Observations of intracellular tension dynamics of MC3T3-E1 cells during substrate adhesion using a FRET-based actinin tension sensor

Junfeng WANG*, Masahiro ITO*, Wenhao ZHONG*, Shukei SUGITA*, Tatsuo MICHIEUE**, Takashi TSUROI**, Tetsuya KITAGUCHI***.**** and Takeo MATSUMOTO***.*****
*Department of Mechanical Engineering, Nagoya Institute of Technology
Gokiso-cho, Showa-ku, Nagoya 466-8555, Japan
E-mail: wangjunfeng35@yahoo.co.jp
**Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo
3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan
***Cell Signaling Group, WASEDA Bioscience Research Institute in Singapore
11 Biopolis Way, #05-02 Helios, Singapore 138667, Singapore
****Comprehensive Research Organization, Waseda University
#304, Block 120-4, 513 Wasedatsurumaki-cho, Shinjuku, Tokyo 162-0041, Japan
*****Department of Mechanical Science and Engineering, Graduate School of Engineering, Nagoya University
Furo-cho, Chikusa-ku, Nagoya 464-8603, Japan (Current)

Abstract
Tension in actin filaments plays crucial roles in multiple cellular functions, although little is known about tension dynamics of cells during adhesion to substrates. In this study, we visualized intracellular tension in actin filaments using a newly developed Förster resonance energy transfer (FRET)-based tension sensor (Actinin-sstFRET-GR). Tension dynamics were monitored during adhesion in MC3T3-E1 mouse osteoblastic cells after introduction of the sensor. Whole cell areas continued to increase from 10 to 180 min after plating and tension was monitored in a typical section close to the bottom, where the mean fluorescence was the largest. Tension on the bottom side was negatively correlated with cell area from 10 to 110 min. Thereafter, this correlation was positive until 180 min. We then analyzed central–peripheral differences in tension close to the bottom. In these experiments, tension in the cell periphery increased during expansion of the area and decreased during contraction. As a result, fluctuations of tension in this area were much larger than those in the central area of the cell. Finally, we analyzed upper–lower differences in tension development during initial adhesion and showed continuous decreases in the lower side of the cell from 10 to 110 min after plating, and decreases in the upper side until 70 min, followed by increases. In this study, we successfully visualized dynamic changes in intracellular tension at the sub-cellular level and found that development of tension during initial adhesion processes is time-dependent and has central–peripheral and upper–lower differences. The present FRET tension sensor may become a powerful tool in studies of cell biomechanics.

Key words: FRET, Tension sensor, Cytoskeleton, Actin filament, α-actinin, MC3T3-E1, Adhesion

1. Introduction
Cells change shape dramatically during adhesion processes. After seeding into culture dishes, they adhere and spread over time (Senju et al., 2009). During this process, cells generate tension with intracellular actin filaments and pull on the dish via focal adhesions, which are mediated by large protein complexes on basal surfaces that maintain cell shape (Goffin et al., 2006). Cells sense mechanical properties of the extracellular matrix and change their shapes to accommodate substrate stiffness. In particular, smooth muscle cells cultured on stiff glass substrate spread greatly with polarity, but adhere poorly to soft polyacrylamide gels and remain rounded (Engler et al., 2004). Mechanical
forces play important roles during adhesion of cells and are required for maintenance of morphology. Thus, measurements of forces applied to and generated by cells are important assessments of these processes.

Several techniques are used to measure forces that are generated by cells. In particular, substrates with polydimethylsiloxane micropillars have been frequently used to measure cell traction forces from deformations of micropillars (Tan et al., 2003; du Roure et al., 2005; Nagayama et al., 2011, 2012). Moreover, polyacrylamide hydrogels with gel-surface-bound fluorescent beads have been used to identify changes in traction forces in cultured cells (Marinković et al., 2012; Delanoë-Ayari et al., 2010), and atomic force microscopy has been used to measure tension of cell membranes (Besc et al., 2003; Spagnoli et al., 2008; Pietuch et al., 2013). Moreover, Sugita et al. (2011) measured local tensions in stress fibers by attaching magnetic particles and applying known forces using an electromagnetic needle. However, none of these methods can be used to measure intracellular forces without disrupting cells.

