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Detection of microtubules *in vivo* using antibody-immobilized nanoneedles

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We present here an alternative, force-based measurement method for the detection of intracellular cytoskeletal proteins in the live cell. High aspect ratio nanoneedles of 200 nm in diameter were functionalized with anti-tubulin antibodies and inserted, using an atomic force microscope (AFM), into live NIH3T3 cells, without affecting cell viability. Force curves were recorded during insertion and evacuation of nanoneedles from the cells, and used to analyse intracellular interactions of the nanoneedles with the microtubule cytoskeleton during evacuation from the cell. Disruption of microtubules led to a correlated time-dependent decrease in the measured intracellular binding forces, pointing to the high-sensitivity and high-specificity of this detection method. This analytical technique allows for real-time evaluation of the microtubule network in the live cell, without the need to use potentially harmful molecular markers as do conventional detection methods, and may prove beneficial in the diagnosis and investigation of cytoskeleton-associated diseases.

[**Key words:** Nanoneedle; Atomic force microscopy; Force measurement; Microtubules; Intracellular protein detection]

Microtubules are hollow, cylindrical filaments of approximately 25 nm in diameter, which are formed by the assembly of tubulin monomers. Individual microtubules originate from the centrosome that is near the nucleus, and can span the entire cell. They play an important role in organelle transport and organization, in cell division and chromosome distribution, and in mechanical stabilization of the cell (1,2). Many diseases, such as cardiovascular syndromes, neurological disorders and cancers, have been associated with cytoskeleton abnormalities (3,4). In particular, microtubules have been previously shown to be implicated in several neurodegenerative diseases (5) such as Alzheimer's disease, the most common cause of dementia among elderly people, in which microtubules disintegration play a major role (6–8).

Various methods are available for the detection of cytoskeletal proteins and evaluation of cytoskeleton integrity in intact cells. Commonly used techniques are immunofluorescence detection (9), or the introduction of cytoskeletal genes fused to fluorescent protein tags into the cell, such as green fluorescent protein (GFP), for visualizing the cytoskeleton in live cells (10). Another method often used is flow cytometry, which measures fluorescent intensity of pre-labelled cells and can be used for the detection of various conditions in health and disease, such as membrane cytoskeleton-associated haemolytic anaemia (11). Beside optical-based techniques, alternative methods exist that rely on detection of changes in the mechanical properties of cells, which are governed by the cytoskeleton. One example is the use of optical tweezers (12), which has been employed for the diagnosis of cancer (13). The main disadvantage of those detection techniques is their dependency on the introduction of potentially harmful markers into the cells, or in the case of mechanical analysis, the inability to directly detect intracellular proteins. Hence, a method that can be used for the direct analysis of intracellular proteins, in the live cell, without the need to introduce potentially harmful marker molecules, have an obvious advantage for diagnosis and research applications. In addition, detection method that does not involve introduction of exogenous marker molecules into the cells allows for further follow-up analyses using the same cells, or even medical transplantation into patients, such as in the case of stem cells.

In the past decade, much research has been done into the development of high aspect ratio cylindrical tips for the use as diagnosis tools or manipulation of single live cells. Carbon nanotubes have been employed for various applications, such as delivery of proteins or plasmid DNA genes using functionalized nanotubes (14–16), or even used as endoscopes for intracellular optical and electrochemical diagnosis (17). Recent studies also presented nanowire platforms that can be used for detection and delivery of visible light inside intracellular compartments (18) or the recording and stimulation of neuronal activity in mammalian neurons (19). The nanoneedle technology for probing intracellular proteins used in this study was previously presented (20,21). The nanoneedles are

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fabricated from a standard silicon atomic force microscope (AFM) cantilevers etched to a cylindrical shape of 200 nm in diameter and a length of about 12 μm. Due to its high aspect ratio and mechanical stiffness, the nanoneedle can be easily repeatedly inserted through the plasma membrane of live cells without affecting cell viability (22,23). Moreover, the surface area of the nanoneedle can be efficiently immobilized with proteins, such as antibodies (20,21), or plasmid DNA (24).

The use of AFM for the measurement of intermolecular interactions, using tips that are functionalized with various proteins or antibodies, have been previously reported (25). The advantages of this method include the ability to measure forces with pico-Newton resolution, and in physiological condition, both *in vitro* and in the live cell. With the use of functionalized AFM cantilevers, binding forces of single receptor-ligand and antibody-antigen were successfully measured (25,26). However, application of this technique is limited to extracellular or membrane-surface proteins, and does not readily allow probing of intracellular components inside a living cell.

In the study presented here, an antibody-functionalized nanoneedle was used to probe microtubules in the live cell, without affecting its viability. This high-specificity and high-sensitivity analytical method allows for real-time evaluation of the microtubule network in the live cell, without the use of potentially harmful molecular markers, and may prove beneficial in the diagnosis and investigation of cytoskeleton-associated diseases.

