

## Traction forces exerted by epithelial cell sheets

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# Traction forces exerted by epithelial cell sheets

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## Abstract

Whereas the adhesion and migration of individual cells have been well described in terms of physical forces, the mechanics of multicellular assemblies is still poorly understood. Here, we study the behavior of epithelial cells cultured on microfabricated substrates designed to measure cell-to-substrate interactions. These substrates are covered by a dense array of flexible micropillars whose deflection enables us to measure traction forces. They are obtained by lithography and soft replica molding. The pillar deflection is measured by video microscopy and images are analyzed with home-made multiple particle tracking software. First, we have characterized the temporal and spatial distributions of traction forces of cellular assemblies of various sizes. The mechanical force balance within epithelial cell sheets shows that the forces exerted by neighboring cells strongly depend on their relative position in the monolayer: the largest deformations are always localized at the edge of the islands of cells in the active areas of cell protrusions. The average traction stress rapidly decreases from its maximum value at the edge but remains much larger than the inherent noise due to the force resolution of our pillar tracking software, indicating an important mechanical activity inside epithelial cell islands. Moreover, these traction forces vary linearly with the rigidity of the substrate over about two decades, suggesting that cells exert a given amount of deformation rather than a force. Finally, we engineer micropatterned substrates supporting pillars with anisotropic stiffness. On such substrates cellular growth is aligned with respect to the stiffest direction in correlation with the magnitude of the applied traction forces.

(Some figures in this article are in colour only in the electronic version)

## 1. Introduction

Mechanics plays a key role in the spatial organization of tissues [30]. Adhesion and migration at multicellular scales are essential for a wide variety of biological processes including tissue formation [22], morphogenetic processes [25, 29], responses to wounds [37], inflammation [38], or tumor metastasis [46]. Cells are subjected to chemical and physical signals from their neighbors, the surrounding fluid and the extracellular matrix (ECM) [8] and integrate these various signals to respond. However, the mechanisms that

underlie how these factors affect the organization of tissues still need to be clarified. Cell migration is commonly understood as the movement of individual cells that has lead to a well-established model whereby cells move via the extension and adhesion ahead of the cell pointed in the direction of migration and the retraction and loss of adhesion of the trailing edge at the rear [28]. The transmission of nanonewton-scale contractile forces required for the translocation of the cell body is generated at specific contact points with the surrounding substrate [26]. In the context of multicellular assemblies, mechanical forces result from a balance between cell–cell and cell-to-substrate interactions.

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Indeed, adherent cells exert strong traction forces at their anchorage sites to the matrix [1, 45, 32] and to neighboring cells [9, 33, 3, 19], respectively through focal adhesions and adherent junctions. Thus adhesion and migration of cell groups require the propagation of mechanical forces within multicellular assemblies to maintain their cohesion. In the case of collective migration, in the absence of extrinsic cues, it has been shown that active traction forces [47] or displacements [37, 24] can be observed many cell rows behind the leading edge, suggesting a mechanical cooperativity over multiple length scales. It appears that guidance within tissues is due in part to a cohesive and coordinated movement due to cell–cell contacts. Furthermore, the mechanical stability of multicellular assemblies also relies on the interactions with ECM [23]. In this context, the reciprocal contributions of cell–matrix and cell–cell contacts in the mechanical stability and migration of cell sheets is crucial but remains an open question.

The physical properties of the surrounding matrix have a large influence on the cell response [48, 14, 17, 10, 21], as well as on regulation, formation or organization of tissues [27, 23, 16]. In particular, collective cell behaviors and traction forces are affected by substrate rigidity [42, 12, 43]. By changing the stiffness of the cellular environment, one would expect to observe a destabilization of cellular assemblies, providing important information about the respective modulation of tension induced by cell–cell and cell–ECM adhesions.

