Focal adhesion displacement magnitude is a unifying feature of tensional homeostasis

Abbreviated title: Substrate stiffness and tensional homeostasis of cells

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Abstract

Tensional homeostasis is widely recognized to exist at the length scales of organs and tissues, but the cellular length scale mechanism for tension regulation is not known. In this study, we explored whether tensional homeostasis emerges from the behavior of the individual focal adhesion (FA), which is the subcellular structure that transmits cell stress to the surrounding extracellular matrix. Past studies have suggested that cell contractility builds up until a certain displacement is achieved, and we thus hypothesized that tensional homeostasis may require a threshold level of substrate displacement. Micropattern traction microscopy was used to study a wide range of FA traction forces generated by bovine vascular smooth muscle cells and bovine aortic endothelial cells cultured on substrates of stiffness of 3.6, 6.7, 13.6, and 30 kPa. The most striking feature of FA dynamics observed here is that the substrate displacement resulting from FA traction forces is a unifying feature that determines FA tensional stability. Beyond approximately 1 μm of substrate displacement, FAs, regardless of cell type or substrate stiffness, exhibit a precipitous drop in temporal fluctuations of traction forces. These findings lead us to the conclusion that traction force dynamics collectively determine whether cells or cell ensembles develop tensional homeostasis, and this insight is necessary to fully understand how matrix stiffness impacts cellular behavior in healthy conditions and, more important, in pathological conditions such as cancer or vascular aging, where environmental stiffness is altered.

Keywords: traction force microscopy, stiffness mechanosensing, cytoskeletal tension, homeostasis, focal adhesions

Introduction

The ability to maintain stable cytoskeletal tension (or prestress) is a unique form of homeostasis that exists at the tissue and organ levels.[1,2] Some cell types, such as smooth muscle cells and fibroblasts, develop homeostatic cell prestress at the single cell level, while endothelial cells require contact with neighboring cells for homeostasis to develop.[3–5] However, the mechanisms that lead to generation of stable tension are unknown. An understanding of these mechanisms may have broad implications for human health and disease, since tensional homeostasis may be lost in diseases such as cancer and is an important feedback mechanism for maintaining normal tissue functions. In this study, we sought to determine whether cells generate tensional homeostasis at a sub-cellular length scale, namely at the level of the focal adhesion (FA). In order to test this, we utilized two cell types across a range of substrate stiffnesses. We used a range of substrate stiffnesses since adherent cells are able to sense the stiffness of their surroundings,[6–9] and an increase in substrate stiffness is known to increase contractility in a number of cell types.[10–16] Furthermore, cell contractility is intimately related to cytoskeletal tension,[1,2,17,18] and past works utilized a range of stiffnesses to conclude that cells may ultimately desire to achieve a set value of substrate strain.[19,20] We sought to determine if temporal fluctuations of FA forces are also impacted by the magnitude of substrate strain.

Fluctuations of cell contractility have been the indirect subject of study for some time. Early demonstrations that cells rearrange extracellular matrix (ECM) proteins at material interfaces are now recognized as the result of contractile focal and fibrillar adhesions that aid in matrix remodeling.[21] Likewise, radially inward forces are applied to new contacts, for example to beads on apical cell surfaces.[22] Indeed, force fluctuations have been identified as an

important mechanism of rigidity sensing. Plotnikov and colleagues qualitatively characterized the FAs of mouse embryonic fibroblasts as either fluctuating or more stable, and both classes of FA dynamics were present on all substrate rigidities tested for this cell type. Furthermore, they also showed that lowering substrate rigidity or downregulating p160-Rho kinase (ROCK) could shift FAs from stable to fluctuating.[23] These studies directly indicate that tensional homeostasis of FAs could depend on substrate stiffness, although these studies did not quantify homeostasis with a quantitative metric and were only assessed in fibroblasts.

Tensional homeostasis is an emergent concept in mechanobiology that has been studied in a variety of contexts. One group of studies has been focused on the conceptual understanding of how breakdown in tensional homeostasis in cells and tissues leads to progression in various diseases, including atherosclerosis and cancer.[1,2,17,18] The other group of studies, which are engineering in nature, have been focused on the response of cells to external disturbances, such as mechanical stretch, which tend to draw cells away from equilibrium states in the absence of feedback mechanisms that maintain cell properties such as cytoskeletal tension.[5,24,25] Relevant to tensional homeostasis are the findings that cytoskeletal tension exhibits the tendency to return to its pre-stretch value, although it may not always achieve this value.[5,24,26] Consequently, this phenomenon has been referred to as tensional buffering, rather than tensional homeostasis.[5] Investigations of our group during past years have been focused on the dynamics aspects of tensional homeostasis. We studied the ability of cells to attenuate temporal fluctuations of cytoskeletal tension as a manifestation of achieving tensional homeostasis.[3,4,26–28] We proposed a new definition of tensional homeostasis as the ability of cells to maintain a consistent level of tension with low temporal fluctuations.[3] As a quantitative metric, we used the coefficient of variation (*CV*), i.e., the extent of temporal variability of a cytoskeletal

tension relative to its mean; the smaller the value of *CV* is, the closer the tension to the state of tensional homeostasis is. Although this approach does not specify a threshold below which tensional homeostasis is achieved, it does permit quantitative comparison to determine how different factors, such as multicellularity, cell type, substrate stiffness, or stretch impact tensional homeostasis. In summary, our studies of tensional homeostasis have led to a quantitative framework for assessing tension stability and provided evidence that cells can maintain their tension state.

One of the striking results of our past work is that *CV* of the cytoskeletal tension progressively decreases with increasing of the mean level of tension. Importantly, this observation has been made at different length scales, including multicellular clusters, isolated cells, and FAs[3] and in different cell types.[4] Saez and colleagues showed more than 15 years ago that epithelial cells tend to deform their substrate by a defined length, rather than by a defined force, and suggested that this deformation-dependent control was important for reinforcement of FAs as they mature.[19] That study is also similar to a more recent mathematical model of cell contraction that identified cell average strain, which was cell type specific, as a relatively stable feature of cells cultured on substrates across a range of stiffnesses.[20]

In this study, we tested the hypothesis that substrate displacement may determine whether FAs can maintain homeostatic tension. We used two cell types, bovine vascular smooth muscle cells (BVSMCs), which exhibit high contractility, and bovine aortic endothelial cells (BAECs), which exhibit lower relative contractility. As an experimental tool, we used micropattern traction microscopy, which utilizes micron-level fluorescent ECM protein dot regular arrays that are micro-printed onto elastic polyacrylamide (PAA) gel substrates, to measure traction fields

generated by single cells and by clusters of cells.[29,30] This approach allows us to track traction forces of isolated, individual FAs formed on the protein dots. We showed previously with immunohistochemistry that FAs only form on the patterned dots because the rest of the PAA substrate is non-adhesive for cells.[31] Since traction forces arise in response to contractile force generation, the temporal variability of the observable and quantifiable traction field is indicative of the temporal variability of the cytoskeletal tension. We found that stability of FA contractile forces is, remarkably, a function of substrate displacement with a threshold, rather than a progressive decrease in temporal fluctuations of traction forces. FAs that displaced the substrate by more than approximately 1 μm had dramatically lower traction force fluctuations, and this trend was present for both isolated cells and multicellular clusters, across two different cell types, and across a range of substrate stiffnesses. This finding is, to our knowledge, the first description of an emergent property of tensional homeostasis that is applicable across a broad range of conditions.

