Evaluation of the actin cytoskeleton state using an antibody-functionalized nanoneedle and an AFM

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ABSTRACT

A cell diagnosis technique was developed, which uses an Atomic Force Microscope (AFM) and an ultrathin AFM probe sharpened to a diameter of 200 nm (nanoneedle). Due to the high aspect ratio of the nanoneedle, it was successfully inserted into a living cell without affecting its viability. Furthermore, by functionalizing the nanoneedle with specific antibodies and measuring the unbinding forces ('fishing forces') during evaporation of the nanoneedle from the cell, it was possible to measure specific mechanical interactions between the antibody-functionalized nanoneedle and the intracellular contents of the cell. In this study, an anti-actin-antibody-functionalized nanoneedle was used to evaluate the actin cytoskeleton state in living cells. To examine the effect of cytoskeleton condition on the measured fishing forces, the cytoskeleton-disrupting drugs cytochalasin D (cytD) and Y-27632 were used, showing a marked decrease in the measured fishing forces following incubation with either of the drugs. Furthermore, the technique was used to measure the time course changes in a single cell during incubation with cytD, showing a gradual time-dependent decrease in fishing forces. Even minute doses of the drugs, the effects of which were hardly evident by optical and fluorescence methods, could be clearly detected by the measurement of nanoneedle–protein fishing forces, pointing to the high sensitivity of this detection method. This technique may prove beneficial for the evaluation of cytoskeleton conditions in health and disease, and for the selection of specific cells according to their intracellular protein contents, without the need for introduction of marker proteins into the cell.

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1. Introduction

The intracellular cytoskeleton has been implicated with many human diseases, including cardiovascular diseases, neurodegeneration, cancer and various skin diseases (Hall, 1998; Ramaekers and Bosman, 2004). In particular, mutations in most human actin isoforms, such as, for example, the α-actin gene (ACTA1), have been linked to various known pathological conditions (Laing et al., 2009) and developmental disorders (Riviere et al., 2012). Currently, methods for diagnosis of cytoskeleton state mainly make use of various imaging techniques, such as flow cytometric tests that measure the intensity of pre-labeled cells for the analysis of various conditions such as hemolytic anemia (King et al., 2000).

Other techniques include mechanical analysis of cell deformability, such as the use of optical stretcher (Guck et al., 2001) for diagnosis of various diseases, among them cancer, by live cell mechanical phenotyping (Remmerbach et al., 2009). These techniques, however, do not allow for the direct detection of intracellular proteins in a living cell, without the use of potentially harmful molecular markers. The ability to analytically diagnose the condition of the actin cytoskeleton in the live cell, without the use of invasive labeling techniques and with high sensitivity, would therefore be of clear advantage.

In recent years, developments have been made in the fabrication of high aspect ratio nanoneedle-shaped tips from various materials, including carbon nanotubes, which can be used for diagnosis and manipulation in live cells (Chen et al., 2007; Singhal et al., 2011; Yum et al., 2009). More recent studies present nanoprobe and nanowires that can record and stimulate neuronal activity in rat cortical neurons (Robinson et al., 2012) or be used to guide and detect visible light inside intracellular compartments (Yan et al., 2012). The technique used in this study for the mechanical probing of intracellular proteins, using an atomic
force microscope (AFM) equipped with a nanoneedle-shaped cantilever that is functionalized with antibodies, has been previously introduced by our group (Mieda et al., 2012). The nanoneedle is fabricated from a standard silicon AFM cantilever that is etched to a cylindrical shape of 200 nm in diameter and a length of about 12 μm. Its high aspect ratio and mechanical stiffness allows repeated penetration through the membrane of the live cell with minimal cell damage (Han et al., 2005; Obataya et al., 2005a). In addition, the surface area of the nanoneedle is large enough to allow efficient immobilization of proteins, such as antibodies (Mieda et al., 2012) or plasmid DNA molecules (Han et al., 2005).

