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Direct observation of dynamic force propagation between focal adhesions of cells on microposts by atomic force microscopy

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We investigated dynamic force propagation between focal adhesions of fibroblast cells cultured on polydimethylsiloxane micropost substrates, by atomic force microscopy. Live cells were mechanically modulated by the atomic force microscopy probe bound to cell apical surfaces at 0.01–0.5 Hz, while microposts served as a force sensor at basal surfaces. We observed that cells exhibited rheological behavior at the apical surface but had no apparent out-of-phase response at the basal surface, indicating that the dynamic force propagating through cytoskeletal filaments behaves in an elastic manner. Moreover, the direction of the propagated force was observed to be intimately associated with the prestress. © *2011 American Institute of Physics*.
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When adherent cells contact a substrate, they form focal contacts, which are adhesion sites at the cell–substrate interface formed by integrin receptors. The cells then form stress fibers that are made of a coalescence of actin filaments to anchor at the focal contacts and increase stiffness in response to stress applied to the integrins. Not only do the actin filaments behave as a prestressed network of discrete elements to stabilize the shape and structure of cells, but also cytoskeletal (CSK) filaments including actin, microtubule, and intermediate filaments affect various physiological cell functions.[1–3] Therefore, it is essential to understand how forces between focal adhesions propagate through CSK filaments.

Direct measurements of static force using a micropipette technique showed that integrins, CSK filaments, and nuclear scaffolds were discretely connected to each other in response to external static forces.[4,5] Force measurements using magnetic microbeads[6–9] and an elastic micropillar[10] revealed that the static forces propagate across discrete CSK elements over long distance through the cytoplasm in adherent cells, indicating that the prestress in the actin bundles is the key determinant of how far a force can propagate; this is known as "action at a distance" behavior.[6–9] Moreover, atomic force microscopy (AFM)[11–14] allowed us to measure static force propagation over distances within cells with precise control of the position and the magnitude of the loading force.

On the other hand, a cell is a soft viscoelastic material[15] whose fluctuations involve highly dynamic and continuous CSK remodeling. A few studies[14,16–18] on the time-dependent mechanical properties of individual focal adhesion have been carried out. However, little is known about how dynamic forces propagate between focal adhesions in cells. Here, we investigate how a discrete force propagates between focal adhesions over distances within cells employing AFM combined with a micropost substrate technique,[19] which enabled us to measure directly the mechanical response related to the CSK filaments at focal adhesions.

As shown in Fig. 1(a), apical and basal surfaces of fibroblast cells were bound to a colloidal bead[20,21] of an AFM cantilever (Olympus, BL-AC40TS) and polydimethylsiloxane (PDMS) microposts,[19,22] both of which were precoated with an adhesive protein fibronectin. The AFM cantilever was oscillated normal to the substrate surface with peak–peak amplitude of ca. 4.5 μm for three periods ($3T$) at frequency f in the range of 0.01–0.5 Hz, using an AFM apparatus (Asylum Research, MFP-3D), while time-series images of microposts were recorded by phase-contrast or bright-field microscopy (Nikon, TE-2000 E) with a long-working-distance objective lens (Nikon, ELWD DLL $60 \times$ C) with a charge-coupled device camera (QImaging, Retiga-4000DC). The bending force F_p applied to the microposts was estimated using the equation $F_p = 3EI\delta/L^3$, where $E = 2.5$ MPa is Young's modulus for PDMS, I is the moment of inertia, L is the length, and δ is the micropost deflection.[19,22] For further details on the experimental methods, the reader can refer to the supplementary information.[23] Figure 1(b) shows an optical microscopic image of the AFM experiments. The dotted line is the outline of the cell adhered to the micropost substrate. Because the apices of PDMS microposts were focused on to estimate the deflections, the cantilever and the cell are blurred in this image. The deflection of several microposts, to which the cell was adhered, was observed. The force estimated from the deflection is shown as arrows in Fig. 1(b). The arrows are directed toward the center of the cell, showing the traction forces of the cell exerted on the microposts.[19] The magnitude of the traction forces varied for each micropost.