Materials and Methods

Cell Culture

Primary BAECs and BVSMCs (Cell Applications) were cultured in Dulbecco's Modified Eagle Medium (Sigma) supplemented with 10% bovine calf serum and 1% antibiotic antimycotic solution (Sigma). Cells were kept in incubators that maintained 5% CO_2 content. Cells under passage 13 were used for the experiments to insure consistent phenotype. Between 18-24 h prior to imaging, cells were seeded onto the fibronectin micropatterned PAA gel at roughly 30×10^3 to 40×10^3 cells per gel, depending upon whether single cells or multicellular clusters were desired. Media was changed 1 h before the start of the experiment.

Polyacrylamide Gel Stiffness

The Elastic modulus (E) of PAA gels can be tuned by changing the acrylamide and bis-acrylamide ratio in the precursor solution. Using the same recipes that we established previously,[29] PAA gels were made with different acrylamide and bis-acrylamide (bis) concentrations as follows: for 3.6 kPa, 10% acrylamide + 0.07% bis; for 6.7 kPa, 10% acrylamide + 0.13% bis; for 13.6 kPa, 10% acrylamide + 0.26% bis, and for 30 kPa, 8% acrylamide + 0.35% bis. We and others have shown previously that these gels exhibit a linearly elastic behavior over a wide range of stresses and strains.[29,32-34]

Micropattern Traction Microscopy

Micropattern traction microscopy was used to measure cell-substrate traction forces.[30] Briefly, Alexa Fluor-488 tagged human fibronectin (Fn) was patterned onto PAA gel surfaces by placing a glass coverslip patterned with Fn on top of the PAA precursor solution. Gel formulation contained 0.002% acrylic acid N-hydroxysuccinimide ester (NHS) (Sigma), which

covalently bonded Fn to the gel as it polymerized. The pattern of choice was an array of 2-μm diameter dots at a 6 μm center-to-center separation. A micropatterned gel was seeded with cells and imaged with an Olympus IX881 microscope and a Hamamatsu Orca R2 camera. An image was taken every 5 min for 2 h. Experiments took place in an environmental chamber that maintains 37ºC, 70% humidity and 5% CO_2.

Images of the cells and the fluorescent Fn dot array were analyzed using custom MATLAB (MathWorks) scripts, as previously described.[29] The program finds the displacement vector (**u**) of the geometrical center of each Fn dot and calculates the corresponding traction force vector (**F**) according to the following formula

$$\mathbf{F} = \frac{\pi E a \mathbf{u}}{2 + \nu - \nu^2}, \tag{1}$$

where a = 1 μm is the radius of the dot markers and ν = 0.445 is the Poisson's ratio of the PAA gel substrate.[29]

Tracking Individual Focal Adhesions

Because cells were only able to form FAs at the Fn dots, we were able to measure the time lapse of traction forces of individual FAs throughout the 2 h observation time. Traction forces under 0.3 nN were removed because below that magnitude the measured force is indistinguishable from background noise. This threshold was chosen based on background displacements measurements made by Polio et al.,[29] and modified to fit the softest substrate of our experiments (3.6 kPa). We defined an FA as mechanical engaged if the magnitude of the traction force associated with it was above the 0.3 nN threshold. If the force dropped below the threshold, we assumed that particular FA had disassociated. Given the 2 h duration of the

experiments and the sampling rate of every 5 min, the shortest and the longest FA lifetimes were ≤ 5 min and 120 min, respectively.

Assessment of Traction Variability

We used *CV* as a metric of temporal variability of individual FA forces, as well as of the traction field of the entire cell. It is defined as the ratio of the standard deviation to the mean. For an individual FA traction force of magnitude F (i.e., $F = \|\mathbf{F}\|$), we obtained the corresponding *CV* of force time lapses (CV_F) as

$$CV_F = \frac{SD_F}{\langle F \rangle}, \tag{2}$$

where SD_F is the standard deviation and $\langle F \rangle$ is the time-average of $F(t)$, where t denotes time. We also computed the coefficient of variation of the local displacement (deformation) of an FA caused by the applied traction force as

$$CV_u = \frac{SD_u}{\langle u \rangle}, \tag{3}$$

where SD_u is the standard deviation and $\langle u \rangle$ is the time-average of $u(t)$, which is the magnitude of the displacement vector (i.e., $u = \|\mathbf{u}\|$). Because $F(t)$ is calculated from $u(t)$ according to Eq. 1, it follows that $CV_u = CV_F$. Thus, in the remaining text we use CV_F for both coefficients of variation of the force and of the displacement.

In the case of the traction field of a cell or cell cluster, we first computed a scalar metric of the magnitude of the traction field (T) as the sum of magnitudes of all FA forces within the cell, at a given t as follows

$$T(t) = \sum_{i=1}^{N(t)} F_i(t), \tag{4}$$

where $N(t)$ is the number of FA forces in the cell at a given t. The corresponding CV of traction field time lapses (CV_T) is then calculated as follows

$$CV_T = \frac{SD_T}{\langle T \rangle}, \tag{5}$$

where SD_T is the standard deviation and $\langle T \rangle$ is the time-average of $T(t)$.

Data Analysis

After thresholding forces < 0.3 nN, the average traction forces $\langle F \rangle$ were sorted in ascending manner. Next we calculated the difference between the highest and lowest values of $\langle F \rangle$ and divided it by 10. Hence, we obtained ten bins of data for $\langle F \rangle$ and the corresponding CV_F. For each bin, we calculated the mean $\langle F \rangle$, the mean CV_F, and the corresponding standard errors for all substrate stiffnesses. We did the same analysis for $\langle T \rangle$ and CV_T of single cells and clusters.

