Evaluation of the insertion efficiencies of tapered silicon nanoneedles and invasiveness of diamond nanoneedles in manipulations of living single cells*

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Summary. We have been developing a low invasive cell manipulation technology based on inserting an ultra-thin needle—"nanoneedle"—into a living cell by using an atomic force microscope (AFM). The nanoneedle, made from a silicon AFM tip by focused-ion-beam etching, has a diameter of several hundred nanometers and a length of about 10 microns. Successful insertion of the nanoneedle into the cell can be confirmed by the appearance of a steep relaxation of repulsive force in the force-distance curve as monitored by the AFM system. This technology, termed "cell surgery", can be applied for the detection of intracellular proteins in a living cell or for highly efficient gene transfer. The present study shows that the durability of a tapered nanoneedle is superior to that of a cylindrical nanoneedle, and that a proper aspect ratio for the tapered nanoneedle must be chosen to maintain sufficient insertion efficiency for a particular target cell: tapered nanoneedles of an aspect ratio over 20 showed high insertion efficiency for various kinds of mammalian cells. We then used diamond for the material of the nanoneedle because its specific properties, such as high stiffness, heat conductivity, and electrical conductivity capacitated by boron doping, were deemed useful for the analysis and manipulation of intracellular phenomena. We compared the capability of the diamond nanoneedle in cell manipulation with that of the silicon nanoneedle. Evaluation of the effect of the former on transcription efficiency and localization analysis of p53 expression revealed the low invasiveness for cell manipulation as was also the case for the silicon nanoneedle. We also succeeded in achieving highly efficient plasmid DNA delivery into a mouse fibroblast C3H10T1/2 using the diamond nanoneedle. The diamond nanoneedle is expected to contribute to the versatility of "cell surgery" technology.

Introduction

Single cell manipulation is one of the most important technologies in the field of life science, especially in biotechnology, diagnostics, and medicine. Yamanaka and his colleagues have recently found that only three or four gene expressions of transcriptional regulators can reprogram cellular properties to the embryonic state, thus generating an induced pluripotent stem (iPS) cell...
(Nakagawa et al., 2008, Takahashi et al., 2006). However, the rate of success of iPS cell generation using plasmid DNAs is very low (Okita et al., 2008), implying that the expression amount of the genes and their expression time must be controlled strictly. If very precise methods could be developed for handling molecules directly in a living cell, we would be able to investigate the mechanisms of reprogramming and differentiation in cells, and might also control the cellular states and their functions.

In order to achieve precise cell manipulation and analysis, we have been developing a mechanically invasive cell manipulation technology which can transfer substances directly into a cell or can extract substances from a cell using an etched ultra-thin needle (nanoneedle) operated by atomic force microscopy (AFM) (Obataya et al., 2005a, b). We have named this technology "cell surgery." In this technique, the size of the inserted material is important to minimize cell damage and to increase the insertion efficiency. Using a nanoneedle, we have succeeded in achieving highly efficient DNA delivery (Han et al., 2008), and monitoring of the effects of a hormonal drug by delivering an estrogen responsive GFP reporter vector into a human breast cancer cell (MCF-7) (Han et al., 2008). We have also performed protein delivery into a living human cervical cancer cell (HeLa) (Obataya et al., 2005c). Thus, this cell surgery technique allows the control of cellular behavior by introducing suitable amounts of genes and should be useful for detecting successive functions of molecules in a cell.

In our previous studies, we have etched a single crystalline silicon AFM tip to make a cylindrical nanoneedle that has a very high aspect ratio of 50 (200 nm diameter and 10 μm length) by using a focused ion beam (FIB). The nanoneedle was moved vertically toward a target cell by the piezo-tube scanner of an AFM. However, when the nanoneedle collides accidentally with the cell-culture dish during the insertion process or a lateral shear force is exerted on the needle, it is easily broken due to its high aspect ratio. To overcome this problem, we changed the shape of the nanoneedle from cylindrical to tapering. We first studied the relationship between needle shape and insertion efficiency using normal silicon nanoneedles since we already confirmed that a needle having either a conical apex or a normal pyramidal tip was difficult to insert into a cell (Obataya et al., 2005a, b).