Here, we have studied the forces exerted by Madin–Darby Canine Kidney (MDCK) epithelial cells using microfabricated substrates covered by an array of flexible micropillars (as previously described [15, 41, 45, 4]). Our approach uses these high density arrays of microfabricated pillars to map the traction forces within epithelial cell sheets [15]. These force sensor arrays ( $\mu$ FSA) are made of elastomeric pillars of well controlled physical and chemical properties [20]. By analyzing the force distributions within multicellular assemblies, we show that their mechanical stability is based on a collective mechanism based on the transmission of the force transmission through cell–cell junctions. We have also studied the influence of substrate rigidity on the intensity of these traction forces: they linearly increase with the substrate rigidity over a broad range of rigidities, as previously shown [42]. This result has been confirmed by the use of regular arrays of soft pillars of oval cross sections (to create an angle-dependent substrate stiffness) inducing an oriented growth of epithelial cells [43]. From these results, we propose a simple model based on the force distribution within cellular assemblies that could explain some aspects of the extension of cell sheets in the stiffest direction of the substrate. Finally, all our data suggest that traction forces as well as the substrate rigidity may play a fundamental role for the integrity of tissue.

## 2. Experimental set-up: fabrication of the micropillar substrates and force accuracy

### 2.1. Fabrication of dense micropillar substrates

During the past ten years, various techniques have been developed to characterize the mechanical forces generated by

cells [8] and/or the influence of the local mechanical properties on cell functions [14]. Mechanical stresses developed by cells are typically in the nanonewton range, and can, for instance, be measured by deformations of soft material cell culture substrates. Most experiments to date use continuous flexible substrates such as polymeric hydrogels of controlled elasticity, or polymeric thin films [32, 1]. The local deformations are measured by analyzing the local displacement of markers (usually latex beads) dispersed in the substrate. But as deformations propagate inside a continuous medium, the relation between bead displacements and forces is difficult to compute [13, 7, 44, 2]. To overcome this difficulty, alternative methods using elastomeric substrates made of a discrete array of vertical microneedles [45, 41, 15] have been proposed. The deflection of each post gives a direct measurement of the local force exerted by the attached cells independently of the forces acting on the neighboring posts. The pillars act as simple independent springs, and the linear theory of elasticity gives their deflection. For a cylinder of radius  $r$  and length  $L$  bent by the application of a force  $F$ , it leads to the following formula:

$$F = \left(3/4\pi E \frac{r^4}{L^3}\right) \Delta x \quad (1)$$

where  $E$  and  $\Delta x$  are respectively the Young modulus and the deflection of the pillar.

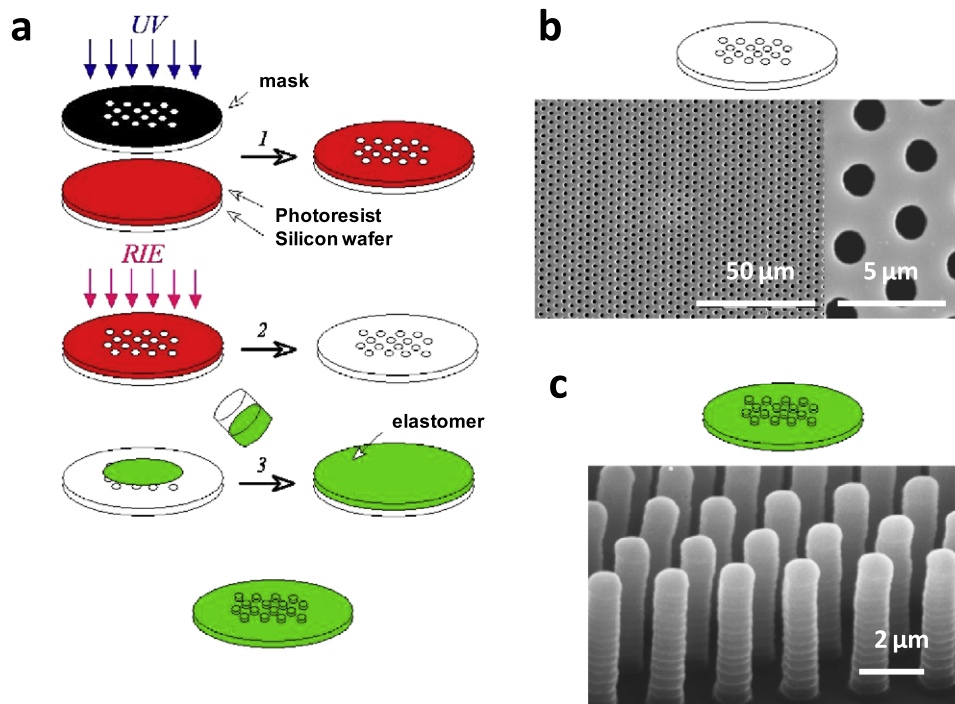
However, the use of discrete substrates may have consequences on cell adhesion and migration. To limit the effects of this drawback we have developed a method allowing one to obtain a very dense array of micropillars with a  $2 \mu\text{m}$  center-to-center spacing for a pillar diameter of  $1 \mu\text{m}$  [15]. The strategy used for the fabrication is standard and combines different steps including photolithography, deep reaction ion etching (DRIE) and soft lithography (figure 1(a)).

