Force-activatable coating enables high-resolution cellular force imaging directly on regular cell culture surfaces

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Abstract
Integrin-transmitted cellular forces are crucial mechanical signals regulating a vast range of cell functions. Although various methods have been developed to visualize and quantify cellular forces at the cell–matrix interface, a method with high performance and low technical barrier is still in demand. Here we developed a force-activatable coating (FAC), which can be simply coated on regular cell culture apparatus’ surfaces by physical adsorption, and turn these surfaces to force reporting platforms that enable cellular force mapping directly by fluorescence imaging. The FAC molecule consists of an adhesive domain for surface coating and a force-reporting domain which can be activated to fluoresce by integrin molecular tension. The tension threshold required for FAC activation is tunable in 10–60 piconewton (pN), allowing the selective imaging of cellular force contributed by integrin tension at different force levels. We tested the performance of two FACs with tension thresholds of 12 and 54 pN (nominal values), respectively, on both glass and polystyrene surfaces. Cellular forces were successfully mapped by fluorescence imaging on all the surfaces. FAC-coated surfaces also enable co-imaging of cellular forces and cell structures in both live cells and immunostained cells, therefore opening a new avenue for the study of the interplay of force and structure. We demonstrated the co-imaging of integrin tension and talin clustering in live cells, and concluded that talin clustering always occurs before the generation of integrin tension above 54 pN, reinforcing the notion that talin is an important adaptor protein for integrin tension transmission. Overall, FAC provides a highly convenient approach that is accessible to general biological laboratories for the study of cellular forces with high sensitivity and resolution, thus holding the potential to greatly boost the research of cell mechanobiology.

Introduction
Cells sense and respond to the mechanical cues of the neighboring cells and surrounding extracellular matrix (ECM). Reciprocally, cells also exert forces on the ECM and transduce mechanical signals to neighboring cells. The cellular forces at the cell–ECM interface are mainly transmitted by membrane protein integrins [1, 2]. Integrin-transmitted forces play both mechanical and regulatory roles in various cellular functions. In the short term, these forces mechanically mediate cell adhesion, polarization, and migration [3–7]. In the long term, these forces are transduced to biochemical signals which are involved in cell proliferation, cancer metastasis [8, 9] and stem cell differentiation [10–12]. Because of the fundamental roles of these forces in cellular functions, measuring and mapping these forces have been long pursued in the study of cell mechanobiology.

The study of integrin-transmitted cellular forces was initiated by Harris et al by plating cells on ultra-soft silicone rubber film and visualizing cellular forces via the substrate strain caused by cellular forces [13]. Currently, the prevailing methods for cellular force mapping are traction force microscopes that function by converting cellular forces to a substrate strain
visible to a microscope. Over the years, traction force microscopy techniques have used the elastic modulus of substrates with micro-patterns [14], microposts [15–17], or embedded microbeads to calibrate and quantify cellular forces. These pioneering techniques have advanced the understanding of cell mechanobiology. However, these force mapping techniques based on force-to-strain conversion have relatively low resolution (2–5 µm) [14] and high technical barriers for universal usage as both sample preparation and data processing require considerable engineering and computer modeling skills. In recent years, a variety of fluorescence-based molecular sensors [15–21] have been developed for the study of integrin molecular tensions and cellular force mapping at the cell–substrate interface. Forster resonance energy transfer-based [22] force sensors have also been developed and applied inside cells for gauging cellular forces [23]. However, all of these methods require considerable bioengineering or surface chemistry skills of the researchers to implement them properly, therefore limiting their applications in general labs. In this consideration, we developed a force-activatable coating (FAC) which has excellent force mapping performance, yet is still highly convenient to be adopted by fellow researchers. FAC is a synthetic molecule that can be activated to the fluorescent state by force (here integrin tension), and enable cellular force mapping directly by fluorescence imaging with high resolution and sensitivity. FAC activation is irreversible and therefore permanently records historic integrin tension events. This signal accumulation process greatly improves the cellular force signal and makes it feasible to perform cellular force mapping using a low-end fluorescence microscope. Moreover, the implementation of FAC requires little surface preparation, as FAC can be directly coated on regular cell culture surfaces fabricated by materials such as glass or polystyrene. The sample preparation only requires one simple step of incubation, thus greatly simplifying the procedure of cellular force mapping to a minimum level. Therefore, FAC will provide a simple yet powerful kit for cellular force study to the field of mechanobiology.