MATERIALS AND METHODS

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-amphotericin B (10 μg/mL and 0.25 μg/mL, GA, Cascade Biologics). One day prior to 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 Chemical Industries, Ltd.) and cultured for 1 day before force measurement experiment. For microtubules disruption, nocodazole (Millipore) was added to the medium.

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, Hitachi High-Tech Science Corp.). Previous studies describe in greater detail the nanoneedle material and shape properties, including the effect of nanoneedle edge profile on penetration success (27,28). Spring constants ($k = 0.3 \pm 0.1$ N/m) were determined using the thermal fluctuation method (29) prior to each experiment. A specially synthesized polymer, PMSiNHS, comprised of 2-methacryloyloxyethyl phosphorylcholine (MPC), γ-methacryloxypropyl triethoxy silane (MPTS), and n-succinimidyloxycarbonyl poly(ethylene glycol) methacrylate (PENHS) was used for surface modification of nanoneedles. The mole fractions of MPC, MPTS, and PENHS in the polymer were 0.86, 0.07, and 0.07, as determined by ^{1}H NMR. Nanoneedles were sequentially cleaned with SPM solution (H_2SO_4:H_2O_2 = 4:1) for 60 min at 50 °C. They were then dipped in an ethanol solution containing 0.5 wt% MPC polymer for 1 min, followed by baking at 70 °C for 4 h. Following washing, nanoneedles were then soaked overnight in PBS with 1 μM anti-tubulin antibody (Anti-α-Tubulin, clone DM1A 05-829, 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 NHS groups, followed by washing with PBS.

AFM force measurements Force measurements were carried out using 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 times at different positions, with 2 μm distance between insertion points. In some cases (about 20%), no force drop was observed in the approach segment of the force-curve, indicating that the nanoneedle failed to insert into the cell. When no force drop was observed, the force-curve was not taken into consideration for the purpose of fishing force analysis. For each experiment, a separate petri dish was used, and a total of 10–15 cells were analysed. The nanoneedle was washed with PBS containing 0.05% Tween for 30 min between experiments, to remove non-specific cell debris absorbed to the nanoneedle surface.

Immunostaining of microtubules 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 100 nM anti-tubulin-Alexa Fluor 488 conjugate (clone DM1A, Millipore) for 1 h in RT. Finally, cells were washed 3 times with PBS and then observed using a fluorescence microscope equipped with a CCD camera system (IX71/DP-30, Olympus Corporation).

Statistical methods Data are presented as mean ± standard deviation (SD), unless stated otherwise. Measurements were analysed using unpaired or paired Student's *t*-tests with statistical significance at $p < 0.05$ or lower.

RESULTS AND DISCUSSION

Preparation of antibody-immobilized nanoneedles and force-curve analysis Standard contact-mode AFM cantilevers were carefully etched, using focused ion beam (FIB), to a cylindrical shape of approximately 200 nm in diameter and 12 μm in length (Fig. 1b). More detailed information regarding the material properties and membrane-insertion characteristics of these nanoneedles can be found in previous studies (22,23,28). Immediately following their fabrication, nanoneedles were incubated in Piranha solution followed by a surface modification step using PMSiNHS, a polymer designed to reduce undesired non-specific adsorption of cellular proteins and lipids (30). Nanoneedles were then covalently immobilized with anti-tubulin antibodies by overnight incubation, and used for cell insertion experiments in the following day. Antibody-immobilized nanoneedles were mounted on the piezo scanner of the AFM and vertically inserted through the plasma membrane and into the cytoplasm of live interphase cells, left inside for 2 s, and then evacuated from the cell. Fig. 1a shows a schematic illustration of the antibody-immobilized nanoneedle inside the cell, and the interactions between the antibodies and microtubules.

The recorded AFM force-distance curves represent the amount of force exerted on the nanoneedle during insertion (approach line) and evacuation (retract line) from the cell as a function of distance (Fig. 1c, 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, d, marked as force drop). Force-distance curves with force drops that are larger than 500 pN were considered to represent a penetration event and were further analysed. During evacuation of the nanoneedle from the cell, unbinding events between the nanoneedle and the cell were recorded. The maximum unbinding force during nanoneedle retraction was calculated for each force-curve, following calibration using the right-hand side of the retraction curve as a baseline, and was termed here fishing force (21). Fig. 1c shows an example of a relatively weak fishing force (green circle) that is 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-tubulin antibodies and intracellular microtubules. Previous study showed a single antibody-antigen binding force to be on the scale of around 200 pN (25). Often, a single insertion followed by evacuation of the nanoneedle from the cell may lead to several peaks, each peak a result of one to several antibody-microtubule unbinding events. The often-observed large peaks, with the magnitude of >1 nN (Fig. 1d), are therefore due to a cumulative unbinding of several antibody-microtubule. However, it is difficult to precisely determine the exact number of antibody-antigen unbinding events, as other non-specific bindings such as, for example, hydrophobic interactions, may contribute to the overall measured fishing force.

Detection of nanoneedle-microtubules interactions in live NIH3T3 cells For the detection of microtubules in live cells,