In recent years, intracellular tension sensors have been developed based on Förster resonance energy transfer (FRET), which comprises energy transfer between two light-sensitive molecules, such as green fluorescent protein (GFP) and red fluorescent protein (RFP). In this example, FRET is excited at its specific fluorescence excitation wavelength, and this excitation energy is then non-radiatively transferred to RFP; GFP returns to the electronic ground state. However, the efficiency of this energy transfer rapidly decreases with the sixth power of the inverse distance between GFP and RFP. FRET was named after the German scientist Theodor Förster (Förster, 1946, 1948), and the first FRET force sensor was named stretch-sensitive FRET (stFRET; Meng et al., 2008) and was based on the cyan fluorescent protein (CFP) Cerulean (Rizzo et al., 2004) and the yellow fluorescent protein (YFP) Venus (Nagai et al., 2002) linked with an α-helix. To improve the sensitivity of stFRET, a spectrin stFRET (sstFRET) sensor was designed by replacing the α-helix with a spectrin repeat (Meng and Sachs, 2011) and was applied to observations of time-course tension changes around focal adhesions during development (Ye et al., 2014). Grashoff et al. (2010) also designed a tension sensor module (TSMod) in which Cerulean and Venus were linked with an elastic linker protein derived from the spider silk protein flagelliform. These authors then inserted the TSMod into vinculin to investigate tension in focal adhesions (Grashoff et al., 2010). All of these FRET sensors comprise CFP and YFP, and previous studies demonstrated that many CFP–YFP reporters suffer from low FRET dynamic ranges and phototoxicity from the CFP excitation light (Malkani and Schmid, 2011; Dixit and Cyr, 2003). However, GFP–RFP pairs such as Clover–mRuby2 conferred greater FRET dynamic ranges (Lam et al., 2012), and the ensuing phototoxicity was limited by the use of excitation light with longer wavelengths.

In this study, we designed a new FRET module sstFRET-GR replacing RFP and YFP pair in sstFRET (Meng and Sachs, 2011) with GFP (EGFP) and RFP (mCherry) pair. The sstFRET-GR can be excited by 488 nm laser which is one of the most common excitation light with longer wavelength and thus low phototoxicity. Subsequently, we inserted it into the actin filament crosslinking protein α-actinin to produce Actinin-sstFRET-GR. Because α-actinin binds actin filaments, it is stretched and shows low FRET when actin filaments are under high tension, and conversely shows high FRET when filaments are under conditions of low tension. We also changed the linker DNA fragment of spectrin linker (Meng and Sachs, 2011) to linker (GPGGA)₈ (Grashoff et al., 2010) and created another sensor ActTS-GR which is successfully applied to the measurement of tension during embryonic development (Yamashita, et al., 2016). In order to evaluate performance of the Actinin-sstFRET-GR, we introduced this sensor into MC3T3-E1 mouse osteoblastic cells and observed intracellular tension dynamics during substrate adhesion.

2. Materials and Methods
2.1 Actinin-sstFRET-GR and the negative control

Figure 1 (a) shows the scheme of the sensors. DNA fragment of spectrin linker (Meng and Sachs, 2011) was cloned into EcoRI/BamHI site of a plasmid. The DNA fragments of mCherry (AgeI/EcoRI) and EGFP (BamHI/NotI) were inserted in N and C-terminus of the spectrin linker, respectively, and named it sstFRET-GR. We then inserted the sstFRET-GR (AgeI/NotI) between the actinin-head domain (1-300aa) and actinin-tail domain (301-892aa) and named it Actinin-sstFRET-GR (GR). We also inserted the sstFRET-GR (AgeI/NotI) into C-terminus of the full length of actinin (1-892aa) as a negative control (NC) which is insensitive to tension. Figure 1(b) shows typical fluorescent images of actin (blue), actinin-EGFP (green), and actinin-mCherry (red) of an MC3T3-E1 cell that expresses GR for 41 h. Actin localization was highly consistent with actinin-EGFP.
2.2 Preliminary confirmation of sensor function