Our other idea to strengthen a nanoneedle was to use diamond as its base material because of diamond’s extreme hardness. The high heat conductivity of diamond, 2000 W/m·K (cf. Si, 150 W/m·K), was also considered useful to control structural changes in molecules immobilized on the needle surface. If an electron conductive boron-doped diamond could be fabricated in the shape of a nanoneedle, the electron conductive nanoneedle might find further use as an electrode to analyze intracellular phenomena electrochemically. In this study to prove the applicability of the diamond nanoneedle, the invasiveness of diamond nanoneedle insertion into mammalian cells was investigated, and DNA delivery to a mouse fibroblast cell using an amine-modified diamond nanoneedle was performed.

**Materials and Methods**

**Cell culture**

Cell lines of mouse embryo fibroblast cells (C3H10T1/2), HeLa cells, human embryonic kidney cells (HEK293), pheochromocytoma cells (PC12), and human bone osteosarcoma epithelial cells (U2OS) were cultivated under routine maintenance in DMEM (Sigma-Aldrich, St. Louis, U.S.A.) supplemented with 10 % fetal bovine serum (FBS) and antibiotics/antimyocytes (GA, Cascade Biologies, Portland, U.S.A.). Human mesenchymal stem cells (hMSC) were cultivated in DMEM supplemented with 15 % α-MEM, normal human neonatal epidermal melanocytes (melanocyte) were grown in a M254S medium supplemented with melanocyte growth serum (HMGS), and MCF-7 were grown in RPMI 1640 supplemented with 10 % FBS and GA.

**Fabrication of silicon nanoneedles and diamond nanoneedles**

As shown in Figure 1A, the cylindrical nanoneedles and tapered nanoneedles were fabricated in the same manner as previously reported (Han et al., 2005, Obataya et al., 2005). For silicon nanoneedles, FIB etching was performed on trigonol pyramid silicon AFM tips (ATEC-Cont, Nanosensors, Neuchatel, Switzerland; spring constant 0.2 N/m). The aspect ratio of a tapered nanoneedle was defined as needle length (10 μm) divided by the diameter at the bottom of the needle. We prepared silicon nanoneedles with the aspect ratios of 20, 10, 5 and 3.3, which were fabricated by adjustment of the angles (θ) to 89.1°, 87.7°, 84.9°, and 82.0°, and were referred to as T20, T10, T5, and T3.3 nanoneedles, respectively. These tapered silicon nanoneedles have a flat end with a 200 nm diameter at the apex. In addition, cylindrical nanoneedles with a flat end of 400 nm or 800 nm in diameter were also fabricated. All nanoneedles were tilted 13° with respect to their axes in order to compensate for
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Invasiveness tests of diamond nanoneedle insertion into a cell

To investigate the invasiveness of diamond nanoneedle insertion into a C3H10T1/2, 4,6-diamido-2-phenylindole (DAPI, Dojindo laboratories, Kumamoto, Japan) exclusion and transcriptional inhibition tests were performed. The DAPI exclusion test was performed in the same manner as previously reported (Han et al., 2005). Immediately before insertion of the silicon and diamond nanoneedles, the medium was replaced with a serum-free one containing 1 μM DAPI. The images were analyzed using the software Image Pro (Media Cybernetics, Maryland, USA).

A transcriptional inhibition test was performed using pd1EGFP (BD Biosciences, Franklin Lakes, USA) and actinomycin D (Wako Pure Chemical Industries Ltd., Osaka). The pd1EGFP-delivered HEK293 cells were treated with 50 μM actinomycin D. After the treatment, fluorescence images were observed and the time course of fluorescence intensity was investigated using Image Pro. As a control, nanoneedles were inserted into single pd1EGFP transfected HEK293 cells and the time course of their fluorescence intensity was also investigated in the same manner.

