Real-time single-cell response to stiffness

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Living cells adapt to the stiffness of their environment. However, cell response to stiffness is mainly thought to be initiated by the deformation of adhesion complexes under applied force. In order to determine whether cell response was triggered by stiffness or force, we have developed a unique method allowing us to tune, in real time, the effective stiffness experienced by a single living cell in a uniaxial traction geometry. In these conditions, the rate of traction force buildup dF/dt was adapted to stiffness in less than 0.1 s. This integrated fast response was unambiguously triggered by stiffness, and not by force. It suggests that early cell response could be mechanical in nature. In fact, local force-dependent signaling through adhesion complexes could be triggered and coordinated by the instantaneous cell-scale adaptation of dF/dt to stiffness. Remarkably, the effective stiffness method presented here can be implemented on any mechanical setup. Thus, beyond single-cell mechanosensing, this method should be useful to determine the role of rigidity in many fundamental phenomena such as morphogenesis and development.

cell mechanics | mechanotransduction | dynamic stiffness control

Living cells are sensitive to their mechanical environment and adapt their activity to it. Many parameters such as forces, deformations, and the geometry and stiffness of the ECM were identified that could trigger cellular functions (1–3). In particular, it was shown that the stiffness of the ECM could influence cell spreading (4, 5), orientation (6), contractility (7, 8), migration (9), and even differentiation (10, 11). These phenomena were mainly attributed to the ability of adhesion complexes to respond to applied forces (12, 13). These complexes, based on integrin transmembrane proteins, transmit forces from inside (cytoskeleton) to outside the cell (ECM) (14, 15) and were thus natural candidates for mechanosensing. On soft substrates, cell contractility could induce high substrate deformation and low generated forces. The adhesion complexes would then be weakly deformed, leading to a weak cell response. On stiff—less deformable—substrates, cell contractility could lead to high stretching of mechanosensory molecules that would activate specific mechanochemical signaling pathways and enhance, in turn, contractility (9, 16, 17).

Following the observation that nonmuscle myosins were needed for stem cells to feel matrix elasticity (11), we have recently investigated the specific role of actomyosin contractility in rigidity sensing. We found that cell response to the rigidity of its environment could reflect the adaptation of the actomyosin machinery to load (8). In this context, it is noteworthy that mechanosensing through adhesion-based signaling pathways and actomyosin-based sensitivity should lead to cell responses occurring at distinct characteristic time scales. At short times, an “instantaneous” actomyosin-dependent response could adapt the cytoskeletal tension, followed, at longer times, by the onset of the regulatory adhesion-based/mechanochemical signaling loops.

In order to decouple the effects of rigidity and force, as well as to reveal the characteristic time scale of cell response to stiffness, we have developed a unique method allowing us to tune, in real time, the effective stiffness experienced by a single living cell,

regardless of the level of the applied force. Here we first present the principle of the effective stiffness technique and the results of a test experiment specifically developed to validate the method. Then we report on single-cell uniaxial traction force measurements revealing the existence of an early mechanical cell response ($t < 0.1$ s) that is unambiguously triggered by stiffness, and not by force.

It must be noticed that the parallel microplates technique used here allows one to measure force components that are normal to the surface of the plates, rather than tangential as usually studied with elastic gel substrates. However, a previous study carried out in the same geometry showed that “normal sensing” was, in fact, qualitatively and quantitatively similar to tangential response to stiffness on 2D gels (8). Moreover, the range of stiffness to which cells are sensitive in parallel plates arrangement corresponds to physiological tissue rigidities (see *Discussion* for details). Hereafter, normal single-cell traction forces are referred to as “forces.”

Results

Principle of the Effective Stiffness Method. In vivo, cell traction forces transmitted through integrins are resisted by the elasticity of the ECM. These forces are measured, in vitro, through the deformation of elastic substrates or probes that mimic ECM rigidity. These probes can basically be considered as springs of defined stiffness (Fig. 1*A* and detailed discussion in *SI Materials and Methods*). In these conditions, cell contraction is equal to probe deformation and is thus directly dependent on its spring constant. The principle of the original method presented here is to decouple probe deformation (i.e., force) from cell contraction (i.e., deformation), allowing one to impose different force-deformation laws corresponding to different effective stiffness values (Fig. 1*B*).

