

VISUALIZATION OF DYNAMIC ORGANIZATION OF CYTOSKELETON GELS IN LIVING CELLS BY HYBRID-SPM*

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Abstract We succeeded in performing of hybrid Scanning Probe Microscopy (hybrid-SPM) in which mechanical-SPM and fluorescence microscopy are combined. This technique is able to measure simultaneously mechanical properties and distribution of cytoskeletons of living cells by using green fluorescent protein. We measured evolution of both local elasticity and distributions of actin stress fibers in an identical fibroblast living in physiological conditions. The SPM experiments revealed that stiffer lines develop in living cells, which correspond to actin stress fibers. The elasticity of the actin stress fibers is as high as 100 kPa. We discuss mechanical effects on the development of actin filament networks.

Keywords Scanning probe microscopy, Green fluorescent protein, Fibroblast, Cell migration, Cytoskeleton, Elasticity

INTRODUCTION

Recently, materials exhibiting self-organization phenomena such as self-assembly membranes have attracted much attention in material science fields. Many researchers have made intensive studies on this theme not only from the stand-point of chemistry but also from that of mathematics or physics. On the other hand, it is well known that the concept of “Self organization” originates from biology and such phenomena are frequently observed in living bodies. In order to produce novel self-organizing materials, it is useful to know how biomolecules or biopolymers cooperate and organize in living systems on a macroscopic scale. Most studies on living systems have been made on a molecular level. It is also essential to investigate cooperative mechanisms in time and space.

Animal cells can move around in tissues, as shown in Fig. 1. The cell locomotion is an important function for maintaining living bodies, such as recovery of wounded parts or development of embryos. The cell locomotion definitely results from cooperation between polymerizing and depolymerizing of cytoskeletons or between forming and breaking adhesion to extra cellular matrix at different parts of a cell. Hence this is a good example to investigate cooperation in living systems. Mechanical intra-tension associated with actin-myosin interaction should be related to cell movements. A conceptual model, named the tensegrity model^[1], is proposed as a mechanism of cooperation among individual actions at different parts of a cell. In this model, there exists a dynamic structure in a cell, which maintains stress balance over a whole cell. The mechanical properties of living cells have attracted much attention. Some experimental methods are proposed to measure local mechanical properties of living cells: cell poking^[2], scanning acoustic microscopy^[3], micropipette aspiration^[4], optical tweezers^[5] and magnetic bead microrheometry^[6]. It has been reported that the elastic response of cells is mainly

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due to the actin filament network^[7]. In order to examine the distribution of cytoskeletons such as actin filaments, cells are usually fixed and cytoskeletons can be observed by immunofluorescence microscopy. Then, there are measurements for spatial and temporal changes neither in mechanical properties nor in distribution of a living cell.



Fig. 1 Fibroblast cells crawling on the glass substrate

Scanning probe microscopy (SPM) is a powerful tool for investigating living systems, because it can image quantitatively surface morphology of cells even under physiological liquid conditions^[8–10]. It also attracts much attention because of its ability to visualize local viscoelasticity over samples. Many kinds of cells were examined by this method, for examples, kidney cells, lung carcinoma and fibroblasts^[11–13]. Recently we found that the elasticity of fibroblasts changes drastically, according to their movement^[14].

In this paper, we described hybrid Scanning Probe Microscopy (SPM) where mechanical SPM and fluorescence microscopy are combined. This is able to visualize simultaneously distributions of both local elasticity and cytoskeleton in living cells under physiological conditions by using the green fluorescence protein (GFP) technique. We examined elasticity and the actin filament network in living fibroblasts. We discuss mechanical effects on the development of actin filament networks in living cells.

MATERIALS AND METHODS

Sample Preparation

Fibroblasts (NIH3T3) were selected for the present measurements. The cells were purchased from RIKEN Cell Bank (Tsukuba, Japan), and cultured in low glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (GIBCO BRL, Basel, Switzerland). The cells grown in plastic flasks were maintained at 37°C and 5% CO₂ in a humidified incubator. The confluent cells were trypsinized and released from the plastic flasks. The cell suspension was plated on a glass petri dish precoated with fibronectin (Boehringer Mannheim, Mannheim, Germany).