The localization of p53 was analyzed by an

Apparatus

A Nanowizard II BioAFM (JPK Instruments, Berlin, Germany) and MFP-1D™/MFP-3D™-Bio Systems (Asylum Research, Santa Barbara, USA) were used for insertion of the tapered nanoneedle into the various cell lines. The probe was placed on the stage of an inverted microscope and was maintained using a heater and a CO2 regulator to ensure the cell conditions. The apparatus was covered with a sound-proofing hood to reduce interference by acoustic noise. The approach and retract velocity was set to 5–10 μm/s in the insertion efficiency experiment. The force-distance curve was performed ten times in every single cell; about 100 force-distance curves were used for the evaluation of insertion efficiency with every tapered nanoneedle. To observe a cell during the AFM operation, a confocal laser scanning microscope (CLSM, FV 300, Olympus, Tokyo) was combined with the AFM.

Fig. 1. A: Schematic representation showing the process of tapered nanoneedle fabrication. An ATEC-Cont tip is etched using a focused ion beam from one direction, rotated 90° to face the corresponding triangular plate, and further etched in the same manner. The aspect ratio is controlled by the change of θ. B: SEM images of various tapered nanoneedles (nanoneedle, T20, T10, T5, T3.3 nanoneedle, and normal tip) Scale bar: 10 μm
**Fig. 2.** A cross sectional image of T10 nanoneedle insertion into a HEK293 cell using a confocal laser scanning microscope (Scale bar: 10 μm) and the force-distance curve during the nanoneedle insertion.

**Fig. 3.** Force-distance curves during the insertion of nanoneedles with different tapered angles into C3H10T1/2 cells. The success or failure of insertion is indicated as ○ or × respectively in each force-distance curve; all of the scales are 1 μm for the X axis and 1 nN for the Y axis. Successful insertion in this study was judged as a drop in magnitude of a force of over 100 pN.
immunostaining method, using a monoclonal anti p53 (human/mouse/rat) antibody (R&D Systems Inc., Minneapolis, USA) and an AlexaFluor488-labelled goat anti mouse IgG antibody (Invitrogen, Carlsbad, USA) toward U2OS cells which were cultured in DMEM on collagen coated glass based dishes for 3 days. One dish was irradiated with UV-C (wavelength of 200–280nm) of 25 J/m² intensity. The other two dishes were used for insertions of a silicon nanoneedle or a diamond nanoneedle. A silicon nanoneedle or a diamond nanoneedle was respectively inserted into a cell for 3 min. After the immunostaining, the treated dishes were also subjected to DAPI staining to confirm the intactness of the cell nuclei.

**DNA delivery using a diamond nanoneedle into a C3H10T1/2**

The DNA delivery was performed in the same manner as previously reported (Han et al., 2008). The surface of the diamond nanoneedle was treated with 2% APTES in ethanol for 1 hour after cleaning with piranha solution (H₂SO₄/H₂O₂, 3/1, v/v) for 15 min at 85°C and NH₄OH/H₂O₂/H₂O (1/1/5, v/v/v) solution for 5 min at 60°C. Then 100 ng/μl of the pEGFP DNA in a Tris-EDTA buffer was dropped onto the cantilever in order to adsorb the DNA on the positively charged surface of the nanoneedle by electrostatic interactions. Before the DNA delivery experiments, we transferred the cultured cells into serum-free medium (opti-MEM, Invitrogen). The DNA-adsorbed diamond nanoneedle was inserted into a C3H10T1/2 and maintained for 3 min to allow the diffusion of DNA into the nucleus. The medium was changed to a growth medium and incubated for 24 h.

**Results**

**Investigation of insertion probabilities of various tapered nanoneedles into various cell lines**

We first tested the strength of a silicon nanoneedle by its lateral movement on the culture dish surface when the nanoneedle directly contacted the dish. The 200 nm-thick cylindrical nanoneedle was broken after a lateral movement of 300 μm under a contact force of 1 μN. However, the tapered nanoneedle with an aspect ratio of 10 (T10 nanoneedle) was not broken even after a lateral movement of 1000 μm under a contact force of 1 μN. From these results, we considered that the mechanical durability of a nanoneedle could be improved by tapering it.