**Methods**

**The principle and implementation of FAC**

FAC is a synthetic material enabling cellular force imaging by one-step surface preparation (figure 1(a)). An FAC molecule consists of one force-reporting domain converting integrin tension to a fluorescent signal and a one adhesive domain enabling FAC coating on glass or polystyrene surfaces via physical adsorption (figure 1(b)). The force-reporting domain is based on the integrative tension sensor (ITS) recently developed by our group for force mapping of single platelets [24]. ITS is an 18 base-paired (bp) double-stranded DNA (dsDNA) decorated with an integrin ligand and a fluorophore-quencher pair. Initially, the fluorophore is quenched by the quencher (black hole quencher 2, BHQ2) with a quenching efficiency of 96.6% [24]. Immobilized on a surface, ITS binds to an integrin and the tension transmitted by the integrin may dissociate the dsDNA and free the fluorophore from quenching, therefore converting the tension signal to a fluorescent signal (figure 1(b)). The tension threshold required for dsDNA dissociation, namely, the tension tolerance ($T_{tol}$) of the dsDNA, is tunable in the range of 10–60 pN and dependent on the DNA structure. $T_{tol}$ is 12 pN for the 18 bp dsDNA in unzipping geometry and 54 pN for the dsDNA in shear geometry (figure 1(c)). Note that these $T_{tol}$ values are nominal values as the application time of integrin tension also matters to the rupture of dsDNA but the time scale is unknown. Prior to this work, ITS was immobilized on a glass surface through biotin–streptavidin interaction [20, 24]. The surface preparation is time consuming, including glass surface cleaning, surface biotinylation, deposition of streptavidin, and eventually ITS immobilization. Recognizing the difficult implementation of ITS and other tension sensors in the field, we synthesized FAC by linking ITS to an adhesive molecule, here bovine serum albumin (BSA). With the FAC, ITS can be directly coated on common cell culture surfaces through physical adsorption, therefore greatly simplifying the process of cellular force mapping (figure 1(d)).

**FAC synthesis**

The FAC synthesis was conducted by three steps as illustrated in schematic 1. First, a fluorphophore-labeled complementary single-stranded DNA (ssDNA) was conjugated to BSA, and then a quencher-labeled complementary ssDNA is conjugated to integrin peptide ligand RGD (arginine-glycine-aspartic acid). These two reagents were then mixed at DNA molar ratio of 1:1.1 and the DNAs hybridize to BSA-ITS as the final product of FAC.

**Conjugation of bovine serum albumin with quencher-labeled ssDNA**

First, we conjugated BSA with fluorphophore-labeled ssDNA. BSA was chosen as the adhesive domain in the FAC because BSA has been proven to be highly adhesive on polystyrene and glass surfaces [25, 26]. BSA can be directly coated on these surfaces by physical adsorption. Moreover, BSA is generally passive and has little influence or toxicity to cells. BSA has been commonly used as blocking material for immunostaining of cells, or carrier protein which protects other proteins from degradation in biological reagents. BSA is also low cost and can be supplied in large amounts.

BSA-ssDNA conjugation was performed through a maleimide–thiol reaction and NHS ester–amine reaction. ssDNAs 5′-/5Cy3/ATG CTG AGG TCG CCG CCC/3ThioMC3-D/-3′ for the synthesis of FAC with $T_{tol}$ = 12 pN and 5′/-/ThioMC6-D/TiCy3/ATG CTG
AGG TCG CCG CCC/-3′ for the synthesis of FAC with $T_{\text{tol}} = 54$ pN were customized and purchased from Integrated DNA Technologies. The /3ThioMC3-D/ and /3ThioMC6-D/ (thiol modification) were deprotected by 10 mM TCEP (Tris(2-carboxyethyl)phosphine hydrochloride) and reacted with the maleimide group of a heterolinker SMCC-sulfo (22622, Thermo Fisher Scientific) which is a hetero-linker with an NHS ester and a maleimide group at two ends, respectively. The molar ratio of ssDNA and SMCC-sulfo was 1:10. Unreacted SMCC linker in the DNA solution was removed by desalting using a desalting column (Bio-spin 6, Bio-Rad). Now, the ssDNA was functionalized with NHS ester and ready for BSA conjugation.
The functionalized ssDNA was mixed with BSA and the NHS ester on the ssDNA reacted with the amine groups present on the BSA molecules, mainly on the amino acid lysines. Eventually, ssDNA was covalently conjugated with BSA. All reactions were done in a phosphate-buffered saline (PBS) buffer.