Sensor functions were checked using bovine aortic endothelial cells (BAECs, CAB30405, TOYOBO, Japan). Cells were seeded on glass bottom dishes (D111400, Matsunami, Japan) coated with fibronectin (100 μg/mL, Sigma, USA) and were cultured in Dulbecco’s modified Eagle’s medium (Wako, Japan) containing 10% fetal bovine serum (Biowest, France), penicillin (100 unit/mL), and streptomycin (100 μg/mL, Sigma) for 24 h. Cells were then transfected with 0.5 μg of GR or NC plasmids using 3 μL of Lipofectamine LTX with Plus Reagent (Invitrogen, USA) and were cultured in Opti-MEM I Reduced Serum Medium (Gibco, USA) for 24–72 h before experiments.

The sensitivity of FRET sensors was initially confirmed following increased intracellular tension under exposure to hypotonic stress in a medium that was diluted to 50% with distilled water. In subsequent experiments, tension was decreased by relaxing actin filaments by adding the ROCK inhibitor Y27632 to a final concentration of 20 nM in the media (Wako). All experiments were performed at room temperature for approximately 40 min under a confocal laser scanning microscope as described in Sections 2.4 and 2.5. Cell images were recorded at 1-min intervals to determine FRET ratios as described in the later sections. Drags were added to the medium at 10 min after the beginning of image acquisition.

2.3 Observations of cell adhesion processes

We used MC3T3-E1 mouse osteoblastic cells (RCB1126, RIKEN Cell Bank, Japan) in previous studies of morphological changes with focal adhesions (Wang et al., 2015) and actin filaments (Wang et al., 2016). In these studies, cells were seeded on plastic dishes in minimum essential medium with alpha modification (αMEM; Wako) supplemented with 10% fetal bovine serum (Biowest), penicillin (100 unit/mL), and streptomycin (100 μg/mL, Sigma). Cells were then transfected with 10 μg of GR or NC plasmids using an electroporation system (NEPA21, Nepa Gene, Japan) and were then cultured in Opti-MEM I Reduced Serum Medium (Gibco) for 24–72 h before experiments. To identify localizations of actin filaments and sensors, we fixed cells using phosphate buffered saline (PBS, Nissui, Japan) containing 3.7% formaldehyde and then stained F-actin with Alexa Fluor 350 Phalloidin (Molecular Probes, USA) diluted in PBS at 1:300. To observe live cells, we labeled cell nuclei with Hoechst33342 (Molecular Probes) diluted in PBS at 1:10000 and trypsinized and then plated cells on glass bottom dishes (D111400) coated with fibronectin (100 μg/mL, Sigma) at 100 cells/mm². Cells were then cultured in a microscope-stage-top incubator comprising a laboratory-made polystyrene foam box with a top heater set at 41°C, bottom heater at 37°C, and bubbling port for 95% air-5% CO₂.

2.4 Live cell imaging

Images were obtained from tops to bottoms of cells with 0.4-μm steps using a confocal laser scanning microscope (FV1200+IX81, Olympus, Japan) with a 100x oil immersion objective (UPLSAP0100XO, N.A. = 1.40, Olympus). A z-drift compensation system (Olympus) was used to correct z-axis drift during time-lapse imaging. The following conditions were used for visualization: Hoechst33342 and Alexa Fluor350 Phalloidin excitation, diode laser LD405
(405 nm, Olympus), emission 430–470 nm; EGFP excitation, Multi-line Ar laser FV5-LAMAR-2 (488 nm, Olympus), emission 505–525 nm; mCherry excitation, 505–525 nm and emission 560–660 nm.