Figure 2(a) shows a typical relation between the displacement of the z-piezo stage for moving the cantilever base z and the loading force F at the apical cell surface during external modulation. Although the profile of F was slightly distorted rather than being a sinusoidal curve, we can see the phase shift ϕ between z and F. Since the contact

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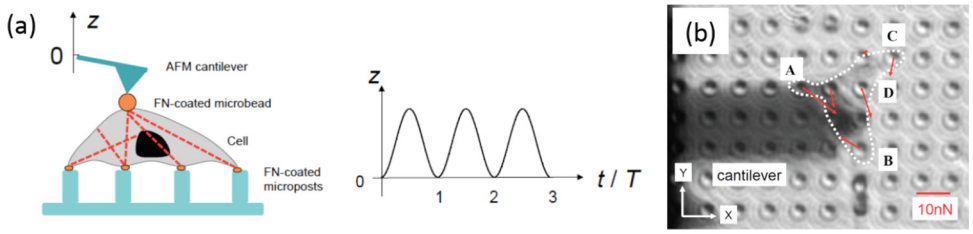

FIG. 1. (Color online) (a) Schematics of the experimental setup. Cells were placed and cultured on PDMS microposts, on which fibronectin was coated. A fibronectin-coated colloidal bead attached on the apex of the AFM tip was bound to the apical cell surface with an initial loading force less than 500 pN for a few tens of minutes. The oscillatory pulling force was applied in the frequency range of 0.01–0.5 Hz for three periods ($0 < t < 3T$) while the deflection of the micropost was measured by phase-contrast or bright-field microscopy. (b) Optical microscopic image of the AFM experiment. An objective lens (60×) with a long working distance was focused on the tops of the microposts. Red arrows indicate cell prestress, estimated from the deflection of microposts (letters A–D).

area between the AFM colloidal probe and the apical cell surface was unknown, the absolute value of the complex elastic modulus E^* could not be determined. Nevertheless, we can still estimate the relative value of E^* using the phase space of F and z if we assume the contact area to be unchanged during external modulation as well as F to be approximately a sinusoidal curve; i.e., the linear viscoelastic properties are satisfied under this condition. In this case, the magnitude of complex modulus $|E^*|$ is proportional to the ratio of stress to strain.[24] Figure 2(b) is a plot of $|E^*|$ normalized by that at $f = 0.01$ Hz as a function of f. It is clearly seen that the relative value of $|E^*|$ follows a single power law, which has been commonly measured at local regions of single cells[25] and in a whole single cell that was largely deformed during stretching.[26] In the present experiments, cell deformation was ca. 4.5 μm, which was between the former and latter experimental conditions, in which cells suffered no fatal damage during force application. The single power law exponent α estimated from Fig. 2(b) was 0.17, which agreed well with previous reports.[25,26]

We observed that some microposts, deflected by the prestress of cells, were further deformed with respect to the external modulation with around 10 nN of the amplitude mentioned above. As shown in Fig. 3, in most cases, a periodic change in force was observed at these frequencies. Interestingly, no apparent phase shift in the force magnitude was observed at these frequencies (Fig. 3 (letter A)). The response of the force at the basal cell surface appeared in-phase even at higher frequency (0.5 Hz) at which the rheological properties of cells were clear for apical surfaces (Fig. 2(b)). The result suggests that forces between focal adhesions of apical and basal cell surfaces are dominantly propagated not in non-discrete cytoplasm but through discrete CSK filaments. Moreover, no correlation between the force magnitude and frequency was seen. This was probably because the CSK filaments were remodeled during long experiments. Thus, we do not discuss the frequency dependence of the amplitude and phase shift measured in the experiments below. Similar behaviors shown in Fig. 3 (letter A) were observed for other microposts (Fig. 3 (letters B-D)). However, it is noted that not all microposts were displaced to the same extent and some microposts had little displacement. Such features are consistent with previous studies using microbeads.[6–9]

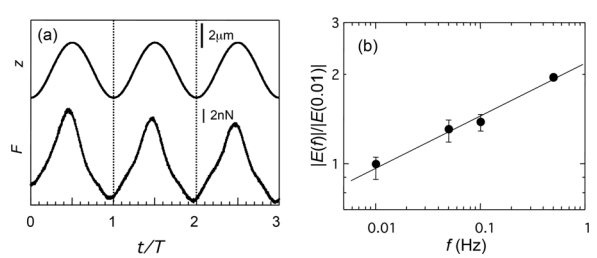

FIG. 2. (a) Typical time series of the displacement of the z-piezo stage for moving the cantilever base z and loading force F at 0.1 Hz ($T = 10$ s). (b) The magnitude of elastic modulus normalized by that at $f = 0.01$ Hz. The magnitude follows a single power law, which is approximately proportional to $f^{0.17}$.