Our fabrication method reduces diffraction effects and allows a better spatial resolution than the one obtained by only standard photolithography to be achieved. A negative replica of the array was fabricated by deep reactive ion etching after a photolithography step (figure 1(b)), was then silanized (to make them anti-adhesive) and covered with a curable silicone elastomer (polydimethylsiloxane, PDMS). After curing, PDMS replicas were peeled off the silicon wafer (figure 1(c)).

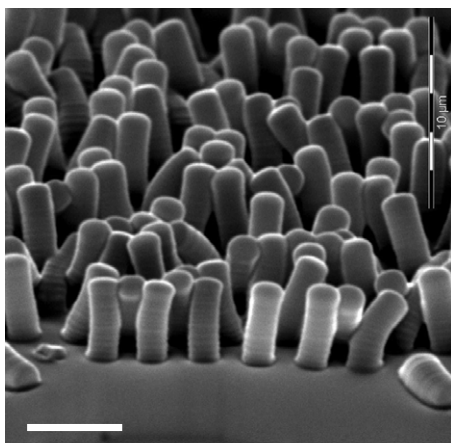
Despite the anti-adhesive treatment of this mold, an important issue concerns the aspect ratio of the pillars: indeed, pillars having an important aspect ratio ( $L/r \geq 6$ ) tend to irreversibly stick to each other during the peeling step [40] (figure 2). To bypass this difficulty, the arrays were peeled off in a liquid (70% ethanol in water) and were kept in water throughout the following steps to damp the relaxation of the elastic energy stored during the peeling process and to avoid further capillary effects sufficient to collapse the pillars.

### 2.2. Force measurements

The geometrical parameters of the pillars are determined by SEM imaging and the Young modulus of the PDMS elastomer is measured to relate the forces to the displacements of the pillars (equation (1)) [15].



**Figure 1.** Microfabrication process. (a) Description of the different steps of the microfabrication procedure; (b) example of the silicon surface obtained after DRIE; (c) example of a molded PDMS micropillar substrate.



**Figure 2.** Scanning electron micrograph (SEM) of collapsed PDMS micro-pillars. Scale bar = 5  $\mu\text{m}$ .

The analysis of the pillar displacements is performed with home-made multiple particle tracking software allowing us to label and detect the deflection of each post over a whole stack of images. This tracking is compatible either with bright field or fluorescent (fluorescently labeled fibronectin coated on the tips of the pillars) microscopy (figure 3). In bright field microscopy, micro-pillars act as waveguides and appear bright whereas the background remains dark. The micro-pillars deflection is then measured with a good accuracy ( $\approx 25$  nm) by determining the center of mass of the corresponding bright pixels. In a similar way, for fluorescently labeled pillars, the routine was used to track the brighter pixels (by thresholding the images). Interestingly, and even though we do not have a

full explanation of this phenomenon, the fluorescent signal is not homogeneous on the top of the pillars and appears brighter on the edges than in the center (figure 3(d)).