Results

We studied the effect of substrate stiffness on tensional homeostasis in isolated cultured cells and multicellular clusters by measuring temporal variability of cellular traction forces on Fn-patterned PAA gels with stiffness of 3.6, 6.7, 13.6, and 30 kPa for BVSMCs and on 3.6, 6.7, and 13.6 for BAECs. Total numbers of cells and FAs under each condition are provided in Table 1. We observed that in both cell types traction forces of larger magnitude were located near the edge of the cells, and traction forces of lower magnitude were located towards the middle of cells (Fig. 1). These findings were consistent with the literature.[32–34] Furthermore, this pattern of traction force distribution remained consistent in multicellular clusters and on all the substrates of different stiffnesses, for both cell types (Fig. 1). Each force vector shown in Fig. 1 represents a single FA, as we demonstrated previously that FAs only form on the dot pattern in this system.[31] Thus, the fundamental measurement in these experiments is FA force, which is tracked for up to 2 h of observation time. Time lapses of magnitudes of FA forces (Fig. 2A) and of their magnitudes normalized by $\langle F \rangle$ (Fig. 2B) for five representative FAs of BAECs on 6.7 kPa PAA substrates exhibited erratic fluctuations over the 2-h observation period. Indeed, FAs may form and disassemble during the course of these experiments, but we limited analysis to a 2-h window. Therefore, we plotted the total numbers and survival rate of FAs that remain intact as a function of time for both BVSMCs (Suppl. Fig. S1A, B) and BAECs (Suppl. Fig. S1C, D).

We first focused on the length scale of single FAs in order to investigate the impact of substrate stiffness on temporal fluctuations of traction forces associated with each FA and whether it could provide a better understanding of temporal fluctuations of the traction field of the cell. We observed that, in both BAECs and BVSMCs, the average CV_F stayed nearly constant as the mean force $\langle F \rangle$ increased until $\langle F \rangle$ reached a threshold value, after which CV_F rapidly

decreased. This behavior was nearly the same in both single cells and in clusters, in BVSMCs and in BAECs. This relationship did not change qualitatively with increasing substrate stiffness; it only got shifted to the right, which was indicative of increasing FA forces due to increasing stiffness of the substrate (Fig. 3A, B).

Since traction forces were calculated as the product between substrate stiffness and the displacement of FAs caused by the corresponding traction force (see Eq. 1), we obtained CV_F versus the average FA displacement magnitude $\langle u \rangle$ relationships. We found that the CV_F versus $\langle u \rangle$ relationships corresponding to different values of substrate stiffness were virtually the same. The threshold of $\langle u \rangle$ after which CV_F rapidly decreases was around 1-2 μm (Fig. 3C, D). According to Eqs. 2 and 3, the dependences of CV_F on $\langle F \rangle$ and on $\langle u \rangle$ shown in Fig. 3 imply that SD_F and SD_u increase, peak (threshold), and then decreases with increasing $\langle F \rangle$ and $\langle u \rangle$, respectively (Suppl. Fig. S2).

Taken together, the above results indicate that increasing FA force and displacement attenuated their temporal fluctuations only after force and displacement reached a threshold value. Importantly, temporal fluctuations of forces and displacements of individual FAs did not appear to depend on the substrate stiffness.

We next obtained CV_T versus $\langle T \rangle$ relationships for single cells and clusters on the 3.6 kPa stiff substrate. Note that $\langle T \rangle$ increased with increasing cluster size since the number of FAs, N, increased (see Eq. 4). We found that the relationship between CV_T and $\langle T \rangle$ in both BVSMCs and BAECs exhibited a decreasing trend (Fig. 4C, D), which was qualitatively different from the behavior observed at the FA level (Fig. 4A, B). Most notably, we did not observe a threshold value of $\langle T \rangle$ after which attenuation of CV_T markedly increased. We attributed the absence of

threshold to the heterogeneity in the traction force distribution (see Fig. 1), which we address in the Discussion.

Discussion

Tensional homeostasis is relatively unstudied at the cellular length scale, although this concept is widely accepted as an essential feedback mechanism at the tissue and organ levels.[1] In this study, we cultured two disparate cell types on substrates with a range of stiffnesses in order to identify common features of tensional homeostasis that apply across a range of cell conditions. We investigated whether fundamental unit of stress application to the substrate, the FA, behaved differently between cell types. The most striking feature of FA dynamics observed here is that the substrate displacement is a unifying feature that determines FA tensional stability. Beyond approximately 1 μm of substrate displacement, FAs, regardless of cell type, whether associated with single cells or cell clusters, or substrate stiffness, exhibited a precipitous drop of CV_F. Traction force, however, depended upon both substrate displacement and substrate material properties, and FAs exhibited a threshold in $\langle F \rangle$ below which CV_F was nearly constant and above which it rapidly decreased, although this threshold value of $\langle F \rangle$ varied between substrates of different stiffness. In contrast, whole-cell traction field variability quantified with CV_T versus $\langle T \rangle$ relationships exhibited no threshold and decayed slowly with increasing $\langle T \rangle$. These findings are both unique and significant since unifying features of tensional homeostasis that apply to more than one cell type or culture condition have remained elusive until now.

Mechanistic Hypotheses

These findings have significant implications for our understanding of the mechanism of tensional homeostasis. We discuss the potential role of FA numbers and arrangement on whole cell homeostasis of tension, how a threshold of substrate displacement may be an indication of FA maturity, how cell regulatory networks may play a role in maintenance of FA behavior, and

how the probabilistic nature of molecular bonds that are needed to generate cell prestress may impact tensional homeostasis below.

Mechanical features of the whole cell or multicellular cluster are clearly dependent upon the arrangement of FAs and contacts with adjacent cells, but the rapid decrease of CV_F after the forces and displacements reached the threshold is not well understood. One possibility may be that after crossing this threshold, the dynamics of actin-myosin bonds[35] and/or of molecular clutches of FAs[36,37] slows down as forces and displacements approach the stalled limit. This threshold behavior, however, was not observed at the whole cell or cell cluster level (see Fig. 4C, D). This may be explained by the presence of competing influences that simultaneously exist at the whole cell and cell cluster level as follows. An increasing number of FAs causes an increase in $\langle T \rangle$ (see Eq. 4), which causes CV_T to decrease (see Eq. 5). This decrease is impeded by heterogeneous distribution of traction forces within cells and cell clusters. Forces of larger magnitudes are located primarily near the cell or cluster edges and forces of lower magnitudes are located primarily in the interior of the cell or cell cluster (see Fig. 1). With increasing number of FAs (e.g., increasing of the cell or cell cluster size), the number of forces of larger magnitudes decreases relative to the number of forces of lower magnitudes. Since forces of lower magnitudes exhibit greater values of CV_F than forces of greater magnitudes, one would expect that CV_T would increase with increasing number of FAs. This, in turn, suggests that metrics of whole cell contractility, such as CV_T, are emergent properties that result from FA dynamics. In addition, it is also known that both substrate stiffness and the density of FAs formed impact traction forces independently.[38] Han et al. showed that FA density was more dominant than cell spread area, and it is important to note that our pattern keeps the FA density constant across all substrates while also limiting FA size, but cell spread area is not controlled.

Finally, a positive correlation between FA traction forces is shown to enhance CV_T and that this effect is cell type-dependent.[28] Therefore, our finding that whole cell metrics emerge from FA dynamics appears to be consistent with the concept of rheostasis proposed by Weng et al.[39] Namely, they showed that rheostasis at the level of FAs drives homeostasis of the whole cell.