Decoupling Force and Stiffness. This method was implemented on a custom-made parallel plates setup (18) used previously to analyze single-cell response to load (19) and the role of actomyosin contractility in rigidity sensing (8). In this technique, a single cell is pulling on two parallel plates, one rigid, the other flexible, and used as a nano-Newton force sensor (20). In the setup used until now, the traction force developed by the cell caused deflection of the flexible microplate toward the rigid one (Fig. 2*A* and *Movie S1*). Thus, the flexible plate deflection $\delta(t)$ is equal to the cell shortening $\Delta L(t) = L_0 - L(t)$ (Fig. 2*A*). For a given plate stiffness k_0 , the force generated by the cell is then $F(t) = k_0\delta(t) = k_0\Delta L(t)$. From the cell point of view, the stiffness of its environment is given by the ratio of the generated force to the corresponding cell shortening $\frac{F(t)}{\Delta L(t)}$, which is obviously k_0 here.

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Fig. 1. Principle of the effective stiffness method. (A) A 2D gel (Left) and a flexible microplate (Right) are representative examples of elastic probes used to measure cell traction forces and the effect of rigidity. Both basically behave as springs (light gray) separating adhesion complexes (red) and resisting cellular contraction. Spring reaction (black arrows) balances cell forces (red arrows). In such setups, probe deformation and cell contraction are equal, and cell behavior is thus dependent on spring stiffness k_0 . In particular, $F_{cell} = k_0 \Delta L_{cell}$, where F_{cell} represents cell traction force and ΔL_{cell} its shortening (contraction). (B) The principle of the effective stiffness method is to decouple probe (black spring) elongation (i.e., force) from cell contraction (i.e., deformation). A double feedback loop independently regulates spring and cell lengths to maintain cell-spring contact in a fixed position. The first feedback signal stretches the spring to balance, in real time, any increment in cell traction force: $dF = dF_{cell} = k_0 dL_{spring}$. Simultaneously, the second feedback signal adjusts cell length to mimic the behavior of a virtual spring of effective stiffness k_{eff} : $\Delta L_{cell} = \frac{dF}{k_{eff}} = \left(\frac{k_0}{k_{eff}}\right) dL_{spring}$. Note that neither the force nor the deformation are maintained constant. Rather, force and deformation evolve continuously following cell response, the only constraint being that the ratio $\frac{dF}{dL_{cell}} = k_{eff}$ is kept constant. In other words, the setup acts as if the cell was compressing a spring of stiffness k_{eff} .

In order to tune the effective stiffness k_{eff} faced by a single cell, our idea was to decouple the force $F(t)$ (i.e., the flexible plate deflection $\delta(t)$) from the cell shortening (i.e., from the change $\Delta L(t)$ in the plate-to-plate distance). To do so, we have implemented an original double output feedback loop on the parallel plates setup [patent (21)]. At the beginning of the experiment (no plate deflection, zero force), the initial position of the flexible plate tip is recorded (18), and its value is used as the set point of the regulation loop (target position to be maintained all over the experiment time span). When the cell pulls on the plates, the servo-controller applies simultaneously two correction commands to avoid flexible plate tip displacement from its initial position. The first correction signal induces a displacement $\delta(t)$ of the basis of the flexible plate, thus bending it to equilibrate the force applied by the cell $F(t) = k_0 \delta(t)$, where k_0 is the true—physical—plate stiffness (Fig. 2D). The second correction signal applies a displacement $D(t)$ to the rigid plate, which induces a cell shortening $\Delta L(t) = D(t)$. The effective stiffness experienced by the cell $\frac{F(t)}{\Delta L(t)}$ is then equal to $k_{eff} = k_0 \frac{\delta(t)}{D(t)}$. Thus, any effective stiffness value can be chosen by tuning of the ratio $\frac{\delta(t)}{D(t)}$.