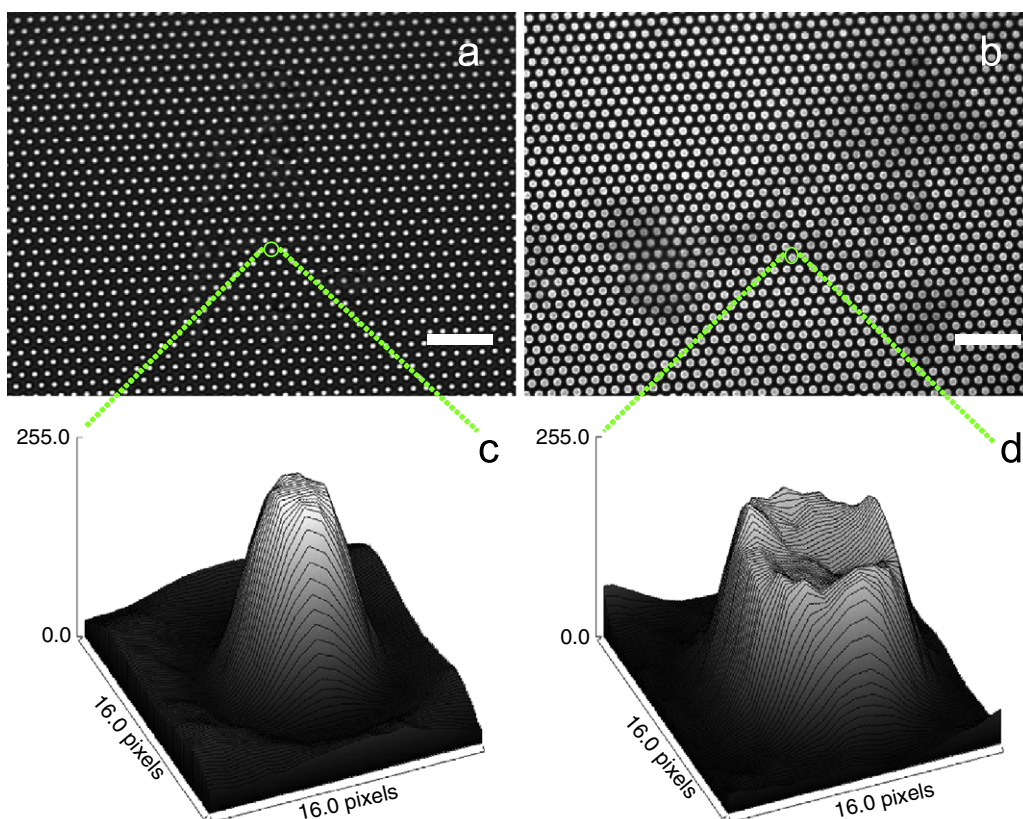
For such an image processing, the main difficulty is to estimate the position of the pillars at rest, to accurately measure their deflection. Our method is based on the regularity of the hexagonal lattice of micro-posts. The position at rest of each micro-pillar covered by cells is determined by computing, with a linear fit, the intersection of the position of the uncovered posts belonging to the same row (3 rows for a hexagonal lattice). It leads to a spatial resolution for the deflection of about 25–30 nm for both bright field or epifluorescence microscopy. This systematic error is estimated from the measurement of the deflections obtained for substrates in the absence of cells.

### 3. Mapping of traction forces exerted by cell assemblies

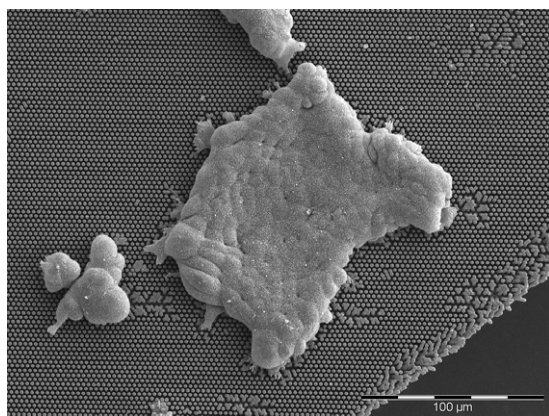
We now focus on the forces exerted by cell assemblies on these microfabricated substrates. Epithelial cells are cultured at subconfluent densities on fibronectin coated pillars until well defined islets of ca 10–20 cells could be defined (figure 4).