Hybrid Scanning Probe Microscopy

Hybrid SPM is an inverted fluorescence microscope equipped with an SPM unit at its stage, as shown in Fig. 2. An instrument composed of TE300 (Nikon Instech Co., Ltd, Japan), an SPA260 and SPI3800 (Seiko Instruments Inc., Chiba, Japan) was used for the fluorescent observation and the SPM measurements, respectively. The SPM unit is loaded separately so as not to interfere with the objective. Thus, both scanning by the SPM unit and fluorescent observation can be done at the same time. A fluorescent image was obtained by a CCD camera during the SPM measurement. The equipped piezo scanner had a maximal *x-y* scan range of 100 μm in each direction and a *z*-range of 10 μm. We used commercially available silicon-nitride cantilevers with an effective length of 320 μm (Thermo Microscopes, Sunnyvale, CA). The typical spring constant of the cantilever was 6 mN/m. To maintain the cells during long time SPM measurements, preheated fresh Ringer's solution was substituted for the culture medium. Topography and elasticity of the living cell were measured in the Force-Mapping mode. The

force-versus-distance curve (force curve) was taken at each position of 64 pixels x 64 lines. Force curves were taken typically at 1.6 Hz with a z range of 1500 nm. It takes approximately 30 min to accomplish the scanning. In order to obtain the Young's modulus and height of the sample at zero loading force, the force curve was fitted to the Hertz model using a non-linear least-squares method. The force mapping was done as described earlier^[15, 16]. Typical fit range was from 50 to 300 pN.

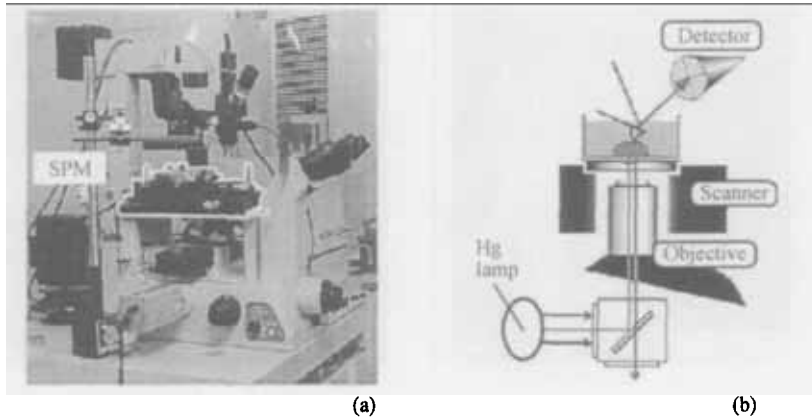


Fig. 2 Side view (a) and schematic drawings (b) of hybrid Scanning Probe Microscope

Immunofluorescence Observation

The identical cells imaged by the SPM were fixed just after the SPM imaging. Fluorescence staining for three types of cytoskeletal filaments was carried out using the usual double staining protocol. Immunofluorescence imaging was performed with a Confocal Laser Scanning Microscope (PCM2000, Nikon). The height resolution of the sectional imaging was about 0.2 μm . The GFP-Actin plasmid (CLONTECH) was transfected into cells, then cells were incubated for at least a day before SPM experiments. Effectene transfect reagent (QIAGEN) was used for the GFP-Actin transfection. Under the present conditions of the transfection of GFP, 30% of the transfected cells can successfully emit fluorescence.

RESULTS AND DISCUSSION

A fibroblast (NIH-3T3) used in the present work show the movement common to most vertebrate cells. The cells cultured on the dish repeatedly crawl like amoebae. When green fluorescence protein (GFP) is transfected to the cells, this sometimes strongly damages development of cytoskeleton networks in the cells and then inhibits their mobility. Actually, there are some aberrant cells in the transfected ones, which show unusual morphology and cannot move. On the other hand, there are some cells resembling control fibroblasts in morphology. They can crawl well on a dish. We selected such well-transfected cells from the transfected cells and compared their mobility and actin filament distribution with those of control ones. Figure 3 shows fluorescence images of distribution of actin filaments in a well-transfected cell and a control one that was fixed and stained by immunofluorescence methods. Actin filament networks develop widely in both cells: Stress fibers extend along the cell movement axis. Such transfected cells can also move as well as non-transfected ones do on a dish substrate. These results guarantee that as long as the transfected cells with usual fibroblast morphology were chosen, transfection of GFP affects neither mobility nor development of the cytoskeleton networks.