Stability of $F(t)$ for those FAs that have reached or exceeded a threshold of substrate displacement may also be a mechanical signature or feature of FA maturity. For molecular insight into cell tension, LaCroix and colleagues recently developed tension sensors for vinculin that require different force magnitudes to achieve the same level of extension, and these vinculin sensors demonstrated that highly loaded FAs on the periphery of mouse embryonic fibroblasts exhibited conserved gradients of vinculin extension, rather than force[40]. This extension-based property of vinculin was also found on soft surfaces, which is qualitatively similar to the extension-based control of tensional homeostasis found here, despite the large differences in extensibility of single molecules relative to the PAA substrate. Furthermore, mechanical tension across vinculin was shown to switch FAs between assembly and disassembly,[41,42] thus making vinculin an exciting candidate transducer of mechanical inputs. These findings may be consistent with results after diminishing cell contractility with drug treatments. Plotnikov and colleagues[23] showed that a low concentration of the ROCK inhibitor Y-27632 led to a loss of more stable FAs and an increase in the proportion of variable tugging FAs in mouse embryonic fibroblasts on substrates from 8 to 32 kPa, which is consistent with our prediction that lower values of substrate displacement would lead to higher CV_F values for FAs. However, these investigators did not assess traction force variability with a quantitative metric. They also found that lowering substrate stiffness causes traction forces to become more dynamic, whereas our results indicate that substrate stiffness does not affect traction force fluctuations. In summary,

our results demonstrate that displacement-based control of FA homeostasis may be unifying across a broad range of cell types.

Tensional homeostasis may also result from the coordinated action of regulatory networks that control myosin activity. FRET-based molecular sensors of Rho GTPases clearly indicate that these kinases fluctuate on fast enough time scales to account for fluctuations in tension measured here (c.f., [43]). For example, extracellular signal-regulated kinase (ERK) was shown to pulse spontaneously or due to cell-to-cell propagation from adjacent cells.[44] In addition, the activity of Src-family kinases (SFKs) such as Fyn may burst on the order of every few minutes, and these fluctuations were shown to vary spatially within single cells.[45] However, Plotnikov and colleagues also showed that serine 19 phosphorylated myosin II regulatory light chain activity was not responsible for an increase in fluctuating FAs,[23] indicating that this may not fully explain FA traction stability. Measurement of the activity of myosin, myosin light chain kinase, or kinases that regulate their activity in control, knockout, and mutant cells while simultaneously measuring cell traction forces may provide insight into this relationship.

Lastly, variability in cell prestress could also result from the probabilistic nature of molecular bonds that hold actin in tension. Modeling approaches that focused on the role of bond mechanics and a molecular clutch at the cell to substrate interface have suggested that substrate rigidity can alter the variability of traction dynamics[36,37]. However, these studies focused on steady state features such as conditions that permit maximum cell traction and are also dependent upon individual bond on- and off-rates.[46] These models may also depend on the mechanical properties of actomyosin networks, which differ within the cytoskeleton of cells and may certainly differ between cell types.[47] Adaptation of these models to study the temporal

dynamics of FA fluctuations may be very informative, and consideration of bond breakage between myosin and actin may also be important for low force-generating FAs since force fluctuations in this regime may result from minifilaments of myosin II, which may even form as a result of these force fluctuations.[48] Regardless, the precipitous drop in CV_F above 1 μm displacement suggests that molecular reinforcement of FAs, as we discussed above, may dominate the behavior of FAs at least in the context of temporal stability.

Implications for Mechanosensing

The finding that tensional homeostasis may derive from FA substrate displacement has implications for our understanding of mechanosensing of the cell microenvironment. These results, combined with our recent finding that traction forces stabilize as cells reorient in the presence of externally applied strain[26], suggests that variability of FA forces may be high during dynamic processes such as migration and cell shape changes. Durotaxis is a dynamic process, and in this context, leading edge FAs will be adherent to stiffer material than those FAs bound to the substrate at the trailing edge. Breckenridge et al. found that NIH 3T3 fibroblasts spanning two rigidities developed stronger traction forces on the more rigid side of the cell[49]. However, whether this leads to lower, higher, or similar substrate displacements from these FAs will depend upon multiple factors, such as the number of FAs that are formed at the leading versus trailing edge. Likewise, the response to externally applied strain requires future investigation. Some FAs could be pulled into high displacement states after imposition of substrate strain, and it is intriguing to consider that strain sensation may stem from those FAs connected to substrates that get extended beyond 1 μm.

Limitations

Several limitations of our approach are noteworthy. First, in tracking individual FAs, we were in fact tracking FA forces. By our definition, if the force applied at an FA dropped below the 0.3 nN experimental threshold,[29] we considered that particular FA to have disassembled. However, it is possible for the FA structure to remain at least momentarily without a measurable force to the substrate. Importantly, this limitation has no impact on the observed dependence of substrate displacement magnitude on CV_F. Second, the Fn dot pattern used in micropatterned traction microscopy spatially restricted where FAs can form. Nevertheless, this had little effect on the observed spatial distribution of traction force magnitude (i.e., large forces near the edges and small forces in the interior), since similar distributions were observed previously on soft substrates with continuous coating of ECM proteins.[32–34] Indeed, this is likely an important feature of this approach, as this allows a more careful examination of the impact of displacement on FA traction variability. In fact, the complex association between FA size, age, and traction force[32,50–52] supports the use of micropatterning to limit FA spread area as was done here. Nevertheless, future studies that track FA maturity based on the association of intracellular accessory proteins while simultaneously monitoring their temporal stability are necessary to determine the molecular origin of FA stabilization that occurs beyond the 1-2 μm threshold.

Summary

This study investigated the effects of substrate displacement on tensional homeostasis of BAECs and BVSMCs and found that substrate displacement is a common feature that dictates whether FAs are variable or stable in their temporal stress. We and others view displacement as a common sense metric in mechanobiology since it suggests a specific deformation of intracellular signaling molecules at the FA that determines whether that FA will be reinforced and stabilized

or immature and fluctuating in substrate stress application. Future studies of stiffness-dependent mechanobiology of cells should focus on holistic metrics of cell dynamics behavior that are assessed from a single FA to whole cell length scales. Furthermore, it is conceivable that these attributes of FAs also play a major role in multicellular ensembles of cells that are critically important to study due to the addition of cell-cell adhesion molecules in their mechanical stability. This understanding across length scales is necessary to fully understand how matrix stiffness impacts cellular behavior in healthy conditions and, more important, in pathological conditions such as cancer or vascular aging, where environmental stiffness is altered.

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References

[1] J.D. Humphrey, Vascular Adaptation and Mechanical Homeostasis at Tissue, Cellular, and Sub-cellular Levels, Cell Biochem. Biophys. 50 (2008) 53–78. https://doi.org/10.1007/s12013-007-9002-3.

