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Stress Relaxation Measurement of Fibroblast Cells with Atomic Force Microscopy

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Abstract

We measured the stress relaxation of mouse fibroblast NIH3T3 cells with an atomic force microscope (AFM) using a sharp silicon tip and a silica bead with a radius of ~1 μm as an indenter. The decay of loading force was clearly observed in NIH3T3 cells at a small initial loading force of ~0.4 nN and was well fitted to the stretched exponential function rather than to a single exponential function. The stretching exponent parameter was ~0.5 for both indenters, indicating that the stress relaxation observed in NIH3T3 cells consisted of multiple relaxation processes. The time-domain AFM technique described in this report allows us to measure directly the relaxation process of living cells in a range from milliseconds to seconds.

KEYWORDS: atomic force microscope, living cells, stress relaxation, viscoelastic properties, colloidal probe.

1. Introduction

Because living cells respond to external mechanical stimuli and emerge various functions such as adhesion, motility, and division, it is important to elucidate their subcellular mechanical properties under physiological conditions. The atomic force microscope (AFM) is one of the most important tools for measuring the elastic and viscoelastic properties of cells at nano- and microscales[1, 2]. The force modulation mode [3-7], which is the basis of measurement of the dynamic response of loading force with respect to an external periodic strain, was used for measuring the complex shear modulus of living cells in the frequency domain [6, 7].

On the other hand, it was reported that time-domain AFM analyses such as creep measurement [8] and stress relaxation measurement[9-11] also allowed us to investigate the viscoelastic properties of living cells. In stress relaxation measurement, [9-11] the cantilever base displacement is kept constant, while loading force is measured as a function of time. Recently, we have shown[11] that the stress relaxation of the human hepatoma cell line, HepG2 cells, can be clearly observed using an AFM tip in the time region from milliseconds to seconds and was well fitted to a stretched exponential function known as the Kohlrausch-Williams-Watts (KWW) function[12, 13] rather than to a single exponential function, suggesting that the relaxation of cells consisted of multiple relaxation processes. However, it has not been clarified whether the relaxation process observed in our previous study[11] is a common feature of living cells.

In general, contact behaviors, such as the contact area between the indenter and the surface and the spatial distribution of stress in the contact area, depend not only on

the loading force applied to the surface, but also on the shape of the indenter. Our previous study[11] showed that loading force affected the relaxation amplitude of cells. However, it has not been clarified how the shape of the AFM indenter affects the stress relaxation measurement of living cells with inhomogeneous structures. In this study, we measured the decay of loading force observed in mouse fibroblast NIH3T3 cells using two types of indenter, namely, a sharp silicon tip and a silica bead.

2. Experimental Procedure

2.1. Cell samples

NIH3T3 cells were cultured on a 35-mm dish (IWAKI, Japan) containing Dulbecco's minimum essential medium (DMEM) (Sigma, U.S.A.) supplemented with 2 mM glutamine, 0.06% (w/v) penicillin, 0.1% (w/v) streptomycin (GIBCO-BRL Life Technologies, U.S.A.) and 10% (v/v) fetal bovine serum (FBS; Atlanta Biologicals, U.S.A.) and incubated at 37°C in a humidified 5% CO_2-containing atmosphere. After incubation on the culture dish for 1 day, NIH3T3 cell samples were used for the AFM experiments.

2. 2. AFM

We used a commercial AFM, MFP-3D AFM (Asylum Research, U.S.A.), which was mounted on an inverted optical microscope, IX-71 (Olympus, Japan). The optical microscope was used to choose a particular cell to be probed with the AFM cantilever (Fig. 1). A small rectangular silicon cantilever, BL-AC40TS (Olympus, Japan) was

used, and the dimensions of the cantilever were 38 μm long, 16 μm wide, 0.2 μm thick, and typically 7-9 μm in tip length with a tip apex half-opening angle, α, of ~ 17.5° [Fig. 2(a)]. The spring constant of cantilevers was determined to be ~0.08 N/m using the thermal fluctuation method[12]. The resonant frequency was ~30 kHz in the culture medium. Loading force was determined by multiplying the cantilever spring constant by its deflection on the basis of Hooke's law. These calibrations were conducted prior to the cell experiments.