Figure 4 shows a topographic image, an elasticity map and the distribution of the actin filament network of identical living cells in the culture medium. The cells were transfected by GFP-actin. Two fibroblasts are seen in the topographic image. The cells adhere thinly on the glass substrate and elongate its lamellipodium. Its height is about 5 μm . In the elasticity map, brighter areas represent stiffer ones. The observed elastic modulus of a cell is not uniform but varies from 1 to 100 kPa depending on the position on the cellular surface. The cell body is rather softer than the surroundings. The stiffer linear structure can be seen clearly in the cell. In the fluorescence

image obtained at the same time, bright lines of GFP-actin run in the cytoplasm. These bright lines agree with the stiffer lines in the elasticity map. These lines correspond to the actin stress fibers.

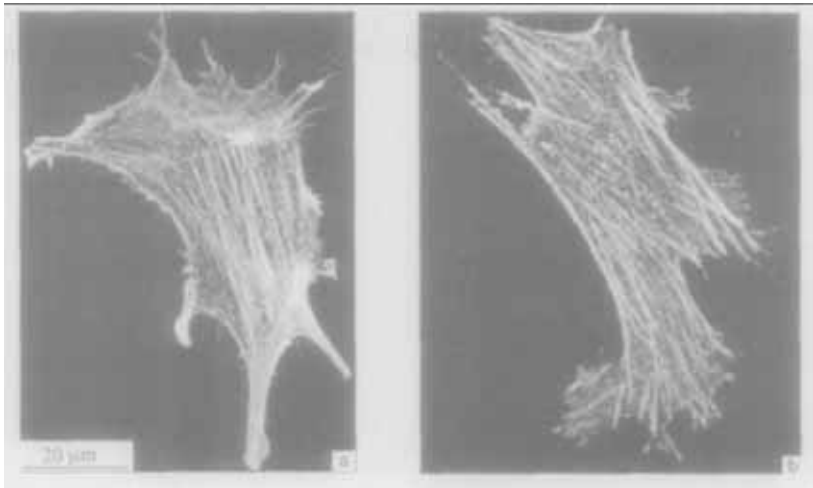


Fig. 3 Comparison of fluorescence images between living GFP-actin transfected cells (a) and fixed cells stained by immunofluorescence imaging method (b)

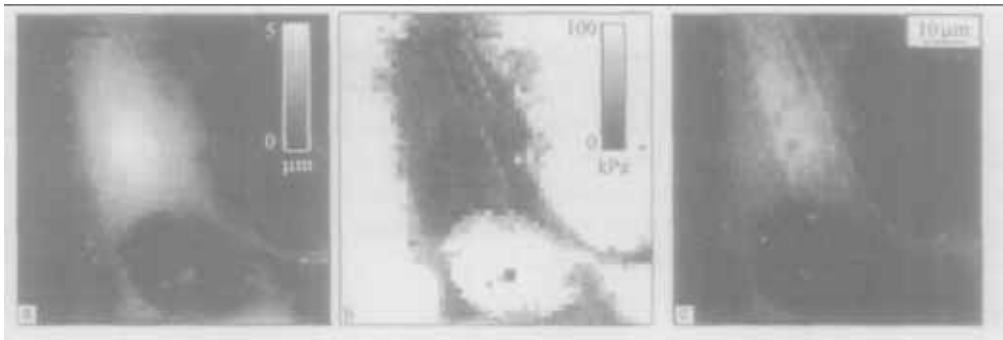


Fig. 4 Topographic (a), elasticity (b) and fluorescence images (c) of living GFP-actin transfected cells, which are captured simultaneously, $80 \times 80 \mu\text{m}^2$

Figure 5 shows time-lapse images of topography, elasticity and distribution of the actin filament network in a part of a fibroblast cell body in culture medium. Its thickness is about 5 μm. The measurements were carried out for 250 min. The cell body moves little and becomes slightly narrower as seen in topographic images. However, the elasticity distribution changes considerably with time: At first, the area at the leading edge is stiffer than the inner parts. After about 100 min, several stiffer lines appear along a direction in the inner parts. The elasticity of the stiffer lines is as high as about 100 kPa. In our previous study^[14], we revealed that the elasticity of the cells changes drastically when the cells begin to move. Especially, the values of the actin stress fibers are lowered from 100 kPa to 10 kPa. Then, the observed elasticity does not only result from the elasticity of the materials but also from external stress acting along the fibers, since the elasticity is estimated by mechanical response to indentation of the SPM cantilever tip. Hence we consider that the values of the elasticity obtained in the present study are affected by stress, because the value is as high as 100 kPa. This implies that the actin stress fibers are developing under the stress in a cell. In other word, the stress may control the development of actin stress fibers, besides signaling molecules such as actin related protein or Rho protein.