AFM has been extensively employed for the investigation of intermolecular interactions both in vitro and in vivo, using tips that are functionalized with various proteins, with its main advantage being the ability to measure forces with pico-Newton precision and in physiological conditions. High sensitivity analysis of receptor–ligand and antibody–antigen interactions has previously been reported, using a functionalized AFM cantilever (Hinterdorfer et al., 1996; Moy et al., 1994). However, this technique is limited to the cell surface and does not readily allow direct mechanical probing of intracellular organelles inside a living cell. By employing an antibody-functionalized nanoneedle for force measurements, as described here, we are able to mechanically probe the intracellular actin cytoskeleton without affecting cell viability. The availability of a high-sensitivity, analytical method that will allow for real-time mechanical evaluation of cytoskeleton state in a living cell, without the need for labeling or transfection procedures, has clear benefits for the research and diagnosis of cytoskeleton-related diseases.

2. Materials and methods

2.1. Cell culture

Mouse NIH3T3 fibroblast cells (Health Science Research Resources Bank) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 10% FBS, 2 mM GlutaMAX, and gentamicin–ampicillin B (10 μg/mL and 0.25 μg/mL, GA, Cascade Biologics). One day prior to the experiment, cells were treated with PBS containing 0.025% trypsin and 0.01% EDTA and then centrifuged to form a pellet. The cell pellet was dispersed and the cells were seeded onto plastic 35 mm culture dishes (Wako Pure Industries, Ltd.) for 15 min, washed in PBS, and treated with 0.5% trypsin–HCl for 1 h. For each experiment, a separate petri dish was used, and a total of 10–15 cells were analyzed. The nanoneedle was washed with PBS containing 0.05% Tween 20 for 30 min between experiments, to remove non-specific cell debris absorbed on the nanoneedle surface.

2.2. Preparation of antibody-functionalized nanoneedles

Nanoneedles were fabricated from pyramidal silicon AFM cantilevers (ATEC-CONT, Nanosensors) and etched to a cylindrical shape of 200 nm in diameter and around 12 μm in length, using a focused ion beam (SMI-500, Seiko Instruments Inc.). Previous studies describe in greater detail the nanoneedle material and shape properties, including the effect of nanoneedle edge profile on penetration success (Kagiwada et al., 2010; Obataya et al., 2005b). Spring constants (k=0.3±0.1 N/m) were determined using the thermal fluctuation method (Hutter and Bechhoefer, 1993) prior to each experiment. A 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer composed of MPC, γ-methacryloyloxypropyl triethoxy silane (MPTS), and n-succinimidylcarbonyl poly(oxyethylene) methacrylate (PENGHS) was used for surface modification of nanoneedles. The molar fractions of MPC, MPTS, and PENGHS in the polymer were 0.82, 0.12, and 0.06, respectively, as determined by 1H NMR. Nanoneedles were sequentially cleaned with SPM solution (H2SO4:H2O2=4:1) for 60 min at 50 °C. They were then incubated in an ethanol solution containing 5 wt% MPC polymer for 2 h, followed by baking at 70 °C for 1 h. Nanoneedles were then soaked overnight in PBS with 1 μM anti-actin antibody (Millipore) at 4 °C. Before force measurements, nanoneedles were soaked for 30 min at room temperature in PBS containing 100 μM ethanolamine (Sigma-Aldrich) for the purpose of blocking unreacted amine groups, followed by washing with PBS. In the antibody-blocking experiments, the anti-actin-anti-body-functionalized nanoneedle was treated with monomeric actin (G-actin) prior to the force measurement experiment. The nanoneedle was immersed in 10 μM actin in G-buffer (2 mM Tris–HCl, 0.2 mM CaCl2, and 0.1 mM ATP; pH 8.0) at room temperature for 1 h and then washed with PBS three times prior to use.