**Conjugation of integrin ligand RGD peptide with quencher-labeled ssDNA**

The conjugation process is similar to BSA-ssDNA conjugation. Instead of BSA, cyclic peptide RGD with an amine group (PCI-3696-PI, Peptides International) was conjugated to a complementary ssDNA: 5′-/ThioMC6-D/GGG CGG CGA CCT CAT/BHQ2/-3′ (customized and purchased from Integrated DNA Technologies). /ThioMC6-D/ was deprotected and functionalized with an NHS ester with the same procedure as that in BSA-ssDNA conjugation. The functionalized ssDNA was mixed with an RGD solution with a molar ratio of 1:4. Finally, conjugated ssDNA-RGD was purified from unreacted DNA and RGD by electrophoresis.

**Synthesis of FAC by hybridizing BSA-ssDNA and RGD-ssDNA**

FAC with $T_{ad} = 12$ pN was synthesized by hybridizing /5Cy3/ATG CTG AGG TCG CCG CCC-BSA and RGD-/GGG CGG CGA CCT CAT/BHQ2/ with a DNA molar ratio of 1:1. FAC with $T_{ad} = 54$ pN was synthesized by hybridizing BSA-/TiCy3/ATG CTG AGG TCG CCG CCC/ and RGD-/GGG CGG CGA CCT CAT/BHQ2/ with a DNA molar ratio of 1:1. With the same protocol, we also synthesized FAC labeled by Cy5 with 12 pN and 54 pN thresholds. After this, BSA-IT5 was synthesized and can be used as an FAC in experiments.

**Preparation of FAC-coated surfaces**

FAC in the amount of 0.1 $\mu$M in PBS was incubated on a glass-bottom Petri dish or a regular polystyrene dish for 20 min. After rinsing with PBS, the dish was ready for cell plating and cellular force mapping.

**Cell plating**

Both CHO-K1 and NIH-3T3 cells are eukaryotic cells. CHO-K1 cells are Chinese hamster ovary cells. This cell line is an epithelial cell line that is extracted from the ovary of Chinese hamster and widely used in biological research. NIH-3T3 cells are fibroblast cells (responsible for synthesizing ECM and collagen) that were extracted from embryo tissue of a Swiss albino mouse. These fibroblast cells are abundant in the connective tissues of animals. CHO-K1 and NIH-3T3 cells were detached from cell culture Petri dishes by an EDTA (ethylenediaminetetraacetic acid) solution (a mild reagent for cell detachment to keep the membrane proteins unscathed). The recipe for the EDTA solution is: 100 ml × HBSS ( Hank’s balanced salt solution) + 10 ml × 1 M HEPES (PH7.6) + 10 ml of 7.5% sodium bicarbonate + 2.4 ml of 500 mM EDTA + 1 L H2O. For both cell lines, the media in the Petri dish was first pipetted out and cells were washed with 1 ml of EDTA solution once. Afterwards, 1 ml of fresh EDTA solution was added to the Petri dish. The Petri dish was incubated at 37 °C for 10 min in an incubator. Next, cells were dispersed by pipetting in a biosafety cabinet and collected in a 1 ml tube. The cell solution was spun down for 3 min at 300 g and cells were resuspended in 1 ml of cell culture medium and plated on the FAC-coated substrates. Serum-free Ham’s F-12K medium (Caisson laboratories Inc, Smithfield, UT) supplemented with L-glutamine, 10% fetal bovine serum (FBS) and 1% penicillin streptomycin (PS) was used for the CHO-K1 cell lines. Serum-free Dulbecco’s Modified Eagle Medium (DMEM) media supplemented with 4.5 g l$^{-1}$ of glucose, L-glutamine, sodium pyruvate (Mediatech Inc., Manassas, VA), 10% calf bovine serum, and 1% PS was used for culturing the NIH-3T3 cells. All these cell lines were plated at a density of about 1000 per mm$^2$ on the FAC substrates.