For instance, applying no displacement to the rigid plate ($D = 0$) leads to infinite k_{eff} (Fig. 2B). Indeed, such conditions correspond to an isometric traction where the cell tetanizes, applying high forces without being able to shorten. This is exactly what would happen if the cell was spreading between two infinitely rigid plates. Conversely, to mimic the behavior of a zero stiffness plate, one has to feedback on the rigid plate only ($\delta = 0$, Fig. 2C). The cell then shortens without applying any

Fig. 2. Technical achievement. (A) The regular parallel plates setup used until now. A cell pulling on a rigid and a flexible glass microplates brings the flexible plate tip toward the rigid one. Flexible plate deflection and cell shortening are equal ($\delta = \Delta L$): The stiffness felt by the cell $\frac{F}{\Delta L}$ is simply that of the flexible plate, $\frac{F}{\Delta L} = k_0$. (B) Infinite k_{eff} protocol. A feedback loop maintains the tip of the flexible plate in a fixed position by applying a deflection δ , balancing thus the force applied by the cell. The plate-to-plate distance is then constant, and the cell tetanizes, applying a force $F = k_0 \delta$ without being able to shorten ($\Delta L = 0$), $k_{eff} = \frac{F}{\Delta L}$ is infinite. (C) $k_{eff} = 0$ conditions. The tip of the flexible plate is maintained in a fixed position by applying a displacement D to the rigid plate. Thus, the cell shortens ($\Delta L = D$) without applying any force ($F = 0$), $k_{eff} = 0$. (D) General case, $0 < k_{eff} < \infty$. Two correction commands are applied simultaneously in order to maintain the position of the flexible plate tip. A displacement D is applied to the rigid plate, whereas a deflection δ is applied to the flexible one. Then, the force generated by the cell is $F = k_0 \delta$, and cell shortening is given by $\Delta L = D$. The value of $k_{eff} = \frac{F}{\Delta L} = k_0 \frac{\delta}{D}$ can then be set by tuning the ratio $\frac{\delta}{D}$ of the correction commands.

force, exactly as if the flexible plate had no measurable stiffness, $k_{eff} = 0$.

Beyond the previous illustrative examples where k_{eff} is constant over the whole experiment, the important point is that k_{eff} can be changed during a given experiment. Indeed, to study the effect of stiffness on cell contractility, one had, until now, to perform many different experiments where different cells were to pull on plates of different stiffness values. Furthermore, in such conditions, the nature of the physical control parameter remained an open question because force, stiffness, and strain are tightly correlated for a regular spring. For instance, using a flexible plate of low stiffness always leads to high cell strain and low generated forces (rigid and flexible plates almost in

Fig. 3. Decoupling force and stiffness. (A) Using a regular elastic probe of fixed stiffness k_0 , it is not possible to decouple the effects of force, stiffness, and strain because these parameters are tightly related. For instance, a flexible plate of low stiffness k_L leads to high cell strain and low traction forces. The cell almost brings the plates into contact, and the force is limited by geometric constraints (flexible plate deflection limited to the initial plate-to-plate distance. See [Movie S1](#)). Conversely, a high plate stiffness k_H leads to low cell strain and high traction forces. The force is then limited to the maximum force the cell can apply, and the plate deflection is weak ([Movie S2](#)). (B) With the effective stiffness method, it becomes possible to decouple the control parameters, in particular force and stiffness. In the example shown here, the effective stiffness k_{eff} is set to a high value k_H in the beginning of the experiment. After the cell traction force has reached a high level F_C , k_{eff} is set to a low k_L value. The cell is then put in very original conditions where it is pulling on a soft substrate while applying high level of force and submitted initially to weak strain.

contact, Fig. 3A; see also [Movie S1](#)). Conversely, high spring constants imply limited cell shortening and high generated forces (Fig. 3A and [Movie S2](#)).

With the original method presented here, we are now able to switch instantaneously ($t = 0.1$ s) the effective stiffness k_{eff} faced by a single living cell during its contraction. Thus, we can decouple the “force” and “stiffness” parameters. For instance, k_{eff} can be set to a high value k_H at the beginning of a given experiment. After the cell has generated a force F_C , k_{eff} could be changed to a low value k_L , leading to an original and informative experiment where the cell would be applying a high level of force on a spring of low stiffness (Fig. 3B). In variable stiffness conditions, k_{eff} is defined as $k_{\text{eff}}(t) = \frac{dF}{d\Delta L}(t) = k_0 \frac{d\delta}{dD}(t)$. Thus $k_{\text{eff}}(t)$ corresponds to the local slope of the $F(\Delta L)$ curve (Fig. 3B), which is determined by the instantaneous ratio $\frac{d\delta}{dD}(t)$ of the two feedback commands.