At 0.01 Hz as shown in Fig. 3 (letter C) and at 0.05 Hz as shown in Fig. 3 (letter D), the response of the force was no longer periodic but more complex. Moreover, force profiles that exhibited periodic responses, as shown in Fig. 3, were asymmetric, and some (depicted by arrows in Fig. 3) showed plateau responses with respect to the lower external forces. This result indicates that the lateral force at the basal cell surface was well propagated when a strong force was applied normally to the apical surface. The heterogeneities of long-distance force propagation may be associated with the deformation of the nucleus[1–5], remodeling of actin filaments in local regions[27] and entanglement of CSK filaments.[28]

Figure 4 shows typical traces of the lateral force vector, subtracting the prestress from the total lateral force applied to microposts during external modulation. The micropost tended to move along the direction of the prestress. The fluctuation of the force propagation appearing at the time scale of measurements was probably due to the spatiotemporal change in the pathway of force propagation from apical to basal surfaces through CSK filaments.

It is valuable to consider the lateral force components applied to the cells during the force oscillation. In this study, the maximum deflection of the cantilever was around 100 nm, a few tens of times smaller than the displacement of the z-piezo stage for moving the cantilever base, i.e., ca. 4.5 μm. Therefore, we considered that the lateral force components could be ignored.

In summary, AFM combined with micropost substrates was developed to measure discrete force propagations over distances within cells between focal adhesions. Employing the method, an external force was applied at an angle normal to cell surfaces, which allows us to prevent any lateral drift of cells. Lateral movement of the microposts provides

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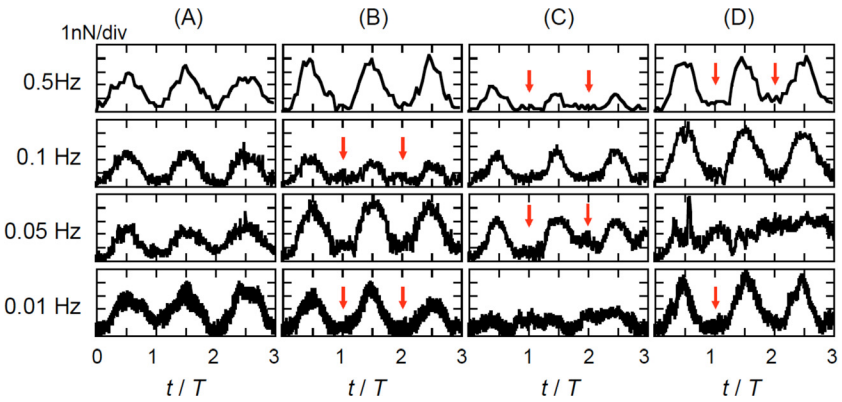

FIG. 3. (Color online) Time series of lateral force magnitude applied to microposts during external modulation at different frequencies by AFM. Letters correspond to those in Fig. 1.

information about force propagation through actin filaments without translational movement of the whole cells. Therefore, the AFM method with a micropost substrate presented here is a useful technique for investigating precisely dynamic features of discrete force propagation between focal adhesions.

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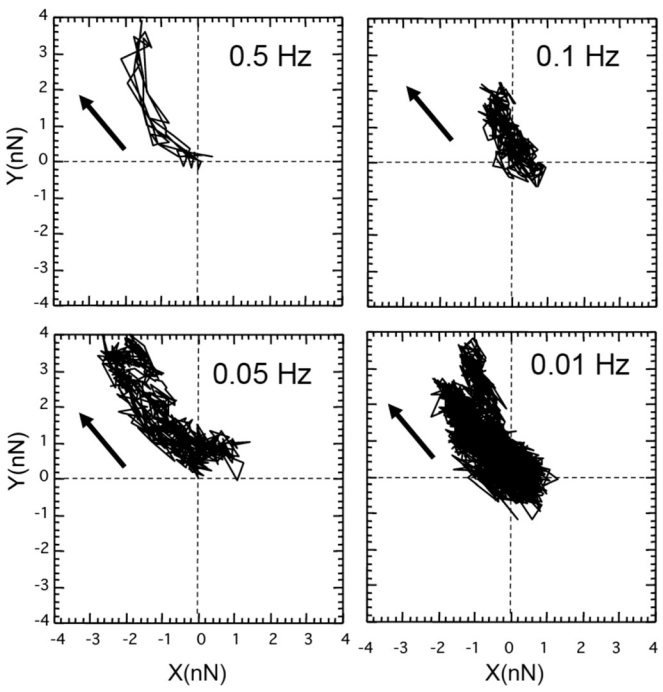

FIG. 4. Typical traces of the lateral force vector, subtracting the prestress from the total lateral force applied to a micropost, in response to external force. Arrows represent the direction of prestress applied to the micropost.

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