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Investigation of penetration force of living cell using an atomic force microscope †

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Abstract

Recently, the manipulation of a single cell has been receiving much attention in transgenesis, in-vitro fertilization, individual cell based diagnosis, and pharmaceutical applications. As these techniques require precise injection and manipulation of cells, issues related to penetration force arise. In this work the penetration force of living cell was studied using an atomic force microscope (AFM). L929, HeLa, 4T1, and TA3 HA II cells were used for the experiments. The results showed that the penetration force was in the range of 2~22 nN. It was also found that location of cell penetration and stiffness of the AFM cantilever affected the penetration force significantly. Furthermore, double penetration events could be detected, due to the multi-membrane layers of the cell. The findings of this work are expected to aid in the development of precision micro-medical instruments for cell manipulation and treatment.

Keywords: Atomic Force Microscope (AFM); Cantilever stiffness; Living cell; Penetration force; Nano-surgery

1. Introduction

Understanding mechanical and damage characteristics of biological tissues is essential for effective surgical treatment and other areas in the medical field. Particularly, the effect of externally applied force on internal organ tissues and cells should be assessed in order to design robotic surgical tools with optimal power. Such information is needed to generate enough force during surgical operations. Also, for development of a robotic micro-endoscope that has a self-propelling capability, the critical traction force between the foot of the robotic endoscope and the organ surface should be optimized to generate enough friction but not high enough to cause tissue damage [1]. Thus, an understanding of mechanical characteristics of biological tissues with respect to externally applied force and the critical force that can cause tissue damage should precede the development of simulation programs used for practicing precise surgery, surgical robots utilized in surgical operation, and a self-propelling robotic micro-endoscope [2].

In addition, information about the forces needed to manipulate cells is important for future medical applications such as transgenesis, in-vitro fertilization, individual cell based diagnosis, and pharmaceutical tests. Also, one of the core applications of nanorobots in the future is medical treatment. These nanorobots aim for direct drug delivery to the cells as well as injecting and destroying the diseased cells moving through the blood vessel [3, 4]. Their functions are to grab, puncture, and destroy the harmful living cells. Thus, to design nanorobots to perform these tasks properly, the external force needed to penetrate cells must be assessed.

Due to advancements in nanotechnology, the study on cell mechanics has been growing. Recently, microinjection using microcapillaries has been widely

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exploited to inject proteins, peptides, and genetic materials [5, 6]. However, damage problems arising from using microcapillaries still remain due to the shape and inaccurate displacement of the device. To overcome these problems, the force needed to penetrate the cell and interactions between the cell and the injecting device should be known in advance.

The atomic force microscope (AFM) is a powerful tool for biological research such as direct force measurement of interaction between liposomes and molecules [7], mechanical properties of living cells [8-12], and penetration forces of living cells [8, 13]. As studies on penetration forces of living cells are beginning to emerge, the previous studies focused on the measurement of penetration force based on the shape of the tip [14, 15]. The penetration force obtained by using a needle-shaped tip was 1-2 nN and the possibility of penetration increased with smaller and cylindrical needles. It was also found that the penetration force increased with the speed of penetration [16].

In this work, the effects of AFM cantilever stiffness on the penetration force characteristics were investigated. Since cantilever stiffness is a mechanical design issue of the measurement device, comparison of the penetration force due to different cantilever stiffness will be important from the user's point of view. Also, analyzing the penetration force characteristics of a cell by using a standard AFM system will be of interest for proper manipulation of the living cell under more realistic and practical conditions.

2. Materials and method

2.1 Preparation of living cell sample

The cell lines used for the experiments were L-929 mouse fibroblast (ATCC®), HeLa human uterine cancer cell (ATCC®), 4T1 mouse colon cancer cell, and TA3 Ha II mouse breast cancer cell. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% Fetal Bovine Serum (FBS) and 1% antibiotics. They were incubated in 5% CO_2 environment at 37.5°C. The cells used in the experiments were all adherent cells. For the experiments, the cells were grown on an 18 mm diameter cover glass (Paul Marienfeld GmbH & Co. KG). The cover glass on which the cells were grown was attached to a metal block. Since the cover glass was transparent, it was difficult to find the sample cells with the AFM optical system. The metal block allowed the sample cells on the cover glass to be observed by reflecting light from its surface. The experiments were conducted in a cell culturing medium to keep the cells alive. The cell culturing medium was put into a Teflon dish and the sample cells on the metal block were immersed in the Teflon dish. As for the AFM system, SPA400 from Seiko Instruments Inc. was used. It was equipped with an electronic control system, and dedicated software was used to record the force-displacement (F-d) curves. From the F-d curves the force interaction between the surface of the sample and the tip could be obtained. A special cantilever holder was adopted for operation in liquid environment. To eliminate the variation in the contact geometry between the tip and the cell during indentation, same pyramidal-shaped Si cantilevers were used for the cell penetration experiments. Cell penetration forces were measured with low stiffness cantilevers of 0.16 N/m, 0.2 N/m, and 0.26 N/m, and a high stiffness cantilever of 23 N/m was used to see the effects of stiffness on the penetration force. The stiffness values were provided by the supplier of the AFM cantilevers (Nanosensors).