The colloidal probe technique[15] has been widely used to measure more quantitatively the mechanical properties of cell surfaces[16] because of the well-defined shape of the probe. We used a silica bead (Catalysts & Chemicals Industries, Japan) whose radius was R= ~1 μm as a force probe. To attach the bead to the cantilever tip, a small amount of low-melting point alloy, U-Alloy47 (melting point 47°C; Niraco, Japan) was placed on the tip, which was heated at a temperature above the melting point. Next, one of the silica beads was placed on the melting alloy deposited on the tip apex and then temperature was decreased below the melting point to fix the bead on the tip. All the fabrication processes were carried out in a scanning electron microscope with a nanomanipulator (Technex Lab., Japan).

2.3. Measurement of stress relaxation

After tapping-mode imaging was carried out at a resonant frequency of the cantilever, stress relaxation measurement was carried out in a central region of a cell to minimize the effect of the solid substrate. Figure 3 shows the schematics of stress

relaxation measurement with AFM. The AFM tip approached the cell surface at a scan rate of ~3 μm/s until the loading force attained a trigger force, F_M, of ~0.4 nN and the cantilever base displacement was fixed at Z_M for a duration, typically 1.5 s. We estimated that the relaxation process appeared at an early stage of <1.5 s because we were not able to calibrate precisely a small drift of the AFM system. After the delay, the AFM tip was retracted at the same scan rate as that in the approaching process. Cantilever deflection signals were sampled with a sampling rate of 100kHz during the force curve measurement. To compare the relaxation properties measured using the silicon tip and silica bead, several NIH3T3 cells cultured in the same day were measured using both indenters.

3. Results and Discussion

Figure 4 shows a typical time course of deflection signals in NIH3T3 cells obtained using the sharp silicon tip. In region I, the AFM tip approached the cell surface. The position where the force abruptly increased represents the contact point between the tip and the surface. In region II, the cantilever base position was kept constant at an initial loading force of ~0.4 nN. The loading force was found to decay, which is attributed to the viscoelastic properties of the cell. In region III, the AFM tip was retracted. The arrow represents the region of adhesive interaction between the tip and the surface.[17] It is noted that there was no marked difference in the baseline of the force between the approach (region I) and retraction (region III) in noncontact regions, showing that the hydrodynamic drag force[18,19] due to the liquid environment was

negligibly small at the present scan rate.

Moreover, we confirmed that when force curve measurement was repeatedly carried out at the same position of the cell surface, the contact point where the cantilever deflection began to increase during the approach was almost unchanged. This indicated that the cell did not exhibit a force-induced irreversible deformation, i.e., cell plasticity[8] at the initial loading force of ~0.4 nN. We observed under an optical microscope that the measured cells were still spreading and alive on the culture dish after the AFM measurements.

Because the observed decay of loading force could not be fitted to a single exponential function, we employed the stretched exponential function[12,13], which is empirically employed to represent the dispersion processes of the system. The functional form is expressed by

$$F(t) = F_r \exp\left[-\left(\frac{t}{\tau}\right)^{\beta}\right] + F_{\infty}, \qquad (1)$$

where F_r is the relaxation amplitude, F_{∞} is the final state of the relaxation process, and τ is the characteristic relaxation time. The β is the stretching exponent with values between zero and unity. The typical stress relaxation processes of NIH3T3 cells measured using the silicon tip and silica bead are shown in Fig. 5, and the fitting results obtained using eq. (1) are shown as a solid line in Fig. 5.