**Co-imaging of FA, F-actin, and cellular forces in fixed cells**

CHO-K1 and NIH-3T3 cells were plated on FAC-coated glass and polystyrene surfaces. The cells were incubated for 2.5 h at 37 °C. For immunostaining, cells were fixed with a 4% paraformaldehyde (catalog No. 15710, Electron Microscopy Sciences) solution for 20 min at room temperature. Cell samples were then rinsed three times with PBS. Next, the cells were permeabilized with 0.5% Triton X detergent at room temperature for 10 min and washed three times with PBS. After cell fixation and permeabilization, 3% BSA was added to the cell samples and the samples were incubated at room temperature for 1 h (or overnight at 4 °C). The cells were then rinsed three times using PBS. This step was followed by the preparation of two antibody solutions. Dilutions of 2.5 $\mu$g ml$^{-1}$ of primary antibody (FAK100, Millipore, mouse anti-vinculin) and 2.5 $\mu$g ml$^{-1}$ of secondary antibody (AP192SA6, Millipore, Donkey antimouse-Alexa647) were prepared using PBS + 0.05% Tween 20. First, the cells were incubated at room temperature in a primary antibody solution for 1–2 h (or overnight at 4 °C). This step was followed by rinsing of the cell samples three times using PBS + 0.05% Tween 20 and 5 min incubation time was maintained throughout three wash cycles. The cell samples were incubated in the secondary antibody solution (previously prepared) at room temperature for 1–2 h (or overnight at 4 °C) followed by the same washing cycle with PBS + 0.05% Tween 20. Next, the cells were incubated in 2.5 $\mu$g ml$^{-1}$ Phalloidin-Alexa (A22287, Invitrogen molecular probes) at room temperature for 20 min. Finally, cell samples were rinsed three times using PBS, and imaging was performed immediately. Phase contrast imaging was performed using a 40× objective lens in a Nikon fluorescence Ti-E microscope.
Co-imaging of talin and cellular forces in live cells

CHO-K1 cells were transfected with talin fused with green fluorescent protein (talin-GFP) (C10611, Thermo Fisher Scientific). Prior to transfection, CHO-K1 cells were passaged for 2 d in multiple wells of a 48 well plate with different dilutions of the original cell solution. On the second day, confluency of cells in each well was monitored with an inverted microscope. One particular well where the cells were around 70% confluent was selected for the talin transfection. The cell media in that well was pipetted out and replaced with a mixture of 500 µl of F-12 K complete media (serum free Ham’s F-12K medium supplemented with L-glutamine, 10% FBS and 1% PS) and 15 µl of a talin-GFP construct. The 48-well plate was placed inside the incubator at 37 °C overnight. After 24 h, the efficiency of the transfection was tested using a Nikon Ti-E fluorescence microscope in the GFP channel.

Results and discussion

FAC converts cell culture surfaces to cellular force imaging platforms

In order to investigate whether FAC functions properly and successfully converts the force signal to a fluorescent signal, we prepared FAC (T_tol = 54 pN) coated glass surfaces and polystyrene surfaces. Next, CHO-K1 and NIH-3T3 cells were resuspended using F-12K complete media and DMEM complete media, respectively, and plated them on these FAC surfaces. The samples were incubated at 37 °C and with 5% CO₂ for 2.5 h. The samples were imaged immediately after incubation with a Nikon Ti-E fluorescence microscope with the thermal control set at 37 °C. Simultaneous phase-contrast imaging and FAC imaging by a Cy3 channel were performed for both cell lines. Figures 2(a)–(d) show the performance of these FACs for cellular force imaging in live cells while figures 2(e) and (f) demonstrate their compatibility with immunostaining. Figures 2(a) and (b) clearly demonstrate that FAC-coated glass surfaces reported integrin-transmitted cellular forces in CHO-K1 and NIH 3T3 cells. FAC without integrin ligand conjugation yielded no fluorescence signal underneath the cells, demonstrating that the fluorescence signal was indeed produced by integrins (supplementary figure 1 (stacks.iop.org/PhysBio/15/065002/mmedia)). Figures 2(c) and (d) demonstrated that FAC-coated polystyrene surfaces also reported cellular forces. We then tested co-imaging of cellular forces and cell structures on FAC-coated glass for both cell lines. Cellular force, focal adhesions (FA) (integrin clusters) marked by immunostained vinculin, and F-actin stained by phalloidin were imaged in the Cy3, YFP, and Cy5 channels, respectively. These experiments demonstrated that FAC can be directly coated on regular cell culture apparatuses which are commonly fabricated with glass or polystyrene. FAC-coated surfaces successfully mapped cellular force directly by fluorescence imaging in both live cells and immunostained cells.