2.3. AFM force measurements

Force measurements were carried out using a Nanowizard II BioAFM (JPK Instruments). Antibody-immobilized nanoneedles were inserted into living cells, left to dwell within the cells for 2 s, and then retracted at a tip velocity of 10 μm/s. The force exerted on the cantilevers was recorded during both insertion and retraction from the cell. For each cell, force measurements were taken 10 or 20 times at different positions, depending on penetration success, with a 2 μm distance between each point of penetration. In some cases, no force drop was observed in the approach segment of the force curve, indicating that there was an insertion failure of the nanoneedle. When no force drop was observed, the curve was not taken into consideration among the successful insertions for the purpose of fishing force analysis. Further explanation is provided in Section 3. For each experiment, a separate petri dish was used, and a total of 10–15 cells were analyzed. The nanoneedle was washed with PBS containing 0.05% Tween 20 for 30 min between experiments, to remove non-specific cell debris absorbed on the nanoneedle surface.

2.4. Fluorescent imaging of the actin cytoskeleton

Cells were fixed with 4% formaldehyde (Wako Pure Chemical Industries, Ltd.) for 15 min, washed in PBS, and treated with 0.5% Triton X-100 (Nacalai Tesque, Inc.) in PBS for 3 min. Cells were then washed in PBS three times and incubated in PBS solution containing 200 nM rhodamine–phalloidin (Millipore) 1 h at RT. Finally, cells were washed three times with PBS and then observed using a fluorescence microscope equipped with a CCD camera system (IX71/DP30, Olympus Corporation).

2.5. Statistical methods

Data are presented as mean ± standard deviation (SD), unless stated otherwise. Measurements were analyzed using unpaired or paired Student’s t tests with statistical significance at p < 0.05 or lower, as stated in the text.

3. Results and discussion

3.1. Detection of the actin cytoskeleton using an antibody-functionalized nanoneedle

Nanoneedles approximately 200 nm in width and with a length of 12 μm were produced as described in Section 2. Details of the material properties and membrane-penetration characteristics of these needles can be found in previous studies (Han et al., 2005; Obataya et al., 2005a,b). One day prior to the force
measurement experiment, anti-actin antibodies were covalently immobilized on the nanoneedle surface, which was previously modified with MPC, a polymer that has an exceptional capability to suppress nonspecific adsorption of cellular proteins and lipids (Goto et al., 2008), as described in Section 2. The antibody-immobilized nanoneedle was set up on the piezo scanner of the AFM, and then inserted into the live cell, left to dwell inside for 2 s, and then evacuated from the cell.

Fig. 1b shows a schematic illustration of the antibody-functionalized nanoneedle as it is inserted into the live cell, and its interactions with the actin filaments. During this process, a so-called 'force–distance curve' was recorded, which shows the force exerted on the nanoneedle during both insertion and evacuation from the cell as a function of distance (Fig. 1c and d). Successful penetrations of the nanoneedle into the cell are easily identified by the appearance of a steep increase in force followed by a distinct 'force drop' in the ‘approach’ segment of the force curve, which is caused by the nanoneedle pushing against and then penetrating through the plasma membrane (Fig. 1c and d). Force–distance curves with force drops that are larger than 500 pN were considered to represent a penetration event and were further analyzed. During evacuation of the nanoneedle from the cell, unbinding events between the nanoneedle and the cell are recorded. The maximum unbinding force is termed here ‘fishing force’ (Mieda et al., 2012). Fig. 1c shows an example of a relatively weak fishing force (green circle) that is probably caused by non-specific interactions between the nanoneedle and cytoskeleton and/or plasma membrane. Fig. 1d, on the other hand, shows an example of a much higher fishing force ( > 1 nN), caused by specific interactions between the immobilized anti-actin antibodies and the intracellular cytoskeleton.

In order to evaluate the relative contribution of antibody–antigen binding to the measured forces, nanoneedles that were not immobilized with antibody molecules were used as a control.

