A novel FRET analysis method for tension dynamics in a single actin stress fiber: Application to MC3T3-E1 cells during movement on a substrate

Junfeng Wang1, Shukei Sugita2, Tatsuo Michiue3, Takashi Tsuboi3, Tetsuya Kitaguchi4, Takeo Matsumoto1,*

1Department of Mechanical Systems Engineering, Nagoya University, Nagoya, Japan
2Department of Electrical and Mechanical Engineering, Nagoya Institute of Technology, Nagoya, Japan
3Department of Life Sciences, The University of Tokyo, Tokyo, Japan
4Institute of Innovative Research, Tokyo Institute of Technology, Tokyo, Japan

Abstract Actin stress fibers (SFs) generate tension and play crucial roles in multiple cellular functions. However, it remains unclear how the tension changes in a single SF during cell movement on a substrate. In this study, we developed a new method to analyze the change in tension in a single SF in a cell with a Förster resonance energy transfer (FRET)-based tension sensor. With this new method, we have evaluated the relationship between the movement of SFs in the bottom of an MC3T3-E1 cell and their FRET ratio change, i.e., the tension change, for the first time to our knowledge. We found that the tension in SFs decreases when they rotate. The tension had no significant correlation with their translation nor with their length change.

Keywords FRET, tension sensor, actin filament, α-actinin, MC3T3-E1

Introduction

Stress fibers (SFs) play crucial roles in producing tension inside and outside of cells, which is involved in many cellular functions, including cell division, formation of pseudopodia and lamellipodia, and locomotion [1]. Thus, it is important to know the mechanical state of SFs. To achieve this goal, several techniques have been used to measure the forces generated by SFs. Cells were cultured on elastic substrates, such as polydimethylsiloxane micro-pillars [2–5] and polyacrylamide hydrogels with gel-surface-bound fluorescent beads [6, 7], and their traction force was measured from deformations of the substrates. Local tension in SFs has been measured by attaching magnetic particles to SFs and by applying known forces using an electromagnetic needle [8]. However, these methods cannot be used to measure intracellular forces acting on the SFs without disrupting cells.

To measure intracellular forces, researchers developed several novel molecular tension sensors based on Förster resonance energy transfer (FRET), which is an energy transfer phenomenon between two light-sensitive molecules, a donor and an acceptor. These sensors include SF tension sensors [9–12] and focal adhesion tension sensors [13, 14]. The FRET ratios were considered to be an index of the tension in SFs or focal adhesions and were calculated as the ratio of the acceptor to donor signal. In most of the studies on these SF FRET tension sensors, the mean FRET ratio of a whole cell or cell population was calculated. To our knowledge, there have been no studies that observed the FRET ratio of a single SF. There are several types of SFs with different structures and behaviors, such as dorsal SF, transverse arc and ventral SF [15]. Changes in tension over time in different types of SFs might have their own characteristics. In this study, we developed a new method for the analysis of the FRET ratio in a single SF and evaluated the time course change of tension in ventral SFs during the movement of MC3T3-E1 cells.

*E-mail: takeo@nagoya-u.jp, TEL/FAX: +81-52-789-2721
Materials and Methods

Tension sensor

The details of the sensor were described previously [12]. Briefly, the EGFP (Donor) molecule and mCherry (Acceptor) molecule were connected with a linker protein spectrin [10] to obtain the tension sensor module. Subsequently, the module was inserted between the actinin-head domain and actinin-tail domain to yield the tension sensor Actinin-sstFRET-GR (GR sensor).

Cell culture and transfection

The mouse osteoblastic cell line MC3T3-E1 (RCB1126, RIKEN Cell Bank, Japan) was used as a test model. Cells were transfected with the plasmid of the GR sensor using an electroporation system (NEPA21, Nepa Gene, Japan) and were then seeded on plastic dishes in minimum essential medium with alpha modification (αMEM; Wako, Japan) supplemented with 10% fetal bovine serum (Biowest, France), penicillin (100 unit/mL), and streptomycin (100 μg/mL, Sigma, Japan) and cultured for 24–72 h before experiment. We trypsinized the cells expressing the GR sensor and plated them on glass bottom dishes (D111400, Matsunami, Japan) coated with fibronectin (100 μg/mL, Sigma, USA) at 100 cells/mm² and cultured them in a humidified 95% air-5% CO₂ incubator at 37°C for 2.5 h. Then, we removed the dish from the incubator and placed it in a microscope-stage-top incubator consisting of a laboratory-made polystyrene foam box with a top heater set at 41°C, a bottom heater at 37°C, and a bubbling port for 95% air-5% CO₂ and cultured the cells for 0.5 h before imaging.