We have first examined the overall pattern of deformation exerted by cell assemblies. Concerning the transmission of forces through epithelial cell groups, we have observed similarities with single-cell experiments, as well as clear discrepancies. For instance, migrating fibroblasts exhibit strong traction forces pointing towards the center of the cell, mostly localized at the anterior and posterior regions, and smaller forces in the central region underneath the





**Figure 3.** Typical images obtained by microscopy to analyze the displacements of the pillars (a) in bright field microscopy and (b) epifluorescence microscopy for pillars whose top is coated with Cy3-labeled fibronectin. Scale bars =  $20 \mu\text{m}$ . Intensity profiles for a pillar observed in bright field (c) and epifluorescence microscopy (d).



**Figure 4.** SEM of MDCK epithelial cells on a micropillar substrate.

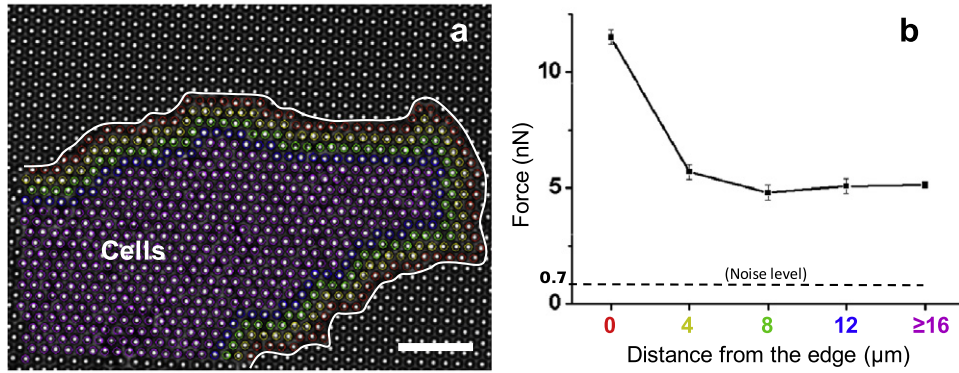
nucleus [32]. These observations have led to the theoretical representation of an adhering cell as a force dipole (a pair of equal and oppositely directed contraction forces) [5, 11]. For cell assemblies, we also observe that the strongest deformations are always localized at the edge of the islands of cells in the active areas of cell protrusions (figure 5). Hence the largest forces are mostly due to the mechanical activity of the edge of the monolayer (figure 5(b)) and they are in average oriented normally to the monolayer edges [15].

For instance, forces at the edge are distributed with an average value around  $12 \text{ nN}$  on a  $23 \text{ nN } \mu\text{m}^{-1}$ -micropillar

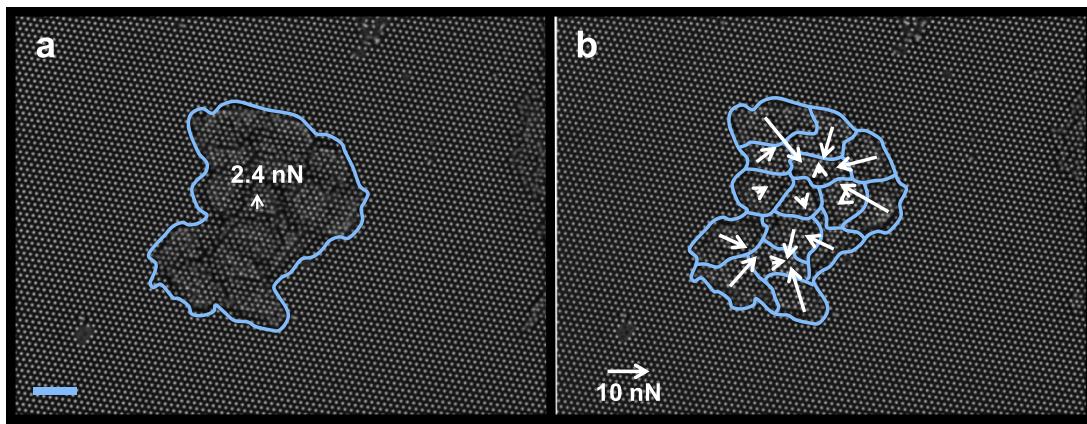
substrate (figure 5(b)). The spatial distribution of the forces inside the monolayer is plotted in figure 5(b) and the average traction force rapidly decreases over less than one cell size from its maximum value at the edge to half this value within the monolayer ( $\sim 5 \text{ nN}$ ), demonstrating that the mechanical activity inside a growing epithelium remains important. A recent study has shown that the traction forces driving the extension of epithelial cell sheets can extend many cell rows behind the leading edge across large distances (around  $200 \mu\text{m}$ ) [47]. Here, since a more rapid decrease of the forces was obtained ( $\sim 5 \mu\text{m}$ ) within epithelial cell assemblies of smaller sizes, it points out that the number of cells interacting with each other, and their density, could impact on the distribution of forces. Additionally, those differences could also be explained by the fact that our  $\mu\text{FSA}$  system is locally reacting and therefore prevents cell–cell mechanical communications through compliant substrates [39]. Further experiments should be done to check the importance of multicellular island size on the transmission of forces.