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**Fig. 1.** (a) FIB image of a nanoneedle etched from a standard AFM pyramidal cantilever. (b) Schematic illustration of an antibody-functionalized nanoneedle insertion into a live cell. (c,d) Typical AFM force–distance curves recorded during approach and retraction of the nanoneedle. A ‘force drop’ identifies the penetration of the nanoneedle through the plasma membrane. A significant ‘fishing force’ peak can be seen in the case of the antibody-functionalized nanoneedle (d) that is absent in the case of the non-functionalized nanoneedle (c). This fishing force results from specific antibody–cytoskeleton unbinding events.

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These nanoneedles underwent the same immobilization protocol as the immobilized nanoneedles, including all surface modifications, aside from the incubation step with the antibody. Fig. 2a shows fishing force measurements in a total of 30 NIH3T3 cells. Each dot represents a force measurement, and each column of dots represents a separate cell. When comparing the fishing forces measured using a non-functionalized needle (left) and those measured using an anti-actin-antibody-functionalized needle (middle), a clear increase in the magnitude of fishing forces can be seen. On average, control (non-functionalized) needles showed a fishing force of $497 \pm 175$ pN (Av ± SD; Fig. 2b, middle) while antibody-functionalized needles showed double the amount of force with $1003 \pm 282$ pN (Av ± SD; Fig. 2b, middle). Reproducibility of these results was confirmed using four separate control (non-functionalized) needles and four antibody-functionalized needles (data not shown). During the experiment, each cell is penetrated 10–20 times, at different locations. However, cell viability was previously shown to be unaffected by repeated nanoneedle penetrations, as measured by the influence on cell doubling time (Mieda et al., 2012) and calcium ion influx (Nakamura et al., 2008).

It is interesting to note the difference in distribution of the measured fishing forces in the case of actin detection in NIH3T3 (presented here) and in the case of the detection of nestin, a type IV intermediate filament protein, in mouse embryonic carcinoma P19 cells, as previously reported by our group (Mieda et al., 2012). The distribution of fishing forces in the case of actin detection reported here is much narrower, with only a few cases of forces lower than 500 pN, unlike that in the case of nestin (Supplementary Fig. S1). This is probably due to the abundance of actin filaments in NIH3T3 cells, in comparison with the more localized morphology of intermediate filaments in P19 cells (Fig. S1), which leads to higher frequency of antibody–antigen unbinding events.

### 3.2. Attenuation of fishing forces observed following nanoneedle blocking with G-actin monomers

In order to confirm that the strong fishing forces observed are indeed caused by antibody-specific unbinding events, the functionalized nanoneedle was immersed in a solution of G-actin monomers (see Section 2) for 1 h prior to the force measurement experiment. If the previously observed fishing forces are the effects of specific antibody–antigen unbinding events, then blocking the antibody using a high concentration solution of G-actin monomers is expected to weaken the measured fishing forces. As can be seen in Fig. 2, following incubation with a 10 μM actin monomer solution, recorded fishing forces were reduced to $473 \pm 114$ pN (Av ± SD; Fig. 2b, right), similar to those seen for a non-functionalized needle, suggesting that the previously observed fishing forces are indeed due to specific antibody–antigen interactions.

### 3.3. Mild disruption of the actin cytoskeleton leads to a marked decrease in measured fishing forces

In order to test the sensitivity of actin detection employed in this method, actin filaments were mildly disrupted using the actin polymerization inhibitor cytoD. Commonly used doses of this drug that induce a visually-detectable effect are usually in the range 1–10 μM (Cooper, 1987); however, even sub-μM concentrations of the drug were previously found to have an effect on the mechanical properties of the cell (Wakatsuki et al., 2001). The success rate of nanoneedle penetration through the plasma membrane greatly depends on the integrity of the actin cortex layer beneath the membrane, as previously shown (Kagiwada et al., 2010). Disruption of the actin cytoskeleton, therefore, reduces the success rate of penetration. In this study, concentrations of cytoD in the range 0.25–10 μM were examined. It was found that at concentrations

