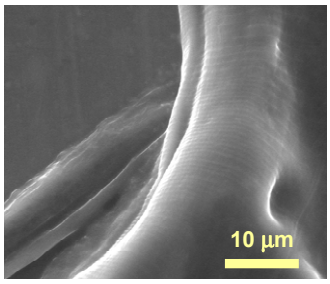
Fig. 5 E-SEM image of *C. elegans* under 600 Pa.

shows their optical microscopic images taken by a stereoscopic microscope. The lengths of *C. elegans* are from 100 µm to 1 mm depending on their growth phases.

Their electron microscopic images are taken under High Vacuum (HV) as shown in Fig. 4. Several micro liter of the solution is dropped on the temperature controlling staged of E-SEM manipulation system by a micropipette. The HV mode is operated with the conditions of the room temperature and 2.03×10^{-3} Pa pressure. Almost all of the *C. elegans* have concave and broken structure under HV mode. The ESEM images are shown in Fig. 5, Fig. 6, and Fig. 7 operated with the conditions of 0.0 °C and 400, 500, and 600 Pa pressures. Almost of the cells keep the round shape structures by water-contained condition with ESEM operation. The surfaces of *C. elegans* are slightly covered by water adsorption under 600 Pa (Fig. 5). By decreasing the pressure to 400 Pa, the surface structures are observed clearly by evaporating the water gradually as shown in Fig. 6 and Fig. 7. The cell surface and inner structures are clearly observed depending on the water absorption by E-SEM (non-coating/non-dyeing/non-special treatment).

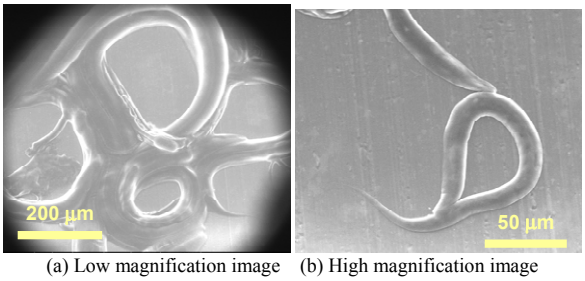


(a) Low magnification image (b) High magnification image

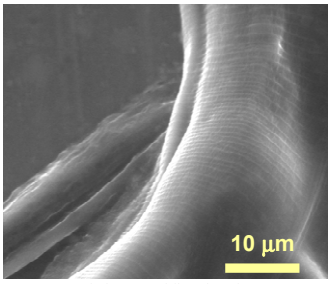


(c) High magnification image

Fig. 6 E-SEM image of *C. elegans* under 500 Pa.



(a) Low magnification image (b) High magnification image

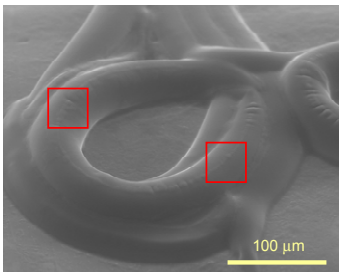


(c) High magnification image

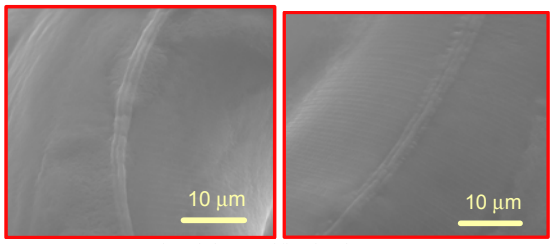
Fig. 7 E-SEM image of *C. elegans* under 400 Pa.

B. E-SEM Observation of C. Elegance under Different Pressures

The fine structures of *C. elegans* were directly observed by E-SEM. Figure 8 show the surface of *C. elegans*. The three nano-lines are observed the side of *C. elegans*. The lines are called “lateral alae” which is marks of the final growth state on the growth phases. They are picked up from the cultured plate and dispersed in pure water. Figure 3 shows their optical microscopic images by a stereoscopic

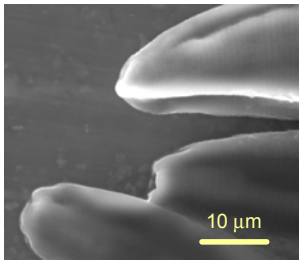


(a) Low magnification images

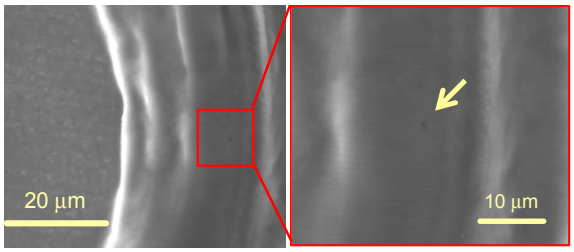


(b) High magnification images

Fig. 8 E-SEM image of *C. elegans* Lateral alae.