2.2 Experimental set-up and method

Fig. 1 shows the schematic of the experimental set-up for measuring the penetration force of living cells. Indentation was performed with an AFM to obtain the F-d curve that provides the relationship between the force applied on the cantilever and the distance of cantilever movement. The tip at the end of the cantilever applies a normal force on the cell surface to cause indentation as the tip is lowered toward the cell. The F-d curve displays the deflection of the cantilever as the substrate moves in the vertical direction driven by a piezoelectric scanner. The piezoelectric scanner moves the sample up and down at a constant speed, which is adjusted by the total moving distance and time. The time refers to the total time for the scanner to complete the up and down motion of the sample.

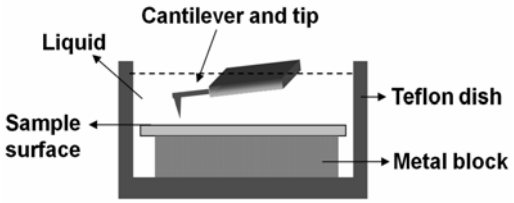


Fig. 1. Schematic of the AFM experimental set-up for measuring the penetration force of living cells.

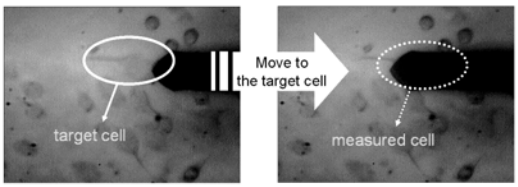


Fig. 2. Top view of cantilever positioned above the measured cell. The left picture shows the target cell and the tip of the cantilever. The right picture shows the tip aligned vertically with the target cell.

Same speed was used in all experiments to eliminate the variation in the viscoelastic behavior of the cell. The F-d curve of living cell was obtained after positioning the cantilever tip above the cell to be measured by using a micro-stage.

Fig. 2 shows the photographs of the cantilever tip and the target cell adhering to the cover glass. The experiment was performed within 3 hours after the cells were taken out from the incubator to minimize the transformation of the cell due to environmental effects. To see the difference between the central region near the nucleus and the outer peripheral region of the cell, measurement locations were chosen in these respective regions.

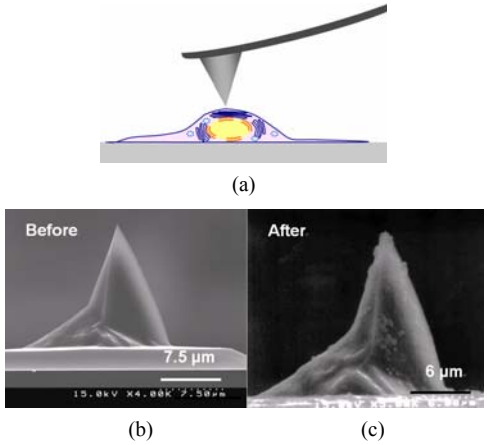
3. Results and discussion

3.1 Range of penetration force

To investigate the penetration force of cells, the adherent cells on the cover glass were measured with Si cantilevers. Fig. 3 shows the schematic of tip contacting the cell and SEM images of the Si tip before and after the penetration experiment. The tip height was 11.5 μm with a radius of about 10 nm. Fig. 3(b) shows that the apex of the tip became contaminated after penetration due to organic materials from the culturing media and the cell surface structure. The measurements were performed with different types of cells. The range of penetration force for each cell line is indicated in Table 1. The range of penetration force of L929 was 3-6 nN and 18-22 nN. It was 2-13 nN for HeLa, 2-17 nN for 4T1, and 3-9 nN for TA3 HA II. Also, it was found that the cells were not penetrated for every indentation. Thus, the percentage of penetrated indention was obtained as well. The reason why the penetration did not occur every time was attributed to the local variation of the cell surface structure. Nevertheless, the percentage of penetration for different cells was also taken as an important data for understanding the penetration characteristics of the cell.

Table 1. Penetration force range and percentage of penetration for different type of cells.