In contrast with single cell, the mechanics of multicellular assemblies is more complex, because each individual cell is physically constrained by its neighbors, and the transmission of forces implies cell–cell contacts.

To test the importance of these cell–cell contacts on the mechanical stability of cell groups, we have determined the vectorial distribution of forces exerted by small islands ( $\sim 10$ – $20$  cells) at the cell-to-substrate interface. A typical example is



**Figure 5.** Traction forces as a function of the distance from the edge of the monolayer. (a) Color mapping of the different rows of micropillars as a function of their respective distance to the cell edge. (b) Average traction force versus distance from the edge. Equidistant posts from the edge are pooled together to calculate the average force for each distance [15]. Scale bar = 20 μm.



**Figure 6.** Distribution of traction forces exerted by an epithelial cell island on a micropillar substrate with a spring constant of 2.1 nN μm<sup>-1</sup>. (a) Bright field image of a MDCK cell assembly on a micropillar array. Sum of the vectorial forces of all the pillars underneath the cells. All the pillars underneath the cells have been selected and the white arrow that corresponds to ~2.4 nN represents  $\sum_i \vec{F}_i$ , where  $i$  is one of the pillar. Scale bar = 20 μm. (b) Resulting vectorial forces of each individual cell delineated in blue within the cellular island.

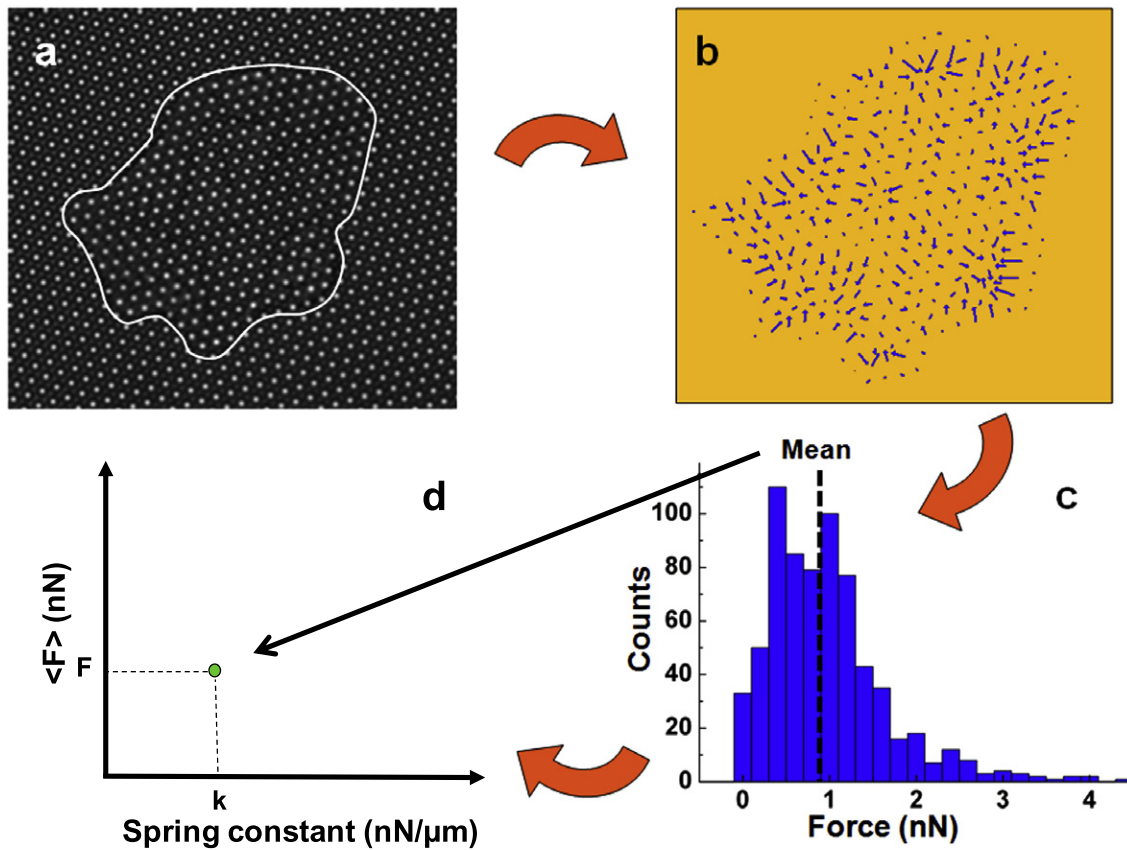
given on figure 6. First it clearly shows that the total intensity of the forces obtained by summing the vector forces of all the underlying pillars vanishes to ~zero. In fact, the overall force is ~2.4 nN (figure 6(a)), which roughly corresponds to the summed error on the positions of all pillars given by  $0.03 \mu\text{m} \times 2.1 \text{ nN } \mu\text{m}^{-1} \times \sqrt{840} = 1.8 \text{ nN}$  (where 0.03 μm is the error on the deflection of each pillar (see before), 2.1 nN μm<sup>-1</sup> the spring constant and 840 the number of pillars underneath the cells). It shows that traction forces are balanced within the cell assembly (figure 6(a)). Moreover this example illustrates the good accuracy of our tracking procedure of the tips of multiple posts. We have analyzed the forces exerted by single cells within the island by delineating the cell–cell boundaries (figure 6(b)). The vector forces exerted by single cells are not