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**Fig. 2.** (a) Fishing forces measured using a non-functionalized needle (left, control), using an anti-actin antibody-functionalized needle (center) and using the same needle but after blocking with G-actin monomers (right). Each dot represents a single fishing force measurement. Each column of dots represents a separate cell. (b) The average measured fishing force. For a non-functionalized needle, the average fishing force was $497 \pm 175$ pN (left), while for the anti-actin antibody-functionalized needle the average fishing force was $1003 \pm 282$ pN (middle, Av ± SD). Following blocking with G-actin monomers, the average fishing force dropped to $473 \pm 114$ pN (Av ± SD; Fig. 2b, right), not a statistically significant difference from the case of the non-functionalized needle ($p > 0.5$). * $p < 0.01$, n=10 cells for each case, with a total of ~300 force curve measurements.

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above 1 μM, penetration rate was reduced below 10%, which was too small for statistical analysis of fishing forces. However, at concentrations of 0.25 μM, sufficient amount of penetration events was observed, with around 40% success rate. Fig. 3a shows the time-dependent effect of cytD (0.25 μM) on the measured fishing forces for one NIH3T3 cell. Remarkably, even at such a low concentration of the drug, and in the time course of a few minutes, fishing forces were reduced by a factor of 2. This is a similar decrease to that seen in the G-actin blocking experiment and in the case of the control, a non-functionalized needle (Fig. 2), suggesting that most of the specific binding forces between the antibody and cytoskeleton were disrupted by the effect of the drug. Fluorescent images of fixed NIH3T3 cells, binding forces between the antibody and cytoskeleton were disrupted functionalized needle (Fig. 2), suggesting that most of the specific G-actin blocking experiment and in the case of the control, a non-reduced by a factor of 2. This is a similar decrease to that seen in the fishing forces, a large number of measurements were acquired, first using untreated cells, followed by incubation with 0.25 μM cytD for 20 min and a second series of measurements with the same nanoneedle. The results show a decrease of ~50% in the average fishing force following incubation with the drug (Fig. 3b and c). In order to ascertain that the decrease in fishing forces did not occur as a result of disruption of antibody molecules by the drug or contamination of the nanoneedle, a subsequent third series of force measurements was acquired using the same needle, but in a new, untreated NIH3T3 dish, showing regeneration of the previously measured fishing forces (Fig. 3b and c). These results, together with the previous force measurements in the case of G-actin blocking, clearly show that the observed fishing forces are due to specific nanoneedle–cytoskeleton interactions.

An additional actin cytoskeleton-disrupting drug, Y-27632, a rho-kinase (ROCK) inhibitor that disrupts the formation of actin stress fibers (Narumiya et al., 2000), was used to evaluate its effect on the measured nanoneedle fishing forces. Effective doses of Y-27632 commonly used are usually around 10 times higher than those used for cytD (Arnsdorf et al., 2009; Narumiya et al., 2000). At a concentration of 2.5 μM, a sufficient penetration rate of >30% was observed. Incubation with Y-27632 led to a decrease of about ~50% in the measured fishing forces, similar to the case of cytD (Fig. 4). The difference between the fishing forces in the case of Y-27632 and cytD was not statistically significant (p > 0.1). Fig. 4c shows fluorescent images of NIH3T3 cells stained with the actin-specific dye rhodamine–phalloidin, after 20 min of incubation with various concentrations of the two drugs. At the lower concentrations of cytD and Y-27632 (0.25 μM and 2.5 μM, respectively), which were used in this study, cell shape and integrity do not seem to be altered much as an effect of the drug, and actin stress fibers can still be clearly seen (Fig. 4c, middle). Although some aggregates of actin are visible, especially in the case of cytD, the overall actin cytoskeleton architecture does not appear to be disrupted and is similar to that of untreated cells (Fig. 4c, top). However, at higher concentrations of cytD and Y-27632 (5 μM and 50 μM, respectively), the actin cytoskeleton architecture is severely disrupted: in the case of cytD, actin filaments are heavily fragmented, with no apparent stress fiber formation and drastically altered cell morphology; in the case of Y-27632, cell shape is less severely altered, but inhibition of actin bundling results in the lack of apparent stress fibers (Fig. 4c, bottom).