Figure 2 shows a confocal laser scanning microscope image of a silicon T10 nanoneedle insertion into a HEK293 cell labeled with a lipophilic tracer, DiO (ex 484/em 501, Molecular Probes, Eugene, Oregon, USA), using an IC3 PE maleimide (Dojindo) labeled T10 nanoneedle. We were again able to judge the nanoneedle insertion into a cell by checking a drop in force in the
Invasiveness of the cylindrical diamond nanoneedle insertion into a cell

Figure 5 shows the results of the DAPI exclusion test, indicating that cell membrane integrity was maintained even during diamond nanoneedle insertion into a C3H10T1/2 cell. The cultured cells were incubated for over 1 h in the DAPI-contained medium during nanoneedle insertion. During this incubation, the fluorescence intensity of the DAPI was monitored. From the time courses of the fluorescence intensity of DAPI, cell viabilities were evaluated. The fluorescence intensity did not increase during the insertion of either the 400 nm silicon nanoneedle or the 400 nm diamond nanoneedle, even for 2 h. However, when the 800 nm silicon or diamond nanoneedle was inserted into a C3H10T1/2, the fluorescence intensity of the cells increased after 1 h. These results indicate that the 400 nm nanoneedles did not decrease in cell viability, and their invasiveness is low enough to enable the insertion for over 1 h, irrespective of the materials from which they are made.

The transcriptional inhibition test was performed using pd1EGFP and actinomycin D. The former contains residues 422–461 of mouse ornithine decarboxylase (MODC) that targets proteins for degradation and results in rapid protein turnover (Li et al., 1998). The latter is a transcriptional inhibitor that acts by binding to G-C bases so that its cyclic polypeptide arms fill the nearby minor groove of DNA (Henry et al., 1985). In Figure 6, we compare the change in the fluorescence intensities (n...
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3) among the pd1EGFP transfectant cells of HEK293: cells not treated (control), cells placed under the AFM apparatus but with no nanoneedle inserted (no insertion), cells subjected to insertion of the 400 nm diamond nanoneedle for 3 min, and cells treated with 50 μM actinomycin D. If the treatment did cause the inhibition of transcription, the fluorescence intensity should decrease in the treated cells because the half life of d1EGFP is one hour. The results showed that the fluorescence intensity of actinomycin D treated cells decreased by about 40 a.u. after 2 h. On the other hand, the fluorescence intensities of control cells, non-inserted cells, and cells inserted with a 400 nm diamond nanoneedle all had similar profiles. Thus, we concluded that diamond nanoneedle insertion for 3 min has almost no influence on the transcription of cells.

We also investigated cellular genomic integrity following insertion of a silicon or diamond nanoneedle into a cell because there is a risk of damaging genomic DNA due to the physical contact of DNA with the nanoneedle during insertion. We investigated the p53 expression level, which increase when DNA is damaged.
(Hasan et al., 2004, Dodson et al., 2006, Suzuki et al., 2007, Lee et al., 2009). We inserted nanoneedles into U2OS cells for 1 h and observed the expression level of p53 by immunocytochemistry (data not shown). The localization of p53 was not changed after insertion of the diamond nanoneedle, which suggests that diamond nanoneedle insertion into a cell does not cause damage to the genomic DNA.

**DNA delivery into a C3H10T1/2 using diamond nanoneedle**

Our goal is the complete handling and control of single cells. For example, cell differentiation could be achieved by controlling gene expressions involved in differentiation processes using the nanoneedle technology. Master genes, e.g., for myoD expression that

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**Fig. 7. Delivery of pEGFP DNA to C3H10T1/2 cell by insertion of the diamond nanoneedle.**

A: SEM image of a diamond nanoneedle. B: Cross sectional CLSM image of diamond nanoneedle insertion into a C3H10T1/2 cell. C, D: Bright field and fluorescence image of the pEGFP DNA-delivered C3H10T1/2 cell after 12 h. E, F: Bright field and fluorescence images of the same portion after 24 h. The pEGFP DNA-delivered C3H10T1/2 cell was divided into two cells.