To confirm that the BSA physical adsorption is strong enough to resist the integrin tension, we prepared a construct to only report the potential BSA detachment by fluorescence. BSA was conjugated with Cy3-labeled ssDNA. The BSA-ssDNA-Cy3 was
annealed with complementary ssDNA-RGD to BSA-dsDNA-Cy3. This construct is similar to FAC, while fluorescence loss is expected to be observed only if BSA is detached from the surface by cells (construct A in figure S2). A control construct was synthesized by annealing BSA-ssDNA and complementary Cy3-ssDNA-RGD (construct B in figure S2). The control construct reports dsDNA rupture by cells through fluorescence loss. Clearly, construct A yielded very little fluorescence signal compared to construct B during cell adhesion, suggesting that potential BSA detachment has a minor influence on the FAC signal even for 54 pN integrin tension mapping (figure S2).

A time-lapse experiment was also conducted to show that BSA was not significantly degraded or spontaneously detached from the surfaces (figure S3).

**Multiplex FAC reports integrin tensions at different force levels simultaneously**

Because FAC has a molecular tension threshold $T_{tol}$ tunable in the range of 10–60 pN, this allows the selective imaging of cellular force consisting of integrin tension at different force levels. Here we demonstrated the co-imaging of integrin tensions at different force levels simultaneously. FACs with $T_{tol} = 54$ and 12 pN, and labeled with Cy3 and Cy5, respectively, were mixed with a 1:1 molar ratio and coated on a glass surface. CHO-K1 cells were plated on the coated glass surface in a complete medium and incubated at 37 °C for 2.5 h. Figures 3(a)–(d) show the phase contrast and cellular force imaging (Cy3 for $T_{tol} = 54$ pN and Cy5 for $T_{tol} = 12$ pN) and the overlay of Cy3–Cy5 channel images for CHO-K1 cells. Integrin tensions above 54 pN and above 12 pN were recorded in the Cy3 and Cy5 channels, respectively. Figure 3(e) demonstrates the intensities of the cellular force signals on the multiplex surface of 12 and 54 pN FACs. The 12 pN FAC signal intensity is about 7.5 times higher than the 54 pN FAC signal intensity, suggesting that a large portion of integrin tension is in the range of 12–54 pN while some integrin tension can go beyond 54 pN.

**FAC-coated surfaces report the efficacy of blebbistatin on cellular force inhibition**

Next, we demonstrated that FAC-coated surfaces can be used to report cell responses to the treatment of reagents disrupting cellular force generation. It is known that actomyosin, the F-actin coupled with myosin II, is an important power source generating cell traction force [27]. In order to show whether the high-level integrin tension (above 54 pN) is generated by actomyosin, we treated CHO-K1 cells plated on 54 pN FAC surfaces with 0 (as a control), 0.01, 0.1, 1, 10, and 50 µM of blebbistatin (myosin II inhibitor), respectively. Figures 4(a)–(f) are FAC images of single CHO-K1 cells under the treatment of blebbistatin at different concentrations. Figure 4(g) shows cellular force intensity versus blebbistatin concentration. Cellular force signal intensity was measured in each of those samples. Force signal intensity was the highest on the control sample and consistently decreased from 4320–24 (average grayscale of the FAC signal as the unit) with increasing blebbistatin concentration. This result suggested that actomyosin is critical for the generation of high-level integrin tension (above 54 pN) in cells. Similarly, we can show the influence of other chemicals or drugs on cellular force generation using FAC as a convenient tool for cellular force imaging.