(a) Lips



(b) Excretory pore

Fig. 9 E-SEM image of *C. elegans* fine strucutures.

microscope. The lengths of *C. elegans* are from 100 µm to 1 mm depending on their growth phases. Figure 9 shows the other fine structures of *C. elegans*. The "Lips" and "Excretory pore" are observed clearly by E-SEM.

IV. Local Stiffness Measurement of *C. elegans* by Nanoprobe through Enviromental-SEM Nanorobotic Manipulation System

The local stiffness of *C. elegans* was measured by buckling of nanoprobe fabricated by FIB etching at the tip of AFM cantilever. As depicted in Fig. 10, the local mechanical

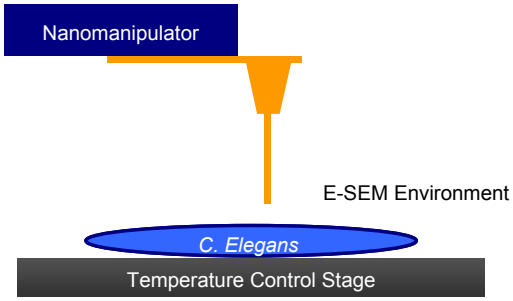


Fig. 10. Local stiffness measurement of single *C. elegans* by nano-probe.

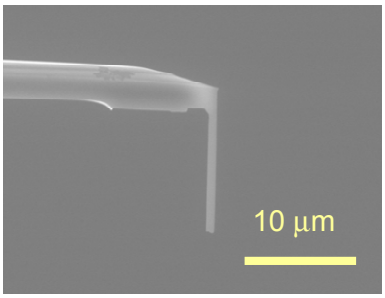


Fig. 11 E-SEM image of fabricated nanoprobe by FIB etching.

stiffness evaluation will be done though the nanoprobes at the tip of an AFM cantilever. The fabricated nanoprobe is shown in Fig. 11 (width a: 725 nm, length: 11.3 µm). The measurement position can be arbitrarily controlled by nanorobotic manipulator inside E-SEM. The local stiffness of *C. elegans* was measured based on the E-SEM observation of their fine structures such as on or around lateral alae.

A. Stiffness Measurement by Buckling of Nanoprobe for Biological Samples

The Hertz-Sneddon models which based on the shape of the tips, i.e. conical, spherical, and cylindrical, were used to calculate the elastic modulus of the cells E_{Cell}. The equations are derived from the classic Hertz's mechanics model for linear elastic material [16].Parameters of E_{Cell}, v, δ, and a are the elastic modulus of cells, the Poisson's ratio (v = 0.5 for soft biological materials [17]) of the elastic half space (cell's surface), the deflection of cells by applied forces, and the radius of a nanoprobe, respectively. Values for δ, and a are obtained from E-SEM images.

Equation (1) is used to calculate the stiffness of the cell from an indentation by the nanoprobe indenter. The nanoprobe has a rectangular cross section. However, it has been shown that the error using the models for non body of revolution is very small as explained in detailed by [18-20].

$$E_{Cell} = \frac{F_{Cell}(1-r^2)}{a\delta} \quad (1)$$

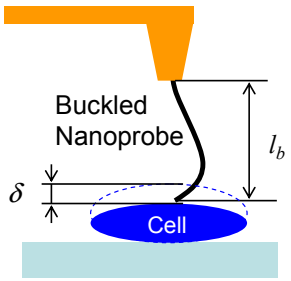
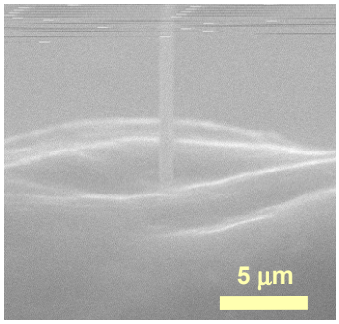
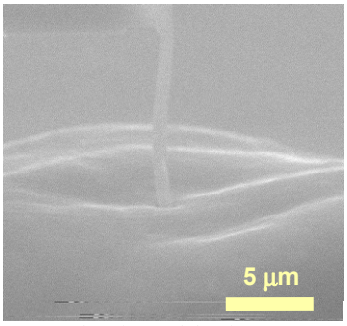


Fig. 12 Local mechanical measurement on the lateral alae of *C. Elegance*.



(a) Before contacting.



(b) After applying force.

Fig. 13 Local mechanical measurement on the lateral alae of *C. Elegance*.