Scale bars: 8 μm (A), 5 μm (B), 100 μm (C, D)
triggers differentiation of C3H10T1/2 cells to functional myoblasts (Rideout III et al., 1994, Wilson et al., 2003, Wilson et al., 2006), represents a highly suitable target for single cell differentiation studies using the nanoneedle technology. Accordingly, we studied the effect of nanoneedle insertion into a DiO labeled C3H10T1/2 using an IC3 PE maleimide labelled diamond nanoneedle (Fig. 7). It was obvious that the diamond nanoneedle was smoothly inserted into the C3H10T1/2 cell. The insertion efficiency of the bare diamond nanoneedle into C3H10T1/2 cell was about 90 %, which was slightly lower than that of the silicon-nanoneedle (95%). This lower efficiency of insertion was presumably due to the higher hydrophobicity of the bare diamond surface. We then tried DNA delivery into a C3H10T1/2 cell using the APTES-modified diamond nanoneedle. Figure 7D shows the strong GFP fluorescence in the manipulated C3H10T1/2 cell after 24 h incubation; the cell displaying GFP fluorescence divided, indicating good cell viability after nanoneedle insertion (Fig. 7E, F). The efficiency of GFP expression relative to the number of manipulated cells was 25 %, which shows more efficient DNA delivery than that of lipofection (13 %).

Discussion

The present study first investigated the shape of the nanoneedle suitable for insertion into cells and revealed that the insertion efficiency of nanoneedles with different aspect ratio varies by cell type. For example, we showed that a nanoneedle with an aspect ratio of 10 (i.e., T10 nanoneedle) is suitable for HeLa cell manipulations.

We previously showed that the insertion efficiency of a nanoneedle having a sharp conical apex was lower than that of a nanoneedle with a flat end (Obataya et al., 2005). To explain this phenomenon, we assume that the larger indentation volume contributes to a higher cortical tension of the cell required to break the plasma membrane. Tapered nanoneedles with smaller aspect ratios having obtuse conical apexes—which should have larger indentation volumes—showed opposite results. In a previous experiment, we also investigated the insertion efficiency of nanoneedles with different apex shapes but with the same 200 nm diameter bottoms, and showed that the apex shape is an important factor in determining insertion efficiency. In the present study, we investigated nanoneedles having identical flat ends of 200 nm and different bottoms of 200 nm (cylindrical), 500 nm (T20), 1 μm (T10), 2 μm (T5) or 3 μm (T3.3) in diameter, resulting in that the thicker bottom nanoneedles showed conspicuously lower insertion efficiencies.

The force drop in the force distance curve is an important factor to recognize the insertion of the nanoneedles into the cell. We therefore investigated the average magnitude of the force drop during the insertion of a cylindrical nanoneedle, T20 nanoneedle, T10 nanoneedle, and normal ATEC-Cont tip into C3H10T1/2 cells. The average force drop was about 2.6 ± 1.8, 1.0 ± 0.7, 0.7 ± 0.5, and 0.6 ± 0.5 nN using the cylindrical nanoneedle, T20 nanoneedle, T10 nanoneedle, and normal tip, respectively. These results showed that the average force drop differs considerably between the cylindrical nanoneedle and the T20 nanoneedle, indicating that the insertion of the cylindrical nanoneedle is much smoother than that of the tapered nanoneedle.

Based on established technology for diamond dry-etching, we have developed a process for diamond nano-tip fabrication in a monolithic cantilever production system. The present study revealed the low invasiveness of diamond nanoneedles etched into 10 μm in length and 200 nm in diameter. We also showed that the surface of the diamond nanoneedle can be silanized for the introduction of cationic charge using APTES to adsorb DNA. It may be thought that, after the release of plasmid DNA, the cationic surface might be harmful because it also interacts with genomic DNA. However, the cell viability after insertion of the APTES-modified diamond nanoneedle was not changed. Thus, our study indicates that the influence of the cationic surface of a diamond nanoneedle on genomic DNA is negligible.

In conclusion, the present study investigated the shape of the nanoneedle for its insertion into cells for manipulation and revealed that the durability of a tapered nanoneedle is superior to a cylindrical nanoneedle and that a proper aspect ratio for the tapered nanoneedle must be chosen to maintain sufficient insertion probability for a particular target cell. We also showed that diamond nanoneedle's invasiveness is low enough to be applied to cell manipulations. We then achieved a highly efficient DNA delivery into a C3H10T1/2 cell by inserting a plasmid-adsorbed diamond nanoneedle. We believe that the diamond nanoneedle will contribute to the enhancement of "cell surgery" technology and be applicable for developing methods of controlled cell differentiation in iPS or other embryonic cells.

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