2.5 Image analysis and FRET ratio calculation

FRET ratios were considered an index of actin filament tension and were calculated as the ratio of acceptor to donor signal (mCherry/EGFP) from raw images using FV10-ASW 4.1 (Olympus). ImageJ ver.1.50b (NIH, USA) software was used to reduce the noise of the FRET ratio image using a median filter. FRET ratios \( r \) of <0.1 and >1.9 were excluded from analyses. FRET ratio images were displayed in a 16-color map. In evaluations of FRET ratios in a typical section close to the bottom (sections 3.1–3.3), we analyzed the slice of FRET ratio images in which mean fluorescence of EGFP was the largest. Subsequently, we evaluated FRET ratios at upper and lower sides of the cell (section 3.4) and then calculated mean FRET ratios from each slice and averaged them from the sixth slice from the top to the slice where the nuclear area was the largest for the upper side of the cell. We discarded the first to fifth slices because these contained multiple no-data points \( r < 0.1 \) and \( r > 1.9 \) in the inside of the cell. To assess the lower side, we used the slice in which the mean fluorescence of EGFP was the largest and its adjacent upper and lower slices, and we presented mean FRET ratios of the three slices (Fig. 6(a)).

2.6 Statistical analysis

Data are expressed as means ± standard deviations (SD). Differences were identified using Student’s paired and unpaired t-tests. Correlations between cell projected area (or cultured time) and FRET ratios were identified with Pearson’s correlation coefficient using the t-test. Differences and correlations were considered significant when \( P < 0.05 \).

3. Results

3.1 Validation of the tension sensor GR and its negative control NC

To confirm that GR responds to tension changes in actin filaments, we measured changes in FRET ratios of BAECs expressing GR following addition of distilled water (tension increase) or Y27632 (tension decrease) to the medium. As a negative control, experiments were performed in cells that were transfected with the NC plasmid. Figure 2(a) shows typical FRET ratio images before and after addition of distilled water. Specifically, yellow areas decreased and green areas increased following addition of water to the medium. Figure 2(b) shows changes in FRET ratios in the rectangle area in Fig. 2(a) and ratios were normalized to those recorded at 1 min. These experiments showed that FRET ratios are almost stable and rapidly decrease upon addition of water. Although there were cell morphological changes after addition of distilled water or Y27632 to the medium, these changes occurred mainly in the cell periphery in 30 min period. Thus the “Measured area” in Fig. 2(a) may show almost the same area of the cell. Figure 2(c, d) show responses of GR and NC to water and Y27632 treatments, respectively. In particular, FRET ratios of GR-expressing cells significantly decreased and increased after treatments with water and Y27632, respectively, although no changes were observed in NC-expressing cells. Similar results were generated in MC3T3-E1 cells (data not shown) and confirmed that GR responds to changes in intracellular tension, whereas NC does not.

(a) Change in FRET ratio image in response to hypotonic stress

(b) Change in FRET ratio
3.2 Correlation of cell areas and FRET ratios during adhesion to substrate

Figure 3(a) shows typical FRET ratio images of an MC3T3-E1 cell from 10 to 110 min after plating on a glass bottom dish. Cell areas increased with time and were recorded and plotted with FRET ratios at 10-min intervals as means ± SD of four cells (Fig. 3(b)). FRET ratios and cell areas were linearly correlated and marked changes in FRET ratios were observed around pseudopods. Asterisk and pound symbols in Fig. 3(a) indicate locations of active expansion and retraction of cell bodies and show that extension and retraction appeared alternately in two locations. In addition, FRET ratios were lower during extension than during retraction. In more detailed experiments, we recorded FRET ratio images at 30-s intervals and presented typical FRET ratio images of an MC3T3-E1 cell at 2–3 h after plating (Fig. 4(a)). In addition, we selected five measurement areas (Boxes 1–5) in the cell periphery, measured areas occupied by the cell body $S$, and calculated mean FRET ratios ($r_m$) for each box as the average for all pixels with valid FRET signals. Correlations between $S$ and $r_m$ in Boxes 1–5 are shown in Fig. 4(c–g) with cell areas and FRET ratios of whole cells (b). As shown in Fig. 4(b), cell areas and FRET ratios were negatively correlated at 2–3 h after plating, but were positively correlated during the early period of adhesion (Fig. 3(b)). In addition, cell areas continued to increase with fluctuations during this period, suggesting that relationships between cell areas and tensions in actin filaments vary between adhesion periods. Specifically, tension decreased with increases in whole cell areas during the initial period of adhesion until 110 min, but was subsequently increased from 120 to 180 min. Similar tendencies were observed in boxes (Fig. 4(c–g)) and in another cell (data not shown). These results indicate that tension of actin filaments is higher in expanded areas and lower in contracted areas.
(a) Typical images of acceptor, donor and FRET ratio