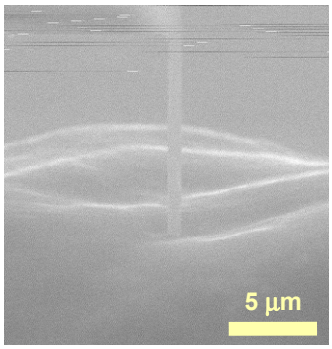
B. Appling Force Measurement by Buckling of Nanoprobe

The applying forces by buckling of nanoprobe are calculated from Euler's equation. The experimental set-up is schematically drawn as Fig. 12. The indentation was carried out until the nanoprobe begun to buckle. Then, the buckled nanoprobe was slowly retracted until the nanoprobe returned to the straight condition.

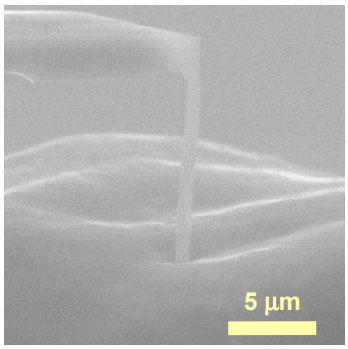
The value of E_P was determined from the Euler buckling equation as shown in (2) where The geometric moment of inertia was obtained from the second moment of area $I = a^4/12$ into in the derivation:

$$F_P = \frac{\pi^2 E_P a^4}{12(cl_b)^2} \qquad (2)$$

where, F_P is the buckling force applied to the nanoprobe, E_P is the Young Modulus of the nanoprobe, l_b the buckled length of the nanoprobe, c is the nanoprobe effective length



(a) Before contacting.



(b) After applying force.

Fig. 14 Local mechanical measurement around the lateral alae of *C. Elegance*.

factor, whose value depends on the conditions of the end support of the nanoprobe and the length of the nanoprobe. The value of the length of the soft nanoprobe was corrected with c: 0.8 for the structure which has one fixed end and the other pinned end [21]. For the experiment, the elastic modulus E_P of silicon nanoprobe is assumed as 100 GPa [22].

C. Local Stiffness Measurement of C. elegans by Nanoprobe inside E-SEM

Figure 13 show the local stiffness measurement on the lateral alae of *C. elegans* by nanoprobe. The position of nanoprobe is set on the lateral alae of *C. elegans* as shown in Fig. 13 (a). Then, the local force is applied on it by nanoprobe as shown in Fig. 13. The nanoprobe is slightly buckled. From these images, the applied force is calculated as 394 µN by the equation (2) from the δ deformation of cell surface. Figure 14 (a) and (b) show the local stiffness measurement around the lateral alae of *C. elegans*. Figure 14 (b) shows after applying the forces by nanoprobe. From these images, the applied force is calculated as 359 µN by the equation (2) from the δ deformation of cell surface. Figure 15 shows the after the indentation experiment by nanoprobe on/around the lateral alae of *C. elegans*.

The elastic moduli on/around lateral alae are calculated from these results as 410/257 MPa. From these results, the elasticity on the lateral alae is 1.6 times higher than it around

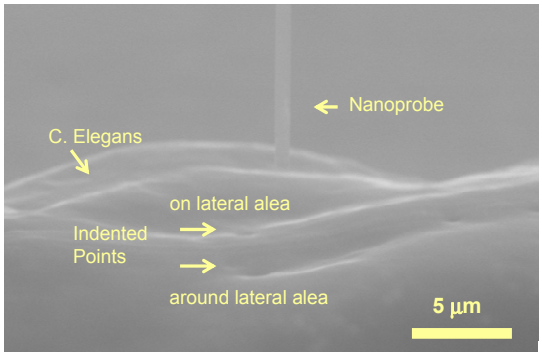


Fig. 15 After the local stiffness measurement on/around the lateral alae of *C. Elegance* (two arrows show the indented points).

lateral alae. This is considered to be caused by the inner structure on the lateral alae [15]. The surface of *C. elegans* is much thicker than other area. This is the first experimental evaluation on the local stiffness of *C. elegans*. Though the proposed evaluation method, wider area on the *C. elegans* will be measured. By changing the nanoprobe sizes, more local information of stiffness will be also obtained.

V. Conclusion

We present the local stiffness measurements of *C. elegans* by the nanorobotic manipulation system inside an E-SEM for the first time. Their fine structures were observed by E-SEM directly; without any drying or dyeing processes. The local stiffness of *C. elegans* was measured by buckling measurement method of the nanoprobe fabricated by FIB etching at the tip of AFM cantilever. The measurement position can arbitrarily be controlled by the nanorobotic manipulator inside the E-SEM. In this work, the local stiffness on or around the lateral alae were measured. From experimental results, the measured elasticity on lateral alae was 1.6 times larger than it around lateral alae. This local stiffness measurement technique can readily be applied to reveal unknown biological local stiffness, cell health conditions and novel cell diagnosis.

Acknowledgment

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