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].

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
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. 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.

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

<table>
<thead>
<tr>
<th>Items</th>
<th>Specifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOFs</td>
<td>Unit1: 4 DOFs (X-Y-Z-γ), Unit2: 3 DOFs (X-Y-Z), Total: 7 DOFs</td>
</tr>
<tr>
<td>Actuators</td>
<td>7 Picomotors™, (Unit1, Unit2)</td>
</tr>
<tr>
<td>Work. Space</td>
<td>~ 16 mm × ~ 16 mm × ~12 mm × ±5°</td>
</tr>
<tr>
<td>Positioning</td>
<td>~ 30 mm (Unit 1, Unit2)</td>
</tr>
<tr>
<td>Resolution</td>
<td>Unit3: 4 DOFs (X-Y-Z-γ), Unit2: 3 DOFs (X-Y-Z), Total: 7 DOFs</td>
</tr>
<tr>
<td>Cooling Stage</td>
<td>Unit3 (Cooling water temp. ± 20 °C)</td>
</tr>
</tbody>
</table>

Environmental Scanning Electron Microscope (E-SEM, FEI, Quanta 600)

<table>
<thead>
<tr>
<th>Vacuum Mode</th>
<th>E-SEM Mode (10 ~ 2600 Pa)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low Vacuum Mode (10 ~ 130 Pa)</td>
</tr>
<tr>
<td></td>
<td>High Vacuum Mode (~10^-4 Pa)</td>
</tr>
<tr>
<td>Acc. Voltage</td>
<td>0.2 ~ 30 kV</td>
</tr>
<tr>
<td>Resolution</td>
<td>3.5 nm (E-SEM Mode)</td>
</tr>
<tr>
<td></td>
<td>15 nm (Low Vacuum Mode)</td>
</tr>
<tr>
<td></td>
<td>3.5 nm (High Vacuum Mode)</td>
</tr>
<tr>
<td>Obs. Space</td>
<td>150 mm × 150 mm × 65 mm</td>
</tr>
<tr>
<td>Max. Obs. Area</td>
<td>Ø0.5 mm (E-SEM Mode)</td>
</tr>
<tr>
<td></td>
<td>Ø18 mm (Low and High Vacuum Mode)</td>
</tr>
<tr>
<td>Detectors</td>
<td>SED, RED</td>
</tr>
</tbody>
</table>

Fig. 3 Optical Microscope 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 ENVIRONMENTAL-SEM

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 ENVIRONMENTAL-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 inner structures are also observed directly by E-SEM under hydropoetic 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

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

The fine structures of C. elegans were directly observed by E-SEM. Figure 8 shows 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 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).
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 stiffness evaluation will be done though the nanopores 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 $E_{Cell}$, $\nu$, $\delta$, and $a$ are the elastic modulus of cells, the Poisson's ratio ($\nu = 0.5$ 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 $\delta$ 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].

$$E_{Cell} = \frac{F_{cell}(1 - r^2)}{a \delta}$$  \hspace{1cm} (1)
B. Applying 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 began 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:

$$F_p = \frac{\pi^2 E_p a^4}{12(c l_b)^2}$$  \hspace{1cm} (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 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
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

The authors are grateful to Prof. K. Matsumoto and Prof. N. Hisamoto at Nagoya University for providing with C. elegans samples and . This work was partially supported by MEXT KAKENHI (17076004) and Japan Science and Technology Agency.

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