(b) Correlation between cell area and FRET ratio
Fig. 3 Changes in cell areas and FRET ratios during the initial phase of cell adhesion. Typical images of acceptor, donor and FRET ratio of an MC3T3-E1 cell from 10 to 110 min after plating on a glass bottom dish (a) and correlations between cell areas and FRET ratios (b). Brightness of acceptor image in (a) was amplified for visibility. Asterisk and pound symbols in (a) indicate locations where active expansion and retraction of cell bodies was observed; bar = 20 μm.

Fig. 4 Correlation between local cell movements and FRET ratios; typical FRET ratio images of an MC3T3-E1 cell 2–3 h after plating (a); correlation of cell areas and FRET ratios in a whole cell (b) and in Boxes 1–5 (c–g); bar = 20 μm.

3.3 Differences in tension dynamics in central and peripheral cell areas

In further studies, FRET ratios were analyzed at 30-s intervals in the central area (red) of an MC3T3-E1 cell and its periphery (white; Fig. 5(a)) after culture for 2 h. The FRET ratio fluctuation \( Q \) (Fig. 5(b)) was calculated as the percentage SD of the mean FRET ratio of peripheral and central cell areas between 2 and 3 h after plating (120 time points). The fluctuation \( Q \) was larger in the peripheral area than in the central area and similar results were obtained in another cell (Fig. 5(c)). These data indicate that tension in the cell periphery is more variable than that in the central area.
3.4 Upper–lower differences in tension during initial adhesion processes

In these analyses (Fig. 6(a)) tension development during initial adhesion processes differed between the upper and lower sides of the cell, as indicated by time course changes in FRET ratios at upper and lower sides of an MC3T3-E1 cell from 10 to 110 min after plating (Fig. 6(b)). Specifically, the FRET ratio of the lower side continuously increased from 10 to 110 min, but increased until 70 min and then decreased in the upper side. These results indicate that tension in actin filaments continuously decreases in the lower side of the cell, but decreases for only 70 min in the upper side, after which it increases.

4. Discussion

In this study, we designed the novel FRET tension sensor Actinin-sstFRET-GR using a GFP (EGFP) and RFP (mCherry) pair. This sensor responded to intracellular tension changes of actin filaments and was successfully introduced into MC3T3-E1 cells for investigations of intracellular tension dynamics during substrate adhesion. The present data show that 1) tension of actin filaments in cells decreases with increases in whole cell areas during the initial 110 min of adhesion (Fig. 3(b)), but increases from 120 to 180 min (Fig. 4(b)). In addition, 2) tension was higher in expanded areas and was lower in contracted areas in the cell periphery (Fig. 4(c–g)), and variations in tension were larger in the periphery than in the central area of the cell (Fig. 5(b, c)). Finally, 3) tension in the upper side of the cell decreased over 70 min and then increased until 110 min, whereas that in the lower side continuously decreased until 110 min (Fig. 6(b)).

Senju and Miyata (2009) reported that actin filaments of Swiss 3T3 fibroblasts mediated different morphological changes between 0–1 h and 2–3 h after plating. Specifically, circular bundles and dorsal stress fibers formed by 1 h after plating, and polygonal and polarized shapes were subsequently formed during the 2–3-h period. Accordingly,
percentages of cells with circular bundles were decreased, whereas those of cells with straight bundles of actin filaments increased during the 2–3-h period. In the present study, similar tension development was observed, with differing tension changes of adhering cells at 10–110 min (Fig. 3(b)) and 120–180 min (Fig. 4(b)) after plating. These data may reflect complex temporal changes in morphology and tension of actin during adhesion processes.