Live cell imaging

The multiarea time-lapse mode of a confocal laser scanning microscope (FV1200 + IX81, Olympus, Japan) with a 100× oil immersion objective (UPLSAPO100XO, N.A. = 1.40, Olympus) and motorized stages (HI1171E11X/F, Prior Scientific, UK) was used for image acquisition. Multi-line Ar laser FV5-LAMAR-2 (488 nm, Olympus) was employed for excitation of EGFP. EGFP (donor) and mCherry (acceptor) images were obtained at 505–525 nm and 560–660 nm, respectively, from the top to the bottom of each cell with a 0.4-μm step. This acquisition process was repeated with a ten-minute interval.

Image analysis and FRET ratio calculation

ImageJ (Ver1.51jB, NIH, USA) was used for image analysis. We used the FRET ratio, which is a ratio of acceptor to donor signal (mCherry/EGFP), to evaluate the tension changes in SF. Figure 1 shows the image processing procedure. The details of all steps are described in Table 1. In the previous study [12], steps (0) to (6) were used, while steps (0), (1), (3), (4), and (7) to (10) were used in this study. We obtained the FRET ratio in a single SF with these steps and

![Image](image_url)

**Fig. 1** Typical image processing procedure. The number of slices used in the process depends on the z step size. The case of three slices is shown. Steps (0) to (6) show the previous method [12], and steps (0), (1), (3), (4), and (7) to (10) show the present method for calculation of the mean FRET ratio at tᵢ.
studied the correlation between the movement of the SF and the change in its FRET ratio. The movement of each SF was parameterized, as shown in Fig. 2. First, we identified the ROI (region of interest) for a SF visually in step (4) on the projected EGFP image in step (3) and calculated the XY coordinates of the centroid of the ROI, the angle of the major axis of the ellipse fitted to the ROI, and the mean FRET ratio of the ROI at each time point with ImageJ. Then, the parameters shown in Table 2 were calculated from these data to evaluate the movement of each SF.

### Statistical analysis

Correlations between the movement parameters $V_i$, $\theta_i$, or $\omega_i$ and the FRET ratios $r_i$, or $\Delta r_i$ of each SF were identified with Pearson’s correlation coefficient using the t-test and were considered significant when $P < 0.05$.

### Results

Figure 3(a–c) show typical images of the maximum intensity projection of the EGFP of an MC3T3-E1 cell cultured for 6 h (a), 9 h (b) and 12 h (c). The amount of cell movement was small and was around 12 μm in 10 h. Change in cell shape was small, either. Fig. 3(d) and (e) show the FRET ratio images with the previous (after step (2)) and new process methods (after step (9)) of an MC3T3-E1 cell at 9 h after plating (Fig. 3(b)). The FRET ratio image obtained by the previous method (d) is smoother than the ones from the new method. It contains signals outside the SFs and does not fit very well with the original EGFP image (b). In contrast, the FRET ratio image with the new method (e) was similar to the EGFP image (b), and it may reflect the dynamics of the SF more faithfully. For example, the SF indicated by the white ellipse was stable for more than 7 h, and thus, its FRET ratio is supposed to be stable. As shown in Fig. 3(f), the time-course change in the FRET ratio was indetical to the ellipse (f) compared with (e). The time-course change in the FRET ratio of each SF in an MC3T3-E1 cell was more stable in the new method than the previous method. Thus, the new method might be more suitable for the analysis of the FRET ratio change in a single SF.

Figure 4 shows typical examples of time-course changes in the parameters of movement ($V_i$, $\theta_i$, $\omega_i$) compared with that of the FRET ratio ($r_i$ and $\Delta r_i$) of a SF in an MC3T3-E1 cell. We performed the same analysis on the other seven SFs in this cell and obtained similar results: although these parameters change over time, there seems to be no significant correlation between the parameters of the cell movement and the FRET ratio, except between $\theta_i$ and $r_i$. Figure 5 shows the time course changes in $\theta_i$, $r_i$, or $\Delta r_i$ for the other seven SFs, and Fig. 6 shows the summary of the correlation between $\theta_i$, $r_i$, and the angle $\theta_i$ of the SF had a significantly positive correlation.

### Discussion

We proposed a new method for the analysis of the FRET ratio in a single SF. In the previous method, the FRET ratio...