Cell type	Range of penetration force	Percentage of penetration
L929	3-6 nN, 18-22 nN	19.6 % (9/46), 21.4 % (6/28)
HeLa	2-13 nN	41.7 % (15/36)
4T1	2-17 nN	5.8 % (4/ 69)
TA3 HA II	3-9 nN	12.8 % (6/47)



(a)

(b) (c)

Fig. 3. (a) Schematic of Si tip contacting the cell and (b) SEM images of Si tip before and after the experiment.

The percentage of penetration of L929 was around 20 %, HeLa was 41.7 % (15/36), 4T1 was 5.8 % (4/69), and TA3 Ha II was 12.8 % (6/47). Generally, the percentage of penetration was not high and the penetration force was in the range of 2~22 nN. In the case of L929, the penetration force range was divided into two regimes. In 9 out of 46 cases the penetration force was in the range of 3~6 nN whereas in 6 out of 28 cases, the range was 18~22 nN. Except for the L929 group with 18~22 nN penetration force, the average penetration force of all cells tested was 5.2 ± 3.4 nN with 0.16 N/m stiffness pyramidal tip.

3.2 Double penetration of cell

Fig. 4 shows a typical F-d curve obtained with the L-929 fibroblast. The x-axis represents the displacement of the tip as it approaches and retracts from the cell surface and the y-axis represents the force between the tip and the cell. The upper curve is the loading curve during indentation and the curve below is

the unloading curve during retraction. The contact point indicated in the figure represents the location where the tip initiates contact with the cell. This can be determined by the point where the force begins to increase.

A cell is a unit system enveloped with cell membrane filled with organelles and cytoplasm. Thus, the composition of a cell is quite complicated with a composite-like structure. As such, penetration of cell membrane and the underlying layers lead to a unique behavior during indentation. The penetration of the cell membrane can be ascertained by noting the sudden jump in the loading curve as indicated in Fig. 4.

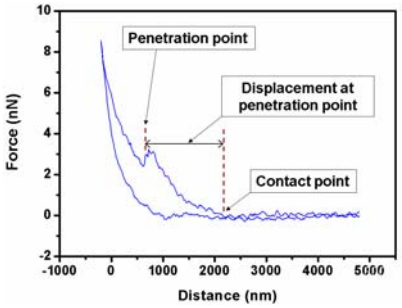


Fig. 4. F-d curve for L-929 fibroblast obtained with an AFM using a low stiffness cantilever (0.26 N/m).

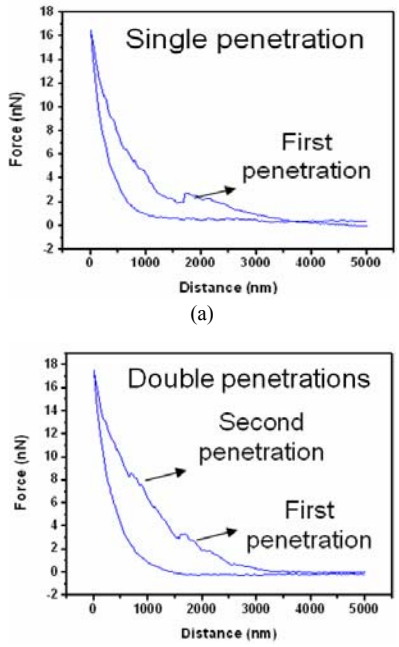


Fig. 5. F-d curves showing (a) single penetration and (b) double penetrations.

One of the interesting findings of penetration tests performed in this work was the double penetration phenomenon of the cell as shown in Fig. 5. As animal cells consist of a number of membranes including outer cell membrane, double envelope of nucleus, lamellar structures of endoplasmic reticulum (ER), and golgi apparatus [17], cells behave differently from general homogeneous materials with respect to indentation and compression. Namely, the compressive force applied by the tip can lead to more than one membrane penetration of the cell. This finding will provide significant insight for accurate manipulation of the living cell as well as precise injection of drugs and genetic materials into the living cell.

3.3 Effects of measurement location

As mentioned before, the range of penetration force of L929 was divided into two regimes: one with 3~6 nN range and the other with 18~22 nN range indicated by #1 and #2, respectively, in Fig. 6. The #1 graph shows a nonlinear increase in force after contact and the contact point is close to the starting point of the measurement. On the contrary, the #2 graph shows a steep increase in force after contact and the contact point is relatively far from the starting point of measurement. The reason for the significant difference between the two groups can be explained by the location of cell indentation. The adherent cell is spread on the cover glass with the peripheral region being thinner than the center or nucleus region. #2 data is from the measurement conducted at the peripheral or lower region of the adherent cell. The force increase after contact at the peripheral region of the cell is steep since it is influenced by the hard substrate. The experimental results indicate that the softer part near the nucleus has a relatively smaller penetration force and the peripheral region has a relatively