SUMMARY

We succeeded in fabrication of a hybrid-SPM, where an inverted microscope equipped with an SPM unit at its stage. This system is able to combine a mechanical SPM with various kinds of optical microscopy, such as

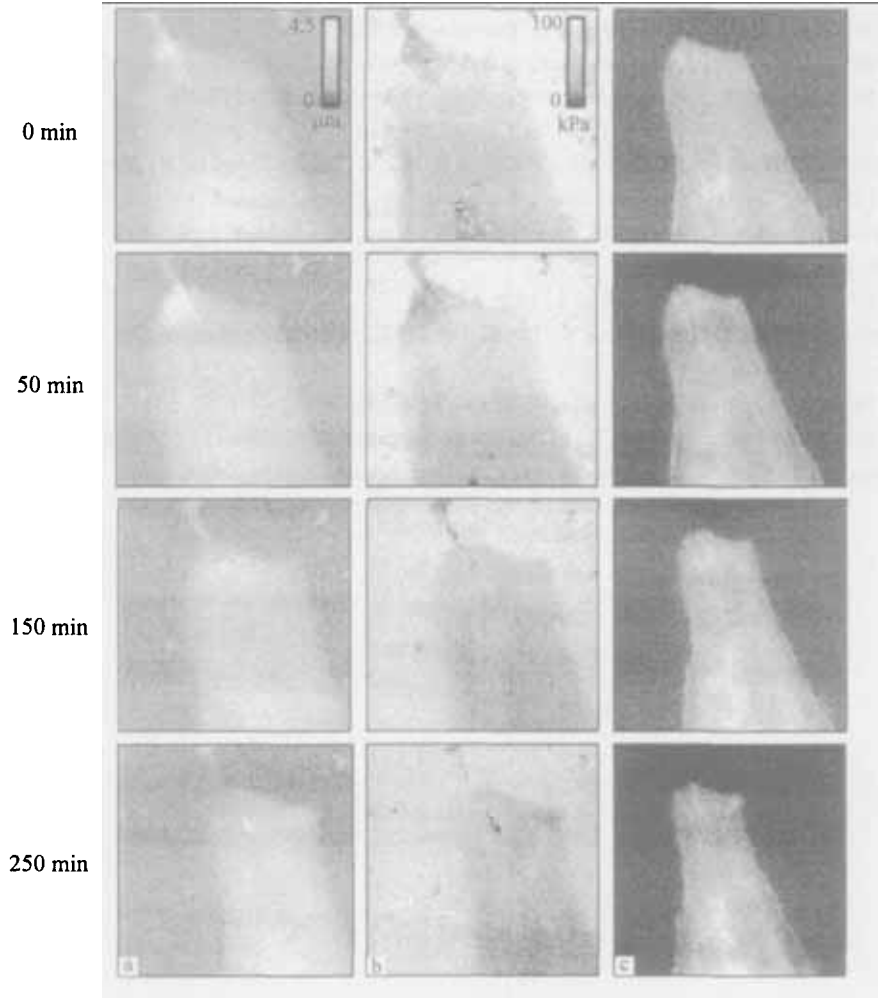


Fig. 5 Time-lapse images p of topographic (a), elasticity (b) and fluorescence images (c) of a living GFP-actin transfected cells
Images were captured for 250 min, $80 \times 80 \mu\text{m}^2$

confocal laser scanning microscopy or surface interference microscopy. We undertook real-time observation of the development of cytoskeleton networks using the GFP transfection technique. We described simultaneous observation of both mechanical properties and distribution of actin networks in fibroblasts living under physiological conditions. The mechanical-SPM experiments for 250 min revealed that the stiffer lines with 100 kPa develop along the direction of cell movement. These stiffer lines correspond directly to actin stress fibers from the fluorescence observation. Thus the present hybrid-SPM is a powerful new instrument for examining spatio-temporal cooperation phenomena in the development of cytoskeletons in living cells.

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