The fitting results of the relaxation process of NIH3T3 cells measured using the silicon tip and silica bead are summarized in Table 1. Taking into account that the surface properties of the silicon tip used in this study are similar to those of a colloidal

silica bead, we can consider that Table 1 shows the probe-shape dependence of the stress relaxation process of NIH3T3 cells at an initial loading force of ~0.4 nN. The average values of the relaxation parameters obtained using the silicon tip and silica bead were almost the same. The τ in NIH3T3 cells was determined to be ~0.18 s, which was shorter than that in HepG2 cells, ~0.5 s [10]. The β in NIH3T3 cells was ~0.5, indicating that the observed relaxation consisted of multiple relaxation processes.

In general, cells are sensitive to the loading force of the indenter. Therefore, it is desirable to measure the mechanical properties of living cells at a loading force as small as possible. In the recent sophisticated force-modulation experiments[6,7], the loading force was fixed at ~0.4 nN, and a small force perturbation was applied in the frequency range of 0.1-100 Hz. As shown in Figs. 4 and 5, the relaxation of the loading force applied to the cell surface could be clearly detected even when the initial loading force was fixed at ~0.4 nN. This indicates that the present time-domain AFM technique for measuring the stress relaxation process of living cells allows us to investigate their viscoelastic properties under the same experimental conditions as those in the force-modulation measurement [6,7].

On the basis of the simple and modified Hertz models[1, 23], the indentation, δ, is expressed as $[\pi (1-\nu^2)F_M/(E\tan\alpha))^{1/2}$ for a conical indenter and $[3(1-\nu^2)F_M/(4ER^{1/2}))^{2/3}$ for a spherical indenter. In the present study, for $\alpha = 17.5^\circ$, R = 1 μm, and F_M = 0.4 nN, δ is roughly estimated to be ~1.2 μm for the conical indenter and ~0.4 μm for the spherical indenter as ν and E are assumed to be 0.5 and 1 kPa, respectively, the latter of which was a typical value of NIH3T3 cells used in the present study. Moreover, the

diameter of the area of contact between the indenter and the cell surface, d, is expressed as $4\delta\tan\alpha/\pi$ for the conical indenter and $2(R\delta)^{1/2}$ for the spherical indenter, from which d is roughly estimated to be ~0.5 μm for the conical indenter and ~1.2 μm for the spherical indenter. Therefore, we concluded that in the case of d and δ to be in the range of 0.4-1.2 μm, the relaxation properties of NIH3T3 cells estimated from the stretched exponential function exhibited no significant difference between the sharp silicon tip and the colloidal silica probe.

Here, we compare the stress relaxation measurement with the conventional force curve measurement, in which there is no duration time between the approaching and retracting processes. The hysteresis of the conventional force curve for living cells has been observed in the contact region.[20-22] Because the observed hysteresis may be mainly attributed to the dissipation of energy to the cells, the relaxation process in principle can be estimated from the hysteresis of the force curve. However, it should be noted that to estimate the stress relaxation of cells from one set of force curve data, the functional form of the relaxation must be assumed. Unless the functional form is assumed, it is necessary to obtain force curves with different loading rates to estimate the stress relaxation of cells. Therefore, we consider that the direct measurement of the stress relaxation process presented here is crucial for understanding the viscoelastic properties of living cells with highly heterogeneous structures and a wide dynamic range.

4. Conclusions

The stress relaxation of mouse fibroblast NIH3T3 cells was measured using a sharp silicon tip and a silica bead with R=1 μm as force probes. The decay (relaxation process) of loading force applied to the cell surface was clearly observed at the initial loading force of ~0.4 nN, which was the same as that used in recent force-modulation mode experiments [6,7]. The relaxation was well fitted to a stretched exponential function. The stretching exponent parameter was ~0.5 for both indenters, indicating that the relaxation observed in NIH3T3 cells consisted of multiple relaxation processes. The time-domain AFM technique described in this report allowed us to investigate the viscoelastic properties of cells in the time range from milliseconds to seconds.

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Figure captions

Fig. 1. Optical image showing the cantilever near the NIH3T3 cell. After the cantilever was roughly placed on the NIH3T3 cell, the tapping mode imaging was carried out to determine the highest part of the cell, around which stress relaxation measurement was carried out.