Method Validation: The Electrostatic Force Assay. In order to test the k_{eff} method, we replaced the usual glass microplates by two parallel metallic plates acting as a capacitor (Fig. 4A). An increasing voltage was then applied, leading to an increasing electrostatic attractive force $F_e(t)$ between the plates. Features of the electrostatic setup were chosen so that forces and plate deflections were equivalent to those observed with single cells (see *Materials and Methods* for details). During force increase, the effective stiffness $k_{\text{eff}}(t)$ of the system was changed, and the flexible plate deflection $\delta(t)$, as well as the rigid plate displacement $D(t)$, were recorded. Fig. 4B displays the results obtained for two successive changes between $k_{\text{eff}} = k_0$ and $k_{\text{eff}} = \frac{k_0}{2}$.

On the one hand, the flexible plate deflection $\delta(t)$ was proportional to the electrostatic force $F_e(t)$, $\delta(t) = \frac{F_e(t)}{k_0}$ whatever the value of k_{eff} . In particular, there was no perturbation to the $\delta(F_e)$ curve during the switching of the k_{eff} value. This result confirmed that the flexible plate deflection $\delta(t)$ constitutes a direct measurement of the force applied to the plates, even when k_{eff} is varied. On the other hand, $D(t)$, which represents the variation of the plate-to-plate distance, was clearly dependent on the actual value $k_{\text{eff}}(t)$. The slope of the $D(F_e)$ curve changed instantaneously from $\frac{1}{k_0}$ to $\frac{2}{k_0}$ when the effective stiffness value was changed from $k_{\text{eff}} = k_0$ to $k_{\text{eff}} = \frac{k_0}{2}$. The local slope $\frac{dD}{dF_e}$ was thus equal to $\frac{1}{k_{\text{eff}}}$ whatever the magnitude of F_e . In other words, the effective stiffness value could be chosen regardless of the overall level of the applied force.

Early single-cell response to stiffness. We then carried out a set of traction force experiments, where we let a single C2.7 myoblast spread between and pull on two parallel glass plates coated with

fibronectin. During a given experiment, the effective stiffness faced by the cell, k_{eff} , was switched many times from a low (5 nN/μm) to a high (90 nN/μm) value (Fig. 5A). For comparison, we have also reported in Fig. 5A two traction force curves measured with the regular setup (no feedback controls, no k_{eff}). The first one was obtained for a plate of low stiffness

Fig. 4. Specific protocol developed to test the k_{eff} method. (A) The glass microplates used with living cells were replaced by metallic ones. The setup acting as a capacitor, applying an increasing voltage to the plates generated an increasing attractive electrostatic force. The k_{eff} technique was tested during force increase, and the flexible plate deflection δ as well as the rigid plate displacement D were recorded. (B) D (yellow circles) and δ (blue squares) as functions of the electrostatic force F_e . δ was proportional to F_e regardless of the value of the effective stiffness k_{eff} (red circles). We found $\delta = \frac{F_e}{k_0}$, with k_0 the true—physical—stiffness of the flexible plate. Thus δ gives a direct measurement of the force applied to the plates. Conversely, the slope of the $D(F_e)$ curve was proportional to $\frac{1}{k_{\text{eff}}}$ as expected: $k_{\text{eff}} = k_0 \frac{d\delta}{dD}$ and thus $D = \frac{F_e}{k_{\text{eff}}}$. Indeed, D is equal to the change ΔL in the plate-to-plate distance and corresponds to the deflection of a virtual spring of stiffness k_{eff} . The $D(F_e)$ curve is an experimental achievement of the concepts represented in Fig. 3B.

technique used previously (Fig. 5A). Thus the original k_{eff} method really allowed us to monitor the cell adaptation to a sudden change in the external stiffness. Indeed, we could perform, on one unique cell, traction force measurements for a broad range of k_{eff} values (Fig. 6A). We found that the results obtained with the k_{eff} protocol were in excellent agreement with those previously obtained with many flexible plates of different stiffness values (Fig. 6B). This observation suggests that mechanical cell response is triggered by the actual stiffness value of its environment and does not depend on the history of cell contraction ($\frac{dF}{dt}$ adapted to stiffness regardless of cell deformation or cell traction force).