---

#### Table 1 Details of the steps shown in Figure 1.

| Step | Operation |
|------|-----------|
| (0)  | Obtain EGFP (donor) and mCherry (acceptor) images in 3–5 slices adjacent to the cell bottom. The number of slices is dependent on the z step size. |
| (1)  | Calculate the FRET ratio (mCherry/EGFP) for each pixel in each slice (0). |
| (2)  | Apply the median filter to each of the FRET ratio images (1) to reduce noise and smooth the edges. |
| (3)  | Apply the maximum intensity projection to the slices of EGFP images (0) to identify a SF. |
| (4)  | Select the ROI (region of interest) for the SF visually on the projected EGFP image (3). |
| (5)  | Obtain the mean value of the FRET ratio in the ROI (4) for each slice. |
| (6)  | Average the mean values (5) to obtain the mean FRET ratio of said SF. |
| (7)  | At each pixel of the EGFP image (0), scan the stack in the z direction to find the pixel with the maximum EGFP intensity, and replace its value with 1 and the others with 0 to make a 3D mask for the specific SF. |
| (8)  | Multiply the stack of the FRET ratio images (1) with the 3D mask (7). |
| (9)  | Project the stack (8) in the z direction to obtain the 2D FRET ratio image of the specific SF. |
| (10) | Obtain the mean FRET ratio of the projected FRET ratio image (9) in the ROI (4). |

---

#### Table 2 List of parameters used to describe the movement of SFs.

| Param. | Description |
|--------|-------------|
| $V_i$  | Velocity of the centroid of the SF from $t_{i-1}$ to $t_i$, i.e., the displacement $X_i$ of the centroid in 10 min [μm/10 min] |
| $\theta_i$ | Angle of the SF at $t_i$ relative to the angle at $t_0$ [deg] |
| $\omega_i$ | Angular velocity of the SF from $t_{i-1}$ to $t_i$ [deg/10 min] |
| $r_i$  | Mean FRET ratio of the SF at $t_i$ [a.u.] |
| $\Delta r_i$ | Change in the mean FRET ratio of the SF from $t_{i-1}$ to $t_i$ [a.u.] |
Fig. 3 Comparison of the present and previous [12] methods. Typical images of EGFP of an MC3T3-E1 cell cultured for 6 h (a), 9 h (b) and 12 h (c), FRET ratio with previous method (d) and the new method (e) at 9 h, and time-course changes in the FRET ratio $r_i$ of the SF indicated by the white ellipse (d) were shown. Bar = 20 μm.

Fig. 4 Comparison of the time-course changes in parameters of movement ($V_i$, $\theta_i$, and $\omega_i$) and of the FRET ratio ($r_i$ and $\Delta r_i$) of SF1 of an MC3T3-E1 cell. Time-course changes in the cell movement parameters shown in (a), (b), and (c) are the same as that of (d), (e), and (f), respectively.
image was smoothed by the median filter, and the ratio in the region of the SF was averaged in each slice and then for all slices to obtain the ratio of the SF. This may contain FRET ratio data that are not from the SF but from the points adjacent to the SF. To obtain the pure FRET ratio only from said SF, we abandoned the median filter and adopted a 3D mask made from the pixel with maximum EGFP intensity in the z direction in the region of said SF. With this method, we obtained a much more stable FRET ratio than that of the previous method for stable SFs (see Fig. 3(f) for an example).

It has been reported that SFs play important roles in cell locomotion [1], and SFs were one of the main components that convey an intracellular tensional force [16]; their tension may have something to do with their movement. We thus studied the possible relationship between their movement and the FRET ratio change. We selected many parameters related to the movement of the SF, including SF velocity $V_i$, its components along the SF and perpendicular to the SF (data not shown), SF length (data not shown), SF angle $\theta_i$, and SF angular velocity $\omega_i$, and tried to find the correlation between these parameters and the FRET ratio $r_i$ and its change $\Delta r_i$. However, we could not find any pairs with a significant correlation, except $\theta_i$ and $r_i$. We have also analyzed the correlation between these parameters by changing their phase difference from +60 min to −60 min to find any possible response with a time delay (data not shown), but these attempts were in vain. These results might indicate that the correlation between the movement and tension change in SFs is weak.

The SF angle $\theta_i$ and FRET ratio $r_i$ had a positive correlation. This means that the tension of the SFs decreases when they are rotating. Although the reason for this correlation remains unclear at this stage, it might be beneficial to the movement of SFs if the tension decreases.

There are several limitations to this study. First, the number of data was limited due to technical difficulties in obtaining long-time observation for >10 h. The fluorescent intensities of the donor and the acceptor were not so strong and decreased gradually due to photobleaching. We might increase the laser intensity or increase the scanning time, but these lead to cell detachment from the substrate, possibly due to phototoxicity. In addition, cells often became out-of-focus due to the drift of the stage. Due to such difficulties, the success rate of the >10 h observation was less than 10%.

Second, it was not easy to identify the ends of each SF clearly as the quality of the EGFP image was not clear enough, and thus, the SF length $L_i$ was not so reliable. In a future study, we need to visualize the focal adhesions (FAs) to determine the length of the SF. This might also be beneficial because we may be able to study the relationship between the tension change in the SFs and the dynamics of the FAs. Third, we focused on the SFs on the bottom of the cells. However, several studies reported that the top and the bottom SFs differ not only in morphological characteristics
but also in tensional behavior [18]. It is important to compare the dynamics of the FRET ratio change in the top and the bottom SFs in detail.