Fig. 2. Scanning electron microscopy images of cantilever probe. (a) Unmodified silicon tip. (b) Colloidal probe, in which a silica bead with a radius of ~1 μm was attached to the tip apex.

Fig. 3. Schematics of stress relaxation measurement with AFM. During the approach (solid line), the AFM tip or the colloidal probe was placed against the cell surface until the loading force attained a preset trigger force, F_M. The position of the cantilever base Z was kept constant, Z_M (sparsely dashed line). The force curve during the retraction (dotted line) was also measured at the same scan rate as that during the approach.

Fig. 4. Time course of deflection (force) signals in one force curve measurement in a NIH3T3 cell with a silicon tip. The data was filtered to remove signal noise. In region I, the AFM tip approached the cell surface. The position where the force abruptly increased represents the contact point between the tip and the surface. In

region II, the cantilever base position was kept constant at an initial loading force of 0.4 nN. The loading force relaxed due to the viscoelastic properties of the cell. In region III, the AFM tip was retracted. The arrow represents the adhesive interaction between the tip and the surface.

Fig. 5 Typical relaxation processes of NIH3T3 cells measured using the silicon tip and silica bead. The data shifted in the vertical direction. Open circles represent the experimental data filtered to remove signal noise. Solid lines show the fitting results obtained using eq. 1.

Fig. 1 T. Okajima et al.

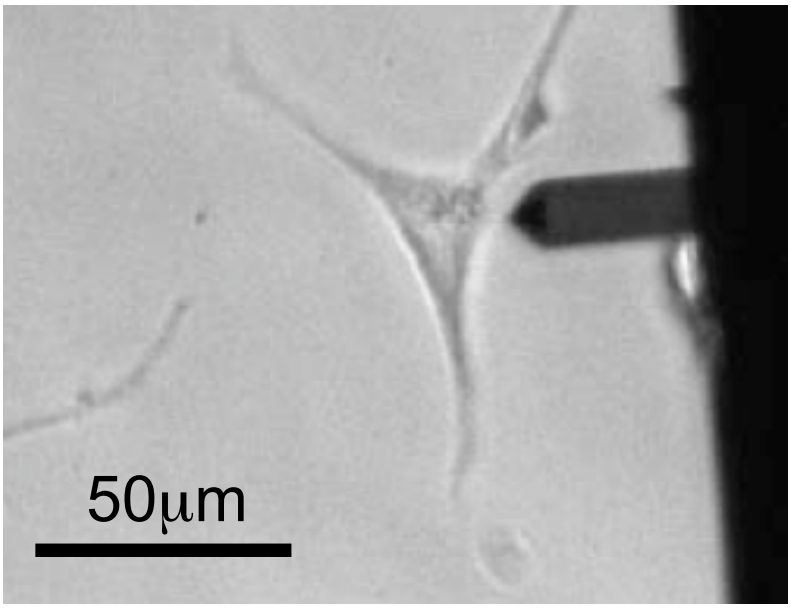
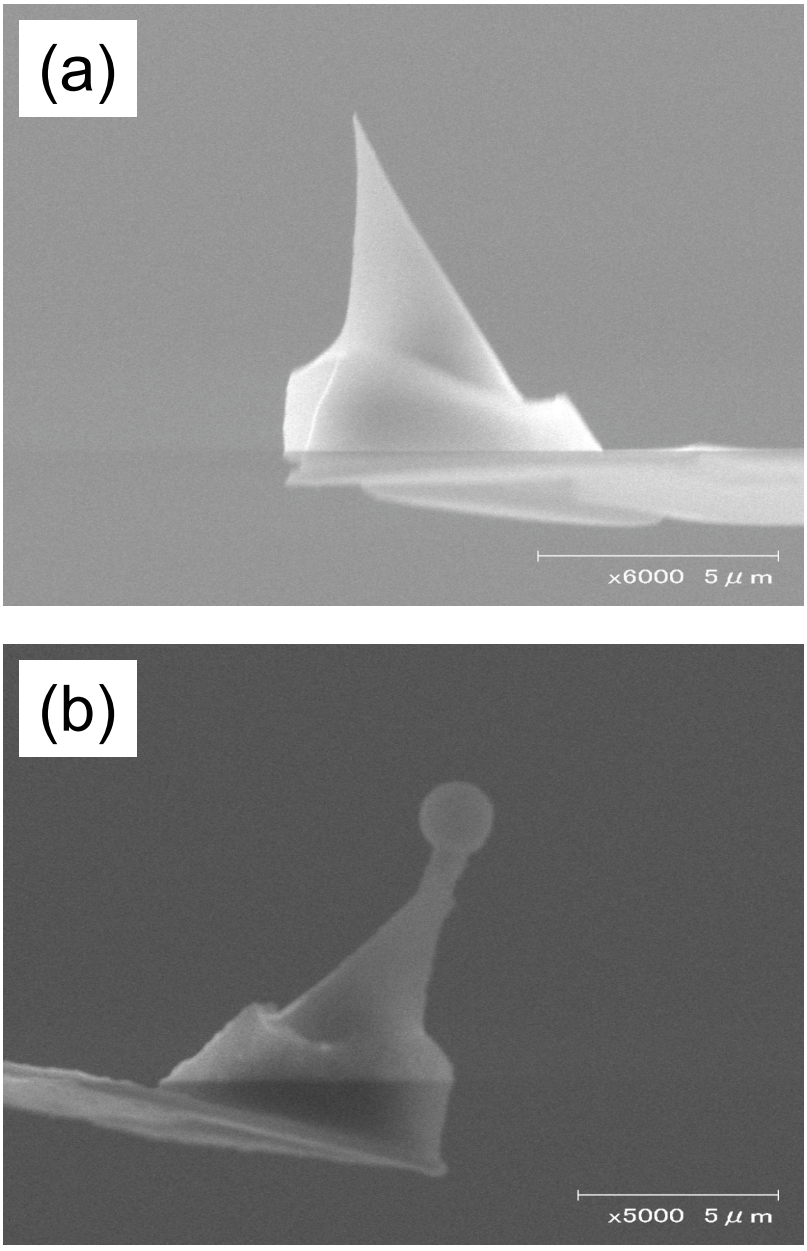