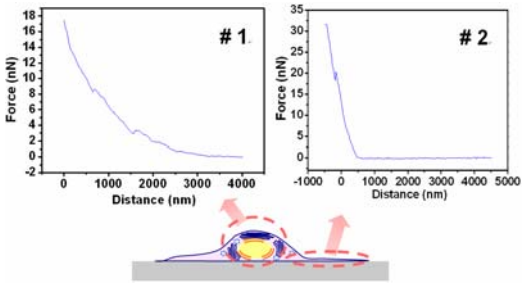


Fig. 6. F-d curve measured at different locations on the cell. #1 at the center region and #2 at the peripheral region.

higher penetration force.

3.4 Effects of cantilever stiffness

The effects of cantilever stiffness of the AFM system on the penetration force were also investigated. In this experiment two cantilevers with stiffness values of 23 N/m and 0.26 N/m were used. The penetration force and displacement between the two different cantilevers were compared. The total travel distance of the cantilever was 10 μm (up and down motion) and the speed was 1 μm/s. Since the speed was the same for both cantilevers, the displacement of the cantilevers for a unit time was the same. Therefore, the stiff cantilever exerted a much higher force than the flexible cantilever for a unit displacement. The stiff cantilever led to a rapid increase in the applied force during indentation, and consequently, penetration was detected at a small displacement after contact as shown in Fig. 7. For the cantilever with 23 N/m stiffness, the displacement at penetration point was 256.5 ± 43.4 nm and the penetration force was 74.7 ± 32.6 nN.

On the other hand, the cantilever with 0.26 N/m stiffness showed fairly different results. Since the increase of applied force per unit displacement was smaller than the stiff cantilever, it was expected that the penetration force would be small and the displacement would be large after contact. The experimental results confirmed this reasoning resulting in 9.5 ± 1.4 nN penetration force and 2357.7 ± 164.4 nm displacement after contact. The flexible cantilever showed significantly smaller penetration force and larger displacement after contact than the stiff cantilever.

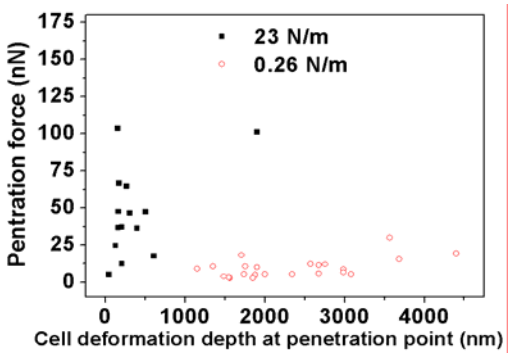


Fig. 7. Distribution of force and cell deformation depth at various penetration points for stiff (23 N/m) and flexible (0.26 N/m) cantilevers.

From the experimental results, it may be concluded that stiffness of the cantilever is a significant factor in the measurement of penetration force of a living cell. It implies that the penetration force may be different depending on the measurement device and penetration condition for the same type of cell. The flexible cantilever is more compliant and thus it is able to respond well to minute force variations. Thus, for high accuracy and repeatability in actual biomedical applications dealing with cells with soft and delicate surfaces, flexible cantilevers are more appropriate for AFM measurements of the cells.

4. Conclusions

Cell penetration forces were measured for different types of cells by using an AFM with various stiffness Si cantilevers. The cells used were L929, HeLa, 4T1, and TA3 HA II. The percentage of penetration for L929, HeLa, 4T1, and TA3 Ha II was 20 %, 41.7 %, 5.8 %, and 12.8 %, respectively. The penetration forces of the cells were in the range of 2~22 nN depending on the type of cell and the condition of indentation such as location. Also, evidence of more than one penetration event could be found during the indentation test, which was attributed to membrane layers of the cell.

The effects of cantilever stiffness on the penetration force were investigated with 23 N/m and 0.26 N/m cantilevers. The flexible 0.26 N/m cantilever showed 9.5 ± 1.4 nN penetration force and 2357.7 ± 164.4 nm displacement after contact, while the stiff 23 N/m cantilever showed 74.7 ± 32.6 nN penetration force and 256.5 ± 43.4 nm displacement after contact. The flexible cantilever was more compliant and thus it was able to respond better to minute force variations.

For high accuracy and repeatability in actual biomedical applications dealing with cells with soft and delicate surfaces, flexible cantilevers are more appropriate. Thus, for AFM measurement of cells the effects of substrate, location of measurement, multilayer membrane structure of cell, and stiffness of cantilever should be carefully considered to obtain reliable and meaningful measurement results.

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