Second, $\frac{dF}{dt}$ was clearly a function of the stiffness $k_{\text{eff}}(t)$, and not of the overall force $F(t)$. For instance, changing k_{eff} from a high to a low value at two different levels of force ($F_C \sim 100$ nN and $2F_C$) led to identical switches from a high to a low $\frac{dF}{dt}$ (Fig. 5A). Thus,

Fig. 6. Comparing effective stiffness measurements to previous results. (A) The effective stiffness method allows one to test the effect of a broad range of stiffness values during the same single-cell traction force experiment. (B) $\frac{df}{dt}$ measurements carried out in these k_{eff} conditions (red circles, 12 cells) were similar to previous results obtained with the regular constant stiffness setup (black squares, ~ 50 cells). In the latter case, many flexible plates and different cells were used to explore the whole range of stiffness values. Thus, cells indeed responded to stiffness, regardless of the history of cell contraction (i.e., regardless of cell deformation and traction force).

early cell response to its mechanical environment is controlled by stiffness, and not by the magnitude of cell-substrate forces. This remark also holds true for the cell strain. Indeed, as cell shortening and force increase are correlated, two different force values correspond to two different cell lengths. Thus, early cell response was not influenced by the cell deformation, but only by the stiffness of its environment.

Third, cell adaptation to k_{eff} appeared to be instantaneous. Indeed, at an acquisition rate of 0.1 s, we could not observe any transient regime, $\frac{dF}{dt}$ (the slope of the traction force curve) being adapted to k_{eff} in less than 0.1 s (see zoom of Fig. 5B). Remarkably, we have also observed this early single-cell response to stiffness for REF52 fibroblasts (Fig. S1). Thus, instantaneous adaptation of $\frac{dF}{dt}$ to stiffness is not a characteristic of the C2.7 myoblasts cell line.

Discussion

Integrated Fast Response to Stiffness. Our findings reveal that a single cell indeed responds to the stiffness of its environment, regardless of the level of cell-substrate or cell-ECM forces. This extremely fast (<0.1 s) integrated cell response to stiffness should be mechanical in nature.

First, mechanochemical regulation loops were shown to be force sensitive (12, 16, 17). On stiff substrates, cell contractility leads to high levels of force applied to the adhesion complexes. Such high forces induce the deformation of sensory molecules (22–24) [or the opening of Ca^{2+} ion channels (9)] that trigger signaling and enhance, in turn, cell contraction. The experiments presented here showed clearly that, at a given level of force, changing the effective stiffness led instantaneously to modification of the cell behavior. Conversely, cell response to a given stiffness value could be reproduced at different levels of the overall force. These observations are incompatible with processes involving the stretching of sensory molecules when submitted to increasing levels of force.

Moreover, mechanochemical signaling cascades and subsequent structural reorganization at the cell scale are characterized by time scales in the minute range (25, 26), much longer than the 0.1 s observed in the k_{eff} experiments presented here. In fact, although mechanochemical signaling can be as fast as 0.3 s at the molecular level (27), amplification and coordination of molecular events to produce a coherent response at the cell scale is a slow process that builds up at least in several minutes.

Normal and Tangential Forces. In the parallel plates setup used here, traction forces and effective stiffness are defined as normal to the surfaces to which cells are adhering. This geometry is different from that studied in usual rigidity sensing experiments where cells are plated and contracting on 2D gels, i.e., with forces tangential to the substrate surface (Fig. 1A; see also *SI Discussion*). However, the relevance of studying cell response to stiffness in the normal direction, as we do here, was, in fact, attested in a previous study carried out in the same parallel plates geometry (8). In this work, there was no effective stiffness control, so different cells were to pull on different flexible plates of various stiffness. This protocol was indeed equivalent to usual procedures involving different cell cultures on 2D gels of various stiffness, and tangential traction force measurements from gel deformation.