Fig. 2 T. Okajima et al.



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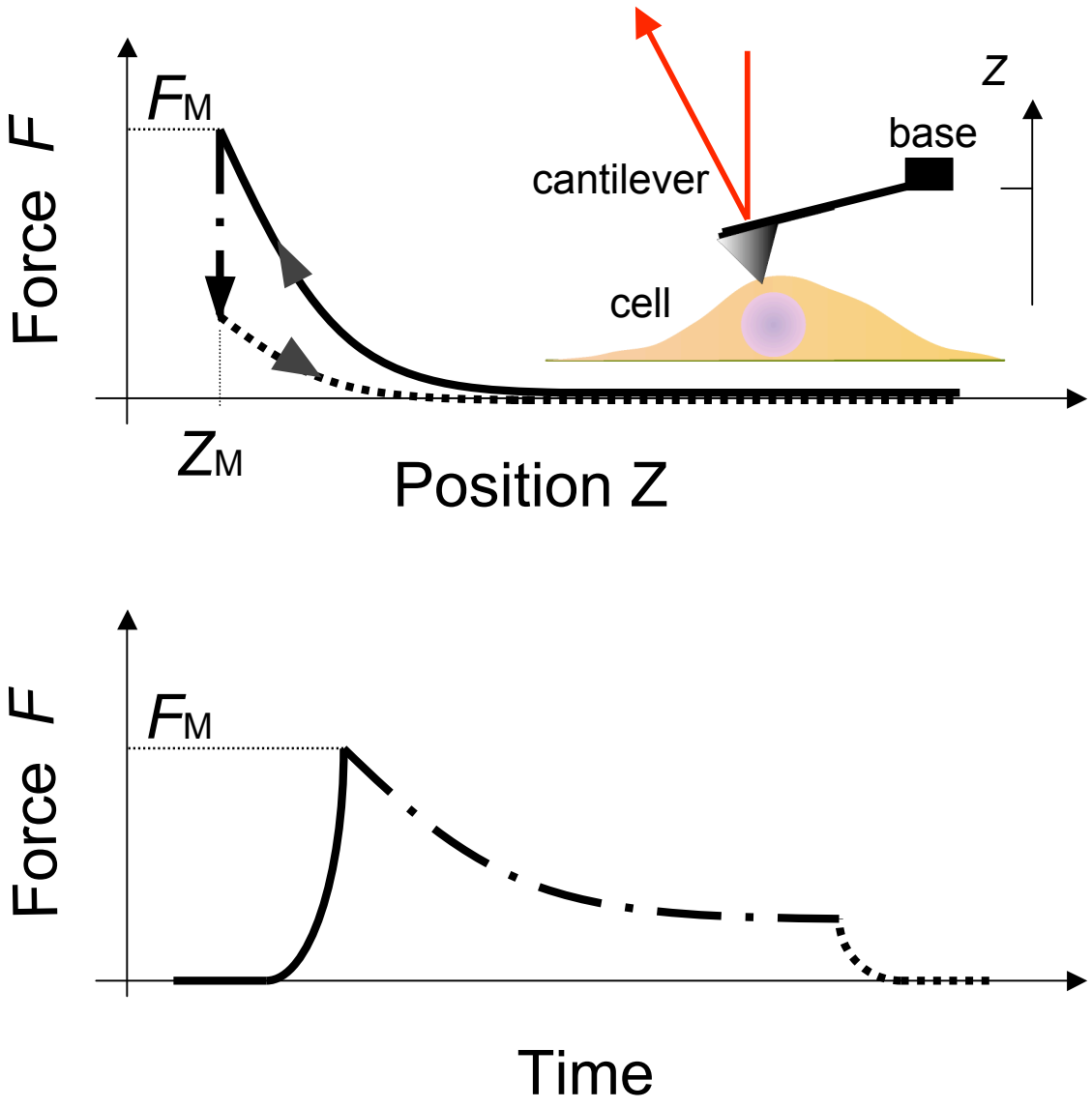

Fig. 4 T. Okajima et al.