[2] S. Chien, Mechanotransduction and endothelial cell homeostasis: the wisdom of the cell, Am. J. Physiol. - Heart Circ. Physiol. 292 (2007) H1209–H1224. https://doi.org/10.1152/ajpheart.01047.2006.

[3] E.P. Canović, A.J. Zollinger, S.N. Tam, M.L. Smith, D. Stamenović, Tensional homeostasis in endothelial cells is a multicellular phenomenon, Am J Physiol Cell Physiol. 311 (2016) C528-35. https://doi.org/10.1152/ajpcell.00037.2016.

[4] A.J. Zollinger, H. Xu, J. Figueiredo, J. Paredes, R. Seruca, D. Stamenović, M.L. Smith, Dependence of Tensional Homeostasis on Cell Type and on Cell–Cell Interactions, Cell. Mol. Bioeng. 11 (2018) 175–184. https://doi.org/10.1007/s12195-018-0527-x.

[5] K.D. Webster, W.P. Ng, D.A. Fletcher, Tensional homeostasis in single fibroblasts, Biophys. J. 107 (2014) 146–155. https://doi.org/10.1016/j.bpj.2014.04.051.

[6] D.E. Discher, P. Janmey, Y.L. Wang, Tissue Cells Feel and Respond to the Stiffness of Their Substrate, Science. 310 (2005) 1139–1143.

[7] B.C. Isenberg, P.A. Dimilla, M. Walker, S. Kim, J.Y. Wong, Vascular smooth muscle cell durotaxis depends on substrate stiffness gradient strength, Biophys J. 97 (2009) 1313–22. https://doi.org/10.1016/j.bpj.2009.06.021.

[8] A.J. Engler, S. Sen, H.L. Sweeney, D.E. Discher, Matrix elasticity directs stem cell lineage specification, Cell. 126 (2006) 677–89.

[9] C.M. Lo, H.B. Wang, M. Dembo, Y.L. Wang, Cell movement is guided by the rigidity of the substrate, Biophys J. 79 (2000) 144–52.

[10] R.L. Urbano, C. Furia, S. Basehore, A.M. Clyne, Stiff Substrates Increase Inflammation-Induced Endothelial Monolayer Tension and Permeability, Biophys. J. 113 (2017) 645–655. https://doi.org/10.1016/j.bpj.2017.06.033.

[11] A.D. Doyle, N. Carvajal, A. Jin, K. Matsumoto, K.M. Yamada, Local 3D matrix microenvironment regulates cell migration through spatiotemporal dynamics of contractility-dependent adhesions, Nat. Commun. 6 (2015) 8720. https://doi.org/10.1038/ncomms9720.

[12] T.R. Polte, G.S. Eichler, N. Wang, D.E. Ingber, Extracellular matrix controls myosin light chain phosphorylation and cell contractility through modulation of cell shape and cytoskeletal prestress, Am. J. Physiol. Cell Physiol. 286 (2004) C518-528. https://doi.org/10.1152/ajpcell.00280.2003.

[13] R. Krishnan, D.D. Klumpers, C.Y. Park, K. Rajendran, X. Trepat, J. van Bezu, V.W.M. van Hinsbergh, C.V. Carman, J.D. Brain, J.J. Fredberg, J.P. Butler, G.P. van Nieuw Amerongen, Substrate stiffening promotes endothelial monolayer disruption through enhanced physical forces, Am. J. Physiol. - Cell Physiol. 300 (2011) C146–C154. https://doi.org/10.1152/ajpcell.00195.2010.

[14] A. Izquierdo-Álvarez, D.A. Vargas, Á. Jorge-Peñas, R. Subramani, M.-M. Vaeyens, H. Van Oosterwyck, Spatiotemporal Analyses of Cellular Tractions Describe Subcellular Effect of Substrate Stiffness and Coating, Ann. Biomed. Eng. 47 (2019) 624–637. https://doi.org/10.1007/s10439-018-02164-2.

[15] D.W. Zhou, T.T. Lee, S. Weng, J. Fu, A.J. García, Effects of substrate stiffness and actomyosin contractility on coupling between force transmission and vinculin-paxillin recruitment at single focal adhesions, Mol. Biol. Cell. 28 (2017) 1901–1911. https://doi.org/10.1091/mbc.E17-02-0116.

[16] J. Huynh, N. Nishimura, K. Rana, J.M. Peloquin, J.P. Califano, C.R. Montague, M.R. King, C.B. Schaffer, C.A. Reinhart-King, Age-related intimal stiffening enhances endothelial permeability and leukocyte transmigration, Sci. Transl. Med. 3 (2011) 112ra122. https://doi.org/10.1126/scitranslmed.3002761.