Conclusions

In this study, we developed a new method for the analysis of the FRET ratio in a single SF. With this new method, we evaluated the relationship between the movement of the SFs and their FRET ratio change for the first time to our knowledge. We found that the tension in SFs decreases when they rotate. The tension had no significant correlation with their translation nor with their length change.

Acknowledgements

This work was supported in part by KAKENHIs (Nos. 15H05860 and 18H03752) and AMED-CREST from the Japan Agency for Medical Research and Development (JP18gm0810005).

References

1. Dennis B. Cell Movements: From Molecules to Motility. Garland Science. 2001; p. 81.
2. Tan JL, Tien J, Pirone DM, Gray DS, Bhadriraju K, Chen CS. Cells lying on a bed of microneedles: an approach to isolate mechanical force. Proceedings of the National Academy of Sciences. 2003; 100(4): 1484–9.
3. du Roure O, Saez A, Buguin A, Austin RH, Chavrier P, Silberzan P, Ladoux B. Force mapping in epithelial cell migration. Proceedings of the National Academy of Sciences. 2005; 102(7): 2390–5.
4. Nagayama K, Adachi A, Matsumoto T. Heterogeneous response of traction force at focal adhesions of vascular smooth muscle cells subjected to macroscopic stretch on a micropillar substrate. J Biomechanics. 2011; 44(15): 2699–705.
5. Nagayama K, Adachi A, Matsumoto T. Dynamic changes of traction force at focal adhesions during macroscopic cell stretching using an elastic micropillar substrate: tensional homeostasis of aortic smooth muscle cells. Journal of Biomechanical Science and Engineering. 2012; 7(2): 130–40.
6. Marinković A, Mih JD, Park JA, Liu F, Tschumperlin DJ. Improved throughput traction microscopy reveals pivotal role for matrix stiffness in fibroblast contractility and TGF-β responsiveness. American Journal of Physiology-Lung Cellular and Molecular Physiology. 2012; 303(3): 169–80.
7. Delanoê-Ayari H, Rieu JP, Sano M. 4D traction force microscopy reveals asymmetric cortical forces in migrating diectostemium cells. Phys Rev Lett. 2010; 105(24): 248103.
8. Sugita S, Adachi T, Ueki Y, Sato M. A novel method for measuring tension generated in stress fibers by applying external forces. Biophys J. 2011; 101(1): 53–60.
9. Meng F, Suchyna TM, Sachs F. A fluorescence energy transfer-based mechanical stress sensor for specific proteins in situ. FEBS J. 2008; 275(12): 3072–87.
10. Meng F, Sachs F. Visualizing dynamic cytoplasmic forces with a compliance-matched FRET sensor. Journal of cell science. 2011; 124(2): 261–9.
11. Yamashita S, Tsuobi T, Ishinabe N, Kitaguchi T, Michiue T. Wide and high resolution tension measurement using FRET in embryo. Scientific Reports. 2016; 6: 28535.
12. Wang J, Ito M, Zhong W, Sugita S, Michiue T, Tsuobi T, Kitaguchi T, Matsumoto T. Observations of intracellular tension dynamics of MC3T3-E1 cells during substrate adhesion using a FRET-based actinin tension sensor. Journal of Biomechanical Science and Engineering. 2016; 11(4): 16-00504.
13. Grashoff C, Hoffman BD, Brenner MD, Zhou R, Parsons M, Yang MT, McLean MA, Sligar SG, Chen CS, Ha T, Schwartz MA. Measuring mechanical tension across vinculin reveals regulation of focal adhesion dynamics. Nature. 2010; 466(7303): 263–6.
14. Kumar A, Ouyang M, Van den Dries K, McGhee EJ, Tanaka K, Anderson MD, Groisman A, Goult BT, Anderson KI, Schwartz MA. Talin tension sensor reveals novel features of focal adhesion force transmission and mechanosensitivity. J Cell Biol. 2016; 213(3): 371–83.
15. Hotulainen P, Lappalainen P. Stress fibers are generated by two distinct actin assembly mechanisms in motile cells. J Cell Biol. 2006; 173: 383–94.
16. Tojkander S, Gateva G, Lappalainen P. Actin stress fibers-assembly, dynamics and biological roles. J Cell Sci. 2012; 125( Pt 8): 1855–64.
17. Wang J, Sugita S, Nagayama K, Matsumoto T. Dynamics of actin filaments of MC3T3-E1 cells during adhesion process to substrate. Journal of Biomechanical Science and Engineering. 2016; 11(2): 15-00637.
18. Nagayama K, Yahiro Y, Matsumoto T. Apical and basal stress fibers have different roles in mechanical regulation of the nucleus in smooth muscle cells cultured on a substrate. Cellular and Molecular Bioengineering. 2013; 6(4): 473–81.