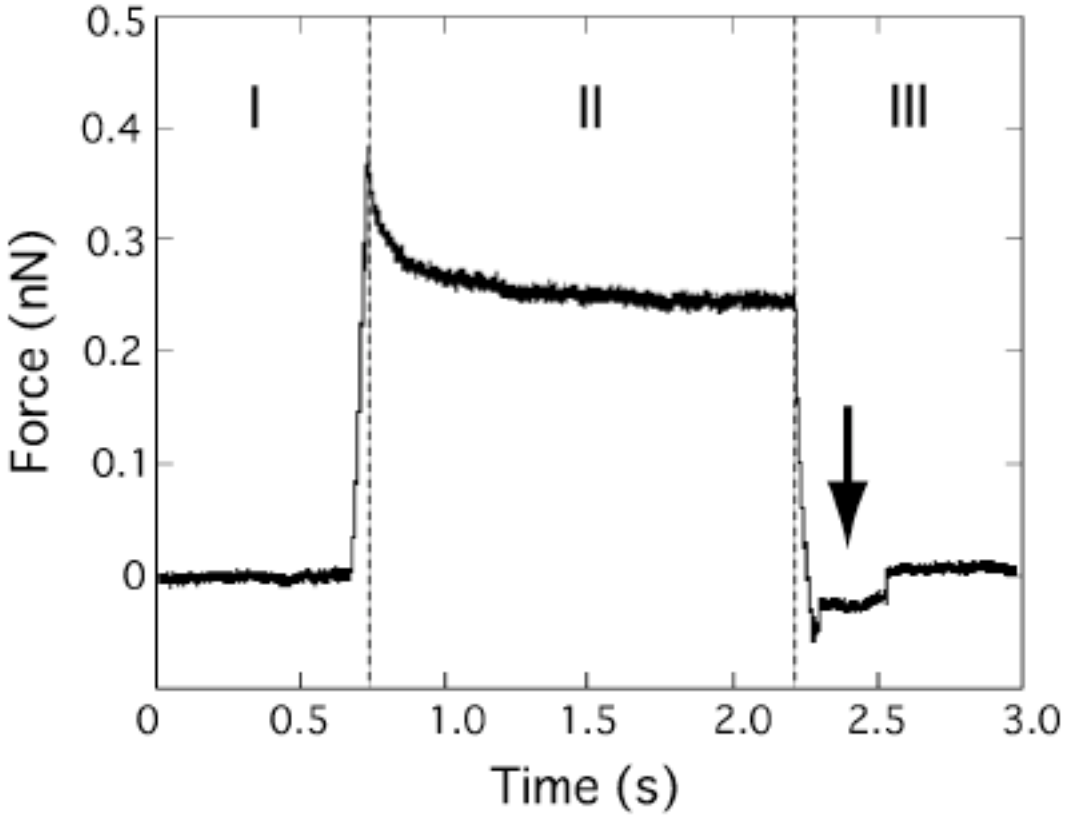

Fig. 5 T. Okajima et al.

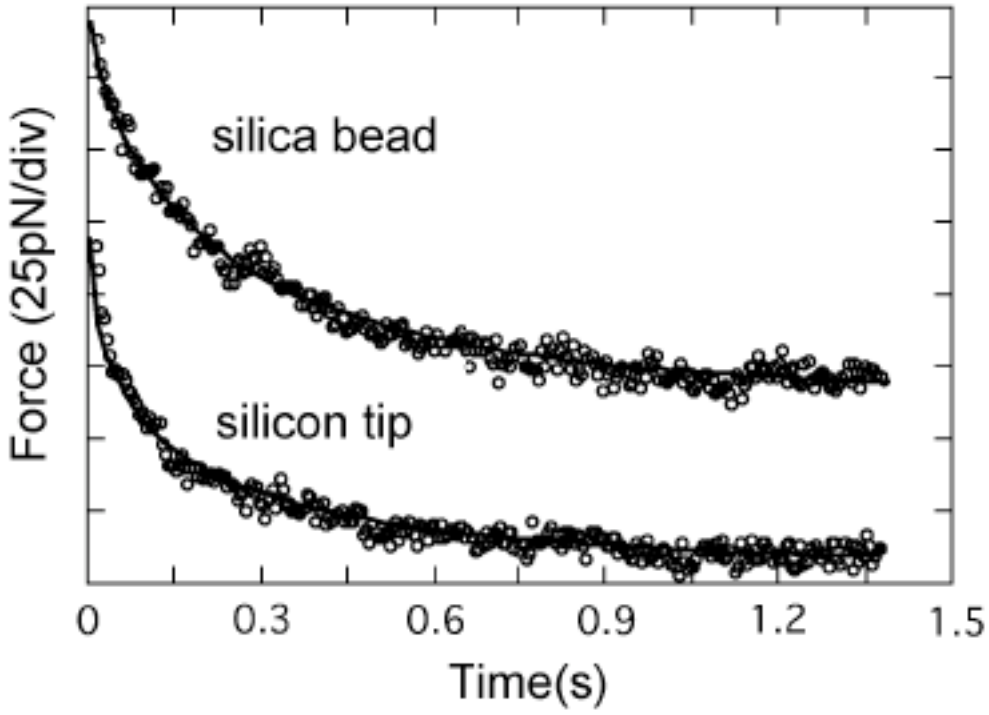

Table 1 T. Okajima et al.

Table 1 Relaxation parameters such as the relaxation amplitude F_r, the characteristic relaxation time τ, and the stretching exponent parameter β obtained in NIH3T3 cells using the silicon tip and colloidal silica probe.

Probe	F_r (pN)	τ (s)	β
Silicon tip	125±18	0.176±0.10	0.52±0.10
Silica bead	124 ±27	0.182±0.07	0.63±0.05