[17] D.T. Butcher, T. Alliston, V.M. Weaver, A tense situation: forcing tumour progression, Nat. Rev. Cancer. 9 (2009) 108–122. https://doi.org/10.1038/nrc2544.
[18] M.J. Paszek, N. Zahir, K.R. Johnson, J.N. Lakins, G.I. Rozenberg, A. Gefen, C.A. Reinhart-King, S.S. Margulies, M. Dembo, D. Boettiger, D.A. Hammer, V.M. Weaver, Tensional homeostasis and the malignant phenotype, Cancer Cell. 8 (2005) 241–254. https://doi.org/10.1016/j.ccr.2005.08.010.
[19] A. Saez, A. Buguin, P. Silberzan, B. Ladoux, Is the mechanical activity of epithelial cells controlled by deformations or forces?, Biophys J. 89 (2005) L52-4. https://doi.org/10.1529/biophysj.105.071217.
[20] S. Lin, M.C. Lampi, C.A. Reinhart-King, G. Tsui, J. Wang, C.A. Nelson, L. Gu, Eigenstrain as a mechanical set-point of cells, Biomech. Model. Mechanobiol. 17 (2018) 951–959. https://doi.org/10.1007/s10237-018-1004-0.
[21] Z. Avnur, B. Geiger, The removal of extracellular fibronectin from areas of cell-substrate contact, Cell. 25 (1981) 121–32.
[22] D. Choquet, D.P. Felsenfeld, M.P. Sheetz, Extracellular matrix rigidity causes strengthening of integrin-cytoskeleton linkages, Cell. 88 (1997) 39–48.
[23] S.V. Plotnikov, A.M. Pasapera, B. Sabass, C.M. Waterman, Force fluctuations within focal adhesions mediate ECM-rigidity sensing to guide directed cell migration, Cell. 151 (2012) 1513–27. https://doi.org/10.1016/j.cell.2012.11.034.
[24] R.A. Brown, R. Prajapati, D.A. McGrouther, I.V. Yannas, M. Eastwood, Tensional homeostasis in dermal fibroblasts: Mechanical responses to mechanical loading in three-dimensional substrates, J. Cell. Physiol. 175 (1998) 323–332. https://doi.org/10.1002/(SICI)1097-4652(199806)175:3<323::AID-JCP10>3.0.CO;2-6.
[25] T. Mizutani, H. Haga, K. Kawabata, Cellular stiffness response to external deformation: Tensional homeostasis in a single fibroblast, Cell Motil. Cytoskeleton. 59 (2004) 242–248. https://doi.org/10.1002/cm.20037.
[26] D. Stamenović, R. Krishnan, E.P. Canović, M.L. Smith, As the endothelial cell reorients, its tensile forces stabilize, J. Biomech. 105 (2020) 109770. https://doi.org/10.1016/j.jbiomech.2020.109770.
[27] S.N. Tam, M.L. Smith, D. Stamenovic, Modeling tensional homeostasis in multicellular clusters, Int J Numer Method Biomed Eng. 33 (2017). https://doi.org/10.1002/cnm.2801.
[28] J. Li, P.E. Barbone, M.L. Smith, D. Stamenović, Effect of correlation between traction forces on tensional homeostasis in clusters of endothelial cells and fibroblasts, J. Biomech. 100 (2020) 109588. https://doi.org/10.1016/j.jbiomech.2019.109588.
[29] S.R. Polio, K.E. Rothenberg, D. Stamenović, M.L. Smith, A micropatterning and image processing approach to simplify measurement of cellular traction forces, Acta Biomater. 8 (2012) 82–88. https://doi.org/10.1016/j.actbio.2011.08.013.
[30] S.R. Polio, M.L. Smith, Patterned hydrogels for simplified measurement of cell traction forces, Methods Cell Biol. 121 (2014) 17–31. https://doi.org/10.1016/B978-0-12-800281-0.00002-6.
[31] S. R. Polio, H. Parameswaran, E. P. Canović, C. M. Gaut, D. Aksyonova, D. Stamenović, M. L. Smith, Topographical control of multiple cell adhesion molecules for traction force microscopy, Integr. Biol. 6 (2014) 357–365. https://doi.org/10.1039/C3IB40127H.
[32] N.Q. Balaban, U.S. Schwarz, D. Riveline, P. Goichberg, G. Tzur, I. Sabanay, D. Mahalu, S. Safran, A. Bershadsky, L. Addadi, B. Geiger, Force and focal adhesion assembly: a close relationship studied using elastic micropatterned substrates, Nat Cell Biol. 3 (2001) 466–72.
[33] J.P. Butler, I.M. Tolić-Nørrelykke, B. Fabry, J.J. Fredberg, Traction fields, moments, and strain energy that cells exert on their surroundings, Am. J. Physiol.-Cell Physiol. 282 (2002) C595–C605. https://doi.org/10.1152/ajpcell.00270.2001.
[34] M. Dembo, Y.L. Wang, Stresses at the cell-to-substrate interface during locomotion of fibroblasts., Biophys. J. 76 (1999) 2307–2316.

[35] H. Parameswaran, K.R. Lutchen, B. Suki, A computational model of the response of adherent cells to stretch and changes in substrate stiffness, J Appl Physiol 1985. 116 (2014) 825–34. https://doi.org/10.1152/japplphysiol.00962.2013.
[36] C.E. Chan, D.J. Odde, Traction dynamics of filopodia on compliant substrates, Science. 322 (2008) 1687–91. https://doi.org/10.1126/science.1163595.
[37] B.L. Bangasser, S.S. Rosenfeld, D.J. Odde, Determinants of maximal force transmission in a motor-clutch model of cell traction in a compliant microenvironment, Biophys J. 105 (2013) 581–92. https://doi.org/10.1016/j.bpj.2013.06.027.
[38] S.J. Han, K.S. Bielawski, L.H. Ting, M.L. Rodriguez, N.J. Sniadecki, Decoupling substrate stiffness, spread area, and micropost density: a close spatial relationship between traction forces and focal adhesions, Biophys J. 103 (2012) 640–8. https://doi.org/10.1016/j.bpj.2012.07.023.
[39] S. Weng, Y. Shao, W. Chen, J. Fu, Mechanosensitive subcellular rheostasis drives emergent single-cell mechanical homeostasis, Nat Mater. 15 (2016) 961–7. https://doi.org/10.1038/nmat4654.
[40] A.S. LaCroix, A.D. Lynch, M.E. Berginski, B.D. Hoffman, Tunable molecular tension sensors reveal extension-based control of vinculin loading, ELife. 7 (2018) e33927. https://doi.org/10.7554/eLife.33927.
[41] D.W. Dumbauld, T.T. Lee, A. Singh, J. Scrimgeour, C.A. Gersbach, E.A. Zamir, J. Fu, C.S. Chen, J.E. Curtis, S.W. Craig, A.J. Garcia, How vinculin regulates force transmission, Proc. Natl. Acad. Sci. 110 (2013) 9788–9793. https://doi.org/10.1073/pnas.1216209110.
[42] C. Grashoff, B.D. Hoffman, M.D. Brenner, R. Zhou, M. Parsons, M.T. Yang, M.A. McLean, S.G. Sligar, C.S. Chen, T. Ha, M.A. Schwartz, Measuring mechanical tension across vinculin reveals regulation of focal adhesion dynamics, Nature. 466 (2010) 263–6. https://doi.org/10.1038/nature09198.
[43] P. Nalbant, L. Hodgson, V. Kraynov, A. Toutchkine, K.M. Hahn, Activation of endogenous Cdc42 visualized in living cells, Science. 305 (2004) 1615–9.
[44] K. Aoki, Y. Kumagai, A. Sakurai, N. Komatsu, Y. Fujita, C. Shionyu, M. Matsuda, Stochastic ERK Activation Induced by Noise and Cell-to-Cell Propagation Regulates Cell Density-Dependent Proliferation, Mol. Cell. 52 (2013) 529–540. https://doi.org/10.1016/j.molcel.2013.09.015.
[45] A. Mukherjee, R. Singh, S. Udayan, S. Biswas, P.P. Reddy, S. Manmadhan, G. George, S. Kumar, R. Das, B.M. Rao, A. Gulyani, A Fyn biosensor reveals pulsatile, spatially localized kinase activity and signaling crosstalk in live mammalian cells, ELife. 9 (2020) e50571. https://doi.org/10.7554/eLife.50571.
[46] A. Elosegui-Artola, E. Bazellieres, M.D. Allen, I. Andreu, R. Oria, R. Sunyer, J.J. Gomm, J.F. Marshall, J.L. Jones, X. Trepat, P. Roca-Cusachs, Rigidity sensing and adaptation through regulation of integrin types, Nat Mater. 13 (2014) 631–7. https://doi.org/10.1038/nmat3960.
[47] S. Lee, E. Kassianidou, S. Kumar, Actomyosin stress fiber subtypes have unique viscoelastic properties and roles in tension generation, Mol. Biol. Cell. 29 (2018) 1992–2004. https://doi.org/10.1091/mbc.E18-02-0106.
[48] J. Grewe, U.S. Schwarz, Mechanosensitive self-assembly of myosin II minifilaments, Phys. Rev. E. 101 (2020) 022402. https://doi.org/10.1103/PhysRevE.101.022402.
[49] M.T. Breckenridge, R.A. Desai, M.T. Yang, J. Fu, C.S. Chen, Substrates with Engineered Step Changes in Rigidity Induce Traction Force Polarity and Durotaxis, Cell. Mol. Bioeng. 7 (2014) 26–34. https://doi.org/10.1007/s12195-013-0307-6.
[50] W. Ronan, A. Pathak, V.S. Deshpande, R.M. McMeeking, J.P. McGarry, Simulation of the Mechanical Response of Cells on Micropost Substrates, J. Biomech. Eng. 135 (2013) 101012. https://doi.org/10.1115/1.4025114.
[51] J.L. Tan, J. Tien, D.M. Pirone, D.S. Gray, K. Bhadriraju, C.S. Chen, Cells lying on a bed of microneedles: an approach to isolate mechanical force, Proc Natl Acad Sci U A. 100 (2003) 1484–9.