In the cell periphery, repeated extension and contraction were observed in some areas (Fig. 3(a)) and in Boxes 1–5 in Fig. 4(a), and tension was higher in expanded areas and was lower in contracted areas (Fig. 4(c–g)). To investigate phase differences between shape and tension changes, we calculated correlation coefficients ($R$) for the relationship between FRET ratios and cell areas at 125–175 min after plating by shifting the timing of FRET ratios by 0.5 min in the range of $-5$ to $+5$ min. In these analyses, the peak $R$ value generally appeared at 0 min (Fig. 7), indicating no phase difference between tension changes and cell area. However, Ye et al. (2014) showed that actinin forces dramatically increase within several minutes of focal adhesion (FA) growth in the leading edge, indicating a phase difference between tension changes and FA growth. However, the relationship between FA morphology, cell area, and tension requires further investigation. Variations in tension were larger in the cell periphery than in the central area, reflecting alternating expansions and contractions in the cell periphery. Moreover, expansion and contraction did not appear in the central area.

Previous studies indicate multiple differences between top and bottom actin filaments, including differing regulation of nuclear shape (Khatau et al., 2009), alignment and tension (Nagayama et al., 2013), and morphological changes during adhesion processes (Wang et al., 2016). However, to the best of our knowledge, no studies have reported tension dynamics of top and bottom actin filaments. In the present study, we observed spatial tension dynamics of cells using a FRET tension sensor and laser scanning confocal microscope and showed tension changes in upper and lower sides of the cell over time. In these experiments, tension in the lower side continuously decreased over 10–110 min, whereas tension in the upper side decreased over 10–60 min and increased from 70 to 110 min. In our previous studies on actin dynamics during adhesion processes (Wang et al., 2016), actin aggregates appeared over the nucleus at 10 min after plating and became smaller over time and then disappeared with the appearance of actin filaments at 60 min. These changes in actin morphology may reflect phasic changes in actin tension.

In this study, we evaluated changes in intracellular tension during adhesion processes using FRET ratios. However, this technique cannot be used to estimate actual tension values, and although difficult, calibrations of FRET ratios with tension would be very helpful. Accordingly, we correlated tension with changes in FRET ratios $Q$, although this value is also qualitative because noise variations from the present observation system have not yet been measured. And also, the $Q$ might be affected by structural heterogeneity of actin filaments themselves although the actin filaments cannot be seen clearly at 10–180 min. The present observations of changes in tension of cells were limited to 3 h after plating, and actin filaments gradually appeared with different morphological changes in upper and lower sides of the cell after 3 h (Wang et al., 2016). Further investigations are required to monitor tension changes for up to 12 h after plating, when adhesion and spreading processes stabilize, and it is expected that clear results would be acquired from tension heterogeneity in actin filaments.

In conclusion, we developed a novel tension sensor, Actinin-ssFRET-GR, comprising GFP-RFP pairs rather than CFP-YFP pairs. The sensor requires 488 nm laser which is one of the most common excitation light with longer wavelength and thus low phototoxicity. One of the reasons we successfully observed intracellular tension dynamics of MC3T3-E1 cells during substrate adhesion for longer period of time might be we could use this 488 nm laser. Intracellular tension dynamics were temporally and spatially heterogeneous, and the relationship between tension in actin filaments and projected cell areas was initially negative and later became positive. Tension fluctuations were larger in the cell periphery than in the central area, and tension in upper and lower sides of the cell showed different changes over time. Taken together, the present data indicate that intracellular tension is highly heterogeneous and is locally controlled and demonstrate the utility of the FRET-based tension sensor in comparison with conventional techniques for studying tension dynamics in the cells, such as micropillar substrate and cell traction force microscopy.
Correlation coefficient $R$ between FRET ratios and cell areas of the whole cell and in Boxes 1–5 from Fig. 5 (a). Extremes of correlation coefficients in positive sides of shifted time analyses indicate changes in FRET ratios with changes in areas.

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