**Real-time cellular force reported by FAC imaging**

Because the dsDNA dissociation by integrin tension in the tension sensor is generally irreversible, FAC records all cellular forces since cell plating. The permanent
fluorescence activation greatly increases the signal-to-noise ratio of cellular force imaging, making it feasible to perform cellular force mapping directly on regular cell culture surfaces. While FAC accumulates force signals to improve the sensitivity, FAC can also be applied to report real-time cellular forces that are newly generated. This real-time cellular force mapping can be simply achieved by recording consecutive force maps by time, and subtracting a previous frame from a current frame of a force map to report the newly generated cellular force signal in the frame interval. In order to demonstrate the capability of FAC reporting real-time cellular forces, CHO-K1 cells were plated on glass surfaces coated with 54 pN FAC labeled with Cy3 and incubated for 1 h. During real-time cellular force imaging, the cell sample was relocated to a microscope and a video was recorded for 1 h with a frame interval of 1 min. Figure 5(a) shows the progression of the fluorescence signal of FAC (in green) with the real-time cellular force displayed in red, scale bar: 10 µm. (b) FAC signal gained in the latest frame. (c) Strength of the FAC signal versus time. The signal strength is the sum of grayscale of all pixels with FAC signals, unit: A.U. (arbitrary unit). (d) Real-time FAC signal strength versus time.
force and real-time cellular force in CHO-K1 cells. This experiment shows that FAC can be used to report both accumulated force signals and real-time cellular forces.

Co-imaging of talin clustering and cellular force contributed by integrin tension above 54 pN
We also demonstrated that FAC can be applied to co-imaging of cellular force and cell structure in live cells. High-level integrin tension (above 54 pN) and talin were imaged simultaneously to report their temporal and spatial correlation. After transfection with a CellLight Talin-GFP (C10611, Thermo Fisher Scientific), CHO-K1 cells were plated on an FAC-coated Petri dish ($T_{tol} = 54$ pN) and incubated at 37°C for 1 h. Subsequently, talin was imaged in a GFP channel and cellular force (integrin tensions above 54 pN) was imaged in the Cy5 channel as shown in figure 6 and supplementary video 2. Real-time imaging of the talin signal and the force signal on the 54 pN FAC-coated surface show that for new cellular adhesions, talin clustering occurs first and high-level integrin tension ($>54$ pN) follows and eventually co-localizes with the talin cluster (figure 6(a)). Figure 6(b) shows that talin clustering inside the cell does not require high integrin molecular tension. Overall, using FAC, we demonstrated that talin clustering occurs prior to the generation of integrin tension above 54 pN.

Conclusion
In summary, we developed FAC to provide a universal approach for cellular force mapping directly by fluorescence imaging. Through multiple tests of FAC with different cell lines on different cellular culture apparatuses, we demonstrated that FAC successfully turns regular cell culture surfaces to cellular force imaging platforms which convert force signals into fluorescence signals and achieve high-resolution imaging on both glass and polystyrene surfaces, the two common surfaces for cell culture. FAC can be designed with a specific molecular tension threshold that enables the selective imaging of integrin tension at different force levels ($>12$ and $>54$ pN in this paper). We also demonstrated that FAC can be used to report cell responses to the treatment of drugs that interfere with cell mechanics. It was shown that the overall intensity of the cellular force signal reported by FAC with $T_{tol} = 54$ pN decreases with increasing concentration of blebbistatin, which inhibits motor protein myosin II, proving that myosin II is critical for the generation of integrin tension above 54 pN. Therefore, FAC can function as a convenient tool to screen reagents that may impact cell mechanotransduction or other biomechanical processes in cells. Moreover, FAC-based cellular force imaging is compatible with cell structural imaging in both immunostained cells and live cells, making FAC highly useful to study the force–structure interplay in cells. By co-imaging talin and cellular force, we revealed that talin clustering consistently precedes the appearance of high level integrin tension (above 54 pN). Taken together, we developed FAC as a powerful yet highly convenient technique for cellular force mapping and quantification. This technique is accessible to any biological laboratory with a common fluorescence microscope, therefore holding great potential to accelerate the research of cell mechanobiology in the field.

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