[52] J. Stricker, Y. Aratyn-Schaus, P.W. Oakes, M.L. Gardel, Spatiotemporal Constraints on the Force-Dependent Growth of Focal Adhesions, Biophys. J. 100 (2011) 2883–2893. https://doi.org/10.1016/j.bpj.2011.05.023.

Table 1. Total numbers of cells (N_{cells}), of FAs (N), and the cluster size (number of cells in the cluster) for each substrate stiffness.

Substrate stiffness (kPa)	Single BVSMC		Cluster BVSMC			Single BAEC		Cluster BAEC		
	N	N_{cell}	N	N_{cell}	*Cluster size*	N	N_{cell}	N	N_{cell}	*Cluster size*
3.6	2538	43	2748	79	2-11 cells	1937	49	909	44	2-5 cells
6.7	992	13	3040	61	2-13 cells	1004	20	7767	213	2-30 cells
13.6	1154	14	791	21	2-9 cells	1088	16	*	*	*
30	2333	15	2083	41	2-7 cells	*	*	*	*	*

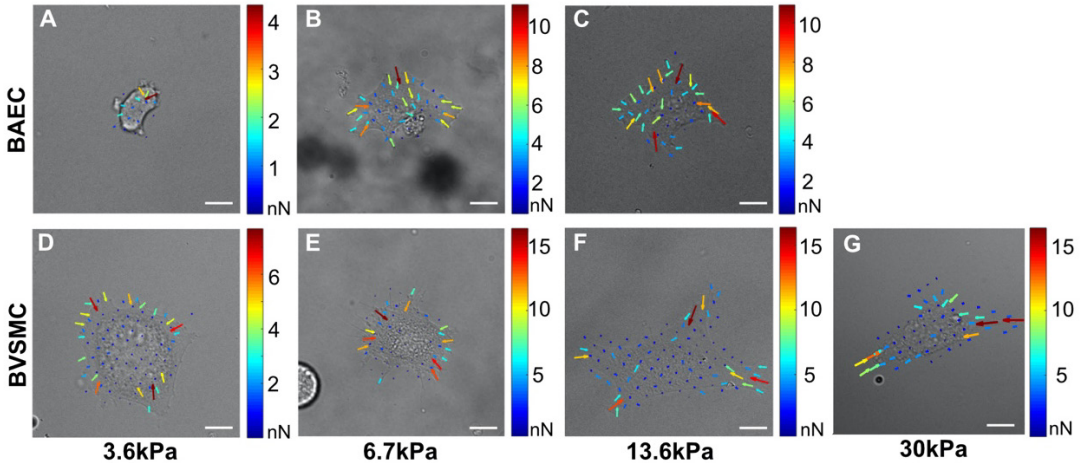
Figure Captions

Figure 1: Images of traction forces obtained by micropattern traction microscopy. Examples of the traction force distribution in bovine aortic endothelial cells (BAECs; upper panels) and in bovine vascular smooth muscle cells (BVSMCs; lower panels) cultured on 3.6 kPa (A and D), 6.7 kPa (B and E), 13.6 kPa stiff gels (C and F), and 30 kPa (G). Scale bars are 15 μm. All forces are in nN.

Figure 2: Temporal fluctuations of focal adhesion (FA) traction forces exhibit erratic behavior. Representative time (t) lapses of magnitudes of FA traction forces [$F(t)$] (A) and of $F(t)$ normalized by its time-average ($\langle F \rangle$) (B). Data are obtained from a cluster of BAECs cultured on a 6.7 kPa stiff substrate, and each trace color represents a different FA.

Figure 3: Focal adhesion traction forces and displacements that reach a threshold value dramatically lower the coefficient of variation of (CV_F) of these forces and displacements. The threshold is shifted to the right with increasing substrate stiffness in both BVSMCs (A) and BAECs (B), but the CV_F versus time-averaged magnitude of the traction force ($\langle F \rangle$) relationship does not change with changes in substrate stiffness. Similar behavior is observed in the CV_F versus time-average magnitude of FA displacement ($\langle u \rangle$) relationship, but in this case, the threshold (1-2 μm) does not depend on the substrate stiffness in both BVSMCs (C) and BAECs (D). The observed relationships were virtually the same in single cells (solid circles, solid lines) and in clusters of cells (open circles, dashed lines). Different colors correspond to different substrate stiffness: 3.6 kPa (black), 6.7 kPa (red), 13.6 kPa (blue), and 30 kPa (green). Data are mean ± standard error.

Figure 4: Dependence of temporal variability of focal adhesion (FA) traction forces on the mean force is qualitatively different from the dependence of the temporal variability of the traction field of single cells and clusters on the mean tension. Coefficient of variation (CV_F) of FA traction forces exhibits a threshold with increasing time-averaged magnitude of the traction forces ($\langle F \rangle$) in single cells (solid circles) and in clusters of cell (open circles) in both BVSMCs (A) and BAECs (B). Coefficient of variation of the traction field (CV_T) does not exhibit threshold with increasing time-averaged magnitude ($\langle T \rangle$) of the traction field in single cells and clusters of cells. Instead, CV_T decreases with increasing $\langle T \rangle$ following roughly a power-law dependence in both BVSMCs (C) and BAECs (D). Measurements are carried out on substrates of 3.6 kPa stiffness. Data are mean ± standard error.