Cell response to stiffness in the normal direction (our data) was, in fact, similar to those obtained with 2D gels. For instance, traction forces were proportional to substrate stiffness as already observed on 2D arrays of pillars (28). Moreover, cells plated on the surface of 2D gels and single cells pulling normally on parallel plates showed responsiveness to rigidity in the same range of elasticities. For instance, we found that maximum myoblasts efficiency in bending their substrate was obtained for plate stiffness of about 100 nN/ μm , corresponding to elastic gels of about

8 kPa Young's modulus (8) (see also *SI Materials and Methods* and Fig. S2), whereas myotube differentiation was optimal on gels with tissue-like stiffness of about 12 kPa (10). Thus, features of rigidity sensing in the normal direction (our measurements) are qualitatively and quantitatively similar to those observed on 2D gels and correspond to a physiologically relevant range of stiffness. This is also true for the real-time single-cell response to stiffness (present study) because dF/dt measured with variable effective stiffness were equal to those previously obtained with different plates of various spring constants (Fig. 6B).

Summary. We developed an original technique allowing us to change, in 0.1 s, the effective stiffness k_{eff} faced by a single living cell, regardless of the level of the applied force (or of the cell strain). In these conditions, measuring the traction force generated by single cells, we found that the rate of force increase was tuned to stiffness in less than 0.1 s and was not dependent on force, nor on strain. It seems unlikely that this instantaneous cell response to the stiffness of its environment could be explained by the force-dependent mechanochemical signaling processes described until now. It should rather be mechanical in nature. Early cell response could, for instance, be explained by the adaptation to stiffness of actomyosin contractility (8). It could also be due to a rigidity calibration mechanism (5, 29).

In fact, early mechanical response and mechanochemical signaling are probably synergistic processes. Instantaneous adaption of the cytoskeletal tension at the cell scale could lead, at longer time scales, to local reinforcement of adhesion complexes and activation of the associated regulatory loops (30). This could be one strategy allowing cells to coordinate local mechanochemical activity at cell-substrate junctions (31). These ideas now need to be put to the test by further investigations combining the original effective stiffness technique presented here and visualization methods (total internal reflection fluorescence microscopy, confocal microscopy, and so forth). Indeed, the k_{eff} method described here can easily be adapted on any mechanical setup involving force measurement through stretching of an elastic probe. It should be a useful tool to determine the role of rigidity or stiffness in many important processes involving mechanosensing, such as plant growth (32), morphogenesis (33), or tissue engineering (34).

Materials and Methods

Electrostatic Setup. When testing the k_{eff} method, our aim was to work with applied forces and flexible plate deflection values characteristic of those observed in single-cell traction force experiments, typically $0 < F < 400$ nN and $0 < \delta < 15$ μm . Thus the geometrical features of the capacitor setup were tuned to fulfill these requirements.

We used two parallel metallic plates separated by a distance d , constituting a capacitor of area A . Applying a tension V leads to an attractive electrostatic force $F_e = \frac{\epsilon A}{2} \left(\frac{V}{d}\right)^2$, where ϵ is the permittivity of air. With $\epsilon \approx 8.85 \cdot 10^{-12}$ F/m, $A = 4 \times 3$ mm², and $d = 300$ μm we could apply forces F_e as high as 500 nN with a tension V less than 30 V.

The flexible metallic plates (~1.5 cm in length, ~4 mm in width) were cut out from an aluminum sheet of thickness $e = 10$ μm . The stiffness of the plates $k_0 = \frac{El^3}{4L^3}$ (E young modulus, l width, e thickness, and L length) was thus about 20 nN/ μm .

We have also verified that k_{eff} absolute values were correctly defined. We have thus compared calibration curves:

- on the one hand, regular flexible plate deflection versus applied force $\delta(F_e)$ for a physical plate stiffness k_0 , and
- on the other hand, plate-to-plate distance (virtual spring deflection) versus applied force $D(F_e)$ for three different k_{eff} values: $k_{\text{eff}} = \frac{k_0}{2}$, k_0 , and $2k_0$.

$D(F_e, k_{\text{eff}})$ curves were in excellent agreement with $\delta(F_e, k_0)$ (Fig. S3) indicating that effective stiffness values k_{eff} were indeed well defined.

Acquisition Rate and Speed of Cell Response. The effective stiffness method (two simultaneous feedback loops) was implemented on an instrument that was described in details in ref. 18. A combination of "heavy" microplates holders and low stiffness piezoelectric actuators leads to a resonance fre-