Both drugs affect the actin cytoskeleton, but in different ways. While Y-27632 inhibits myosin–actin interaction and by that inhibits actin filament bundling and the formation of stress fibers, cytD directly interacts to inhibit actin filament polymerization. In both cases, a similar decrease of ~50% in the measured fishing forces suggests that interactions of the nanoneedle with actin stress fibers might be the main contributor to the measured fishing forces.

Fig. 3. (a) Time course effect of cytD (0.25 μM) on the measured fishing forces on a single cell. (b) Fishing forces measured using an anti-actin-antibody-functionalized needle in untreated NIH3T3 cells (left), after a 20-min incubation of the cells with cytD (0.25 μM; middle), and in a new untreated dish (right), using the same anti-actin-antibody-functionalized needle. (c) Effect of the cytD on the measured fishing forces. For untreated cells, the average fishing force measured here was 1110 ± 215 pN (left). Following a 20-min incubation with cytD (0.25 μM) fishing forces dropped to 492 ± 104 pN (middle, Av ± SD). With the same needle, but a new untreated dish, fishing force rose back to an average of 1055 ± 114 pN (Av ± SD) (*p < 0.01, n=8 cells for each case, with a total of ~200 force curve measurements).
forces. For untreated cells, the average fishing force was 1110 \text{pN} (left), or Y-27632 (right) (Av ± SD, right). (c) Fluorescent images of NIH3T3 cells stained with rhodamine-phalloidin, following incubation with different concentrations of either cytD (left) or Y-27632 (right) (*p < 0.01, n=8 cells for each case, with a total of ~200 force curve measurements). Scale bars are 10 \mu m.

Fig. 4. (a) Fishing forces measured using an anti-actin-antibody-functionalized needle in untreated NIH3T3 cells (left), after a 20-min incubation of the cells with cytD (0.25 \mu M; middle), and in a new untreated dish (right), using the same anti-actin-antibody-functionalized needle. (b) Effect of cytD and Y-27632 on the measured fishing forces. For untreated cells, the average fishing force was 1110 ± 215 \text{pN} (left), for cytD (0.25 \mu M) 492 ± 104 \text{pN} (middle), and for Y-27632 (2.5 \mu M) the average fishing force was 386 ± 59 \text{pN} (Av ± SD, right).

Naturally, an exact quantitative analysis of the filamentous state of the actin cytoskeleton cannot be directly deduced by the measurement of fishing forces at this stage. This is mainly because of the existence of large variations in fishing forces, which are due to the inherent heterogeneous distribution of the cytoskeleton architecture throughout the living cell. Nonetheless, a qualitative analysis of the cytoskeleton state is possible, as can be seen from the strong correlation between the measured fishing forces and incubation time with the actin-disrupting drug cytD (Fig. 3a).

4. Conclusions

In this study, a minimally invasive method for the detection of intermolecular forces in-vivo was presented. The combination of an AFM, a highly sensitive device for measuring intermolecular forces, together with an ultra-thin antibody-functionalized needle, can provide real-time quantitative information about the intracellular contents of a living cell. Here, a nanoneedle functionalized with anti-actin antibody was shown to detect minute alterations in the cytoskeleton architecture, the measured fishing forces were still significantly reduced, pointing to the high sensitivity of intracellular protein detection that is feasible by this method. The use of a nanoneedle for probing the intracellular environment of the cell may prove to be a useful analytical tool, which can provide real-time quantitative information about the state of the intracellular environment of the cell, without the need to expose the cell to any potentially harmful marker molecules and without affecting cell viability. Interestingly, even at low doses of the drug, where fluorescence staining examinations cannot reveal substantial alterations in the cytoskeleton architecture, the measured fishing forces were still significantly reduced, pointing to the high sensitivity of intracellular protein detection that is feasible by this method.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bios.2012.06.044.

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