A
B
C
D
E
F
G
BAEC
BVSMC
4
3
2
1
nN
10
8
6
4
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10
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4
2
nN
6
4
2
nN
15
10
5
nN
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15
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5
nN
3.6kPa
6.7kPa
13.6kPa
30kPa

Fig. 1

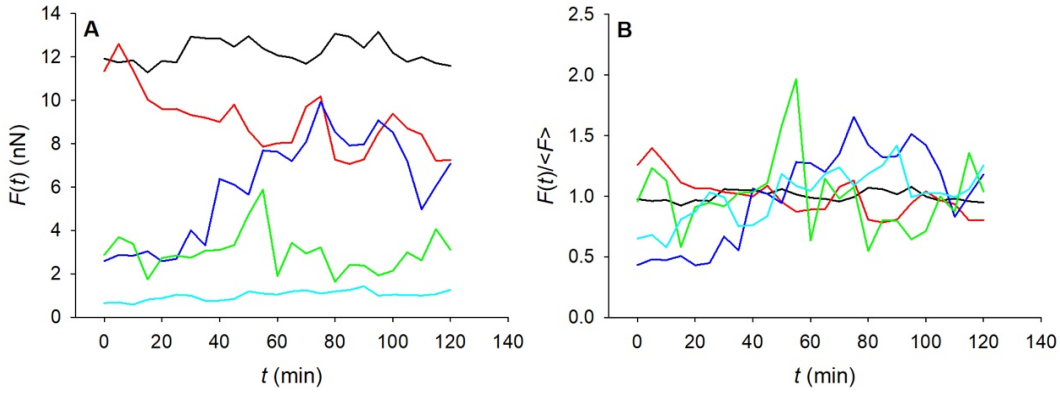
A
B
F(t) (nN)
F(t)/<F>
t (min)
t (min)
0
2
4
6
8
10
12
14
0.0
0.5
1.0
1.5
2.0
2.5
0
20
40
60
80
100
120
140

Fig.2

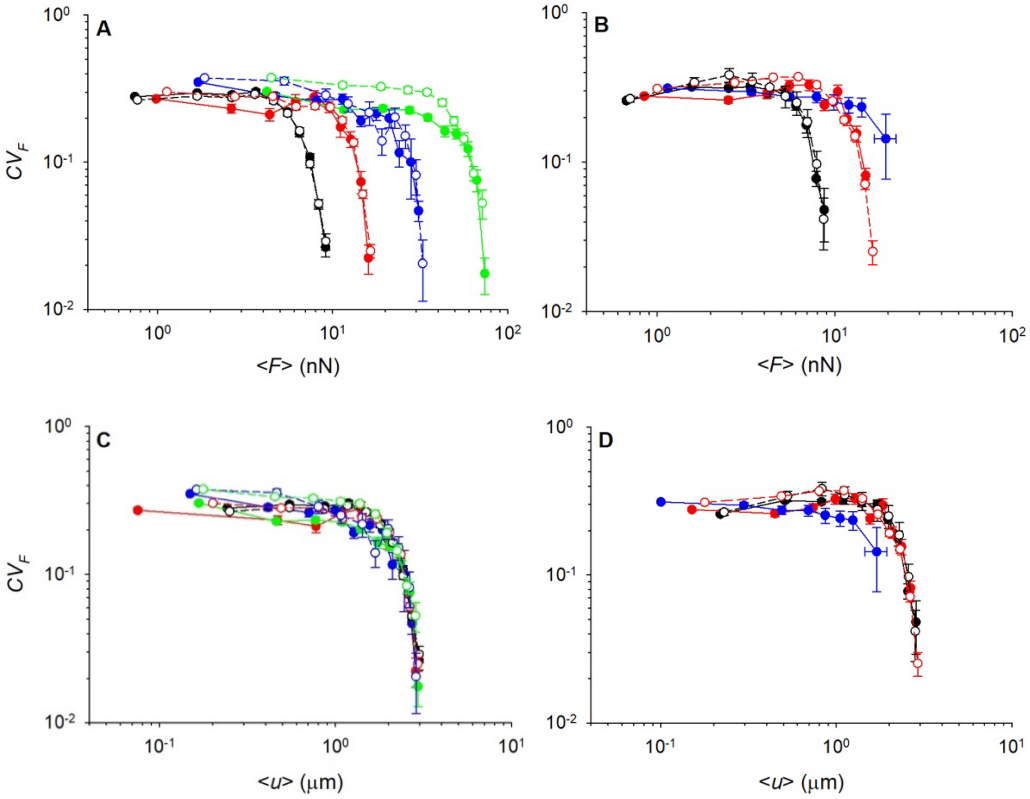

Fig. 3

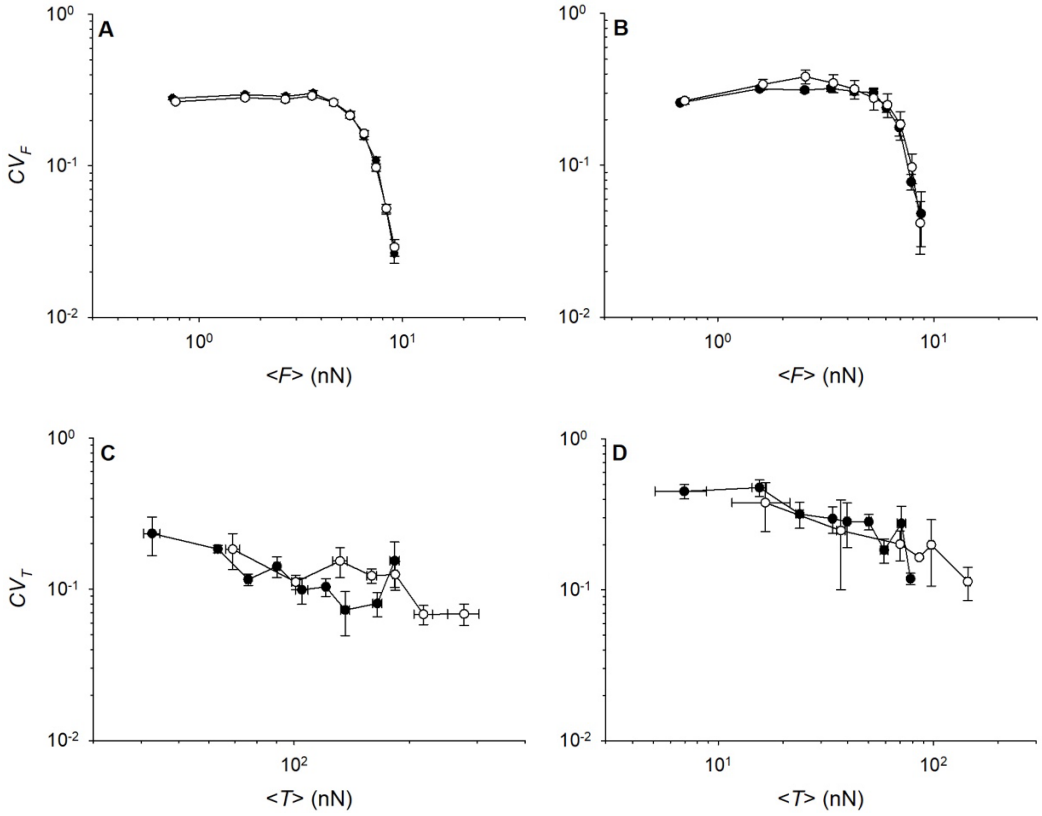

Fig. 4