equal to zero, as would be expected for individual cells with no contact with their neighbors, but instead vary from a few up to tens of nNs. According to Newton’s laws, such forces can be seen as forces exerted by all the neighbors on the selected cell. Our assay allows us to test if the adhesion of epithelial cell groups relies on the mechanical activity of individual cells or on the cohesion through cell–cell junctions. Our findings demonstrate that the forces exerted by cell groups

are not balanced locally (figure 6(b)) as they are at the scale of the group (figure 6(a)), demonstrating the transmission of these forces through cell–cell junctions. Altogether, our results hint that the stress developed within epithelial cell sheets may be the result of a collective behavior and that the cohesion of cell groups results in a reciprocal modulation of the tension induced by cell–cell and cell–ECM adhesions.

#### 4. Influence of the substrate stiffness on epithelial cell traction forces and growth

As collective cell movements and the mechanical stability of multicellular assemblies are affected by substrate rigidity [36, 12], one would expect to observe changes in the growth of epithelial cells, in their adhesion state and the value of the tension transmitted through cell–cell junctions with the stiffness. To do so, we have plated MDCK cells on microfabricated surfaces with pillars of different spring constants that are obtained by changing the geometrical parameters of the pillars (length and radius) according to equation (1). We have varied the dimensions of the posts from 1 to 2 μm in diameter and





**Figure 7.** Determination of the force–stiffness relationship. (a) Typical optical image of a MDCK cell island on micropillars. (b) Spatial distribution of vector forces. (c) Each histogram of force distribution gives the average value of the forces,  $\langle F \rangle$ , which is plotted as a function of the spring constant of the pillars (d). The distance between two consecutive pillars is  $3 \mu\text{m}$ .

from 2 to  $7 \mu\text{m}$  in height, leading to a wide range of spring constants, from  $\sim 1$  to  $200 \text{ nN } \mu\text{m}^{-1}$  [42, 20]. We have performed experiments on small islands of subconfluent MDCK cells containing 10–20 cells. For each experiment, images were captured over time periods of several hours. The forces detected for all the pillars were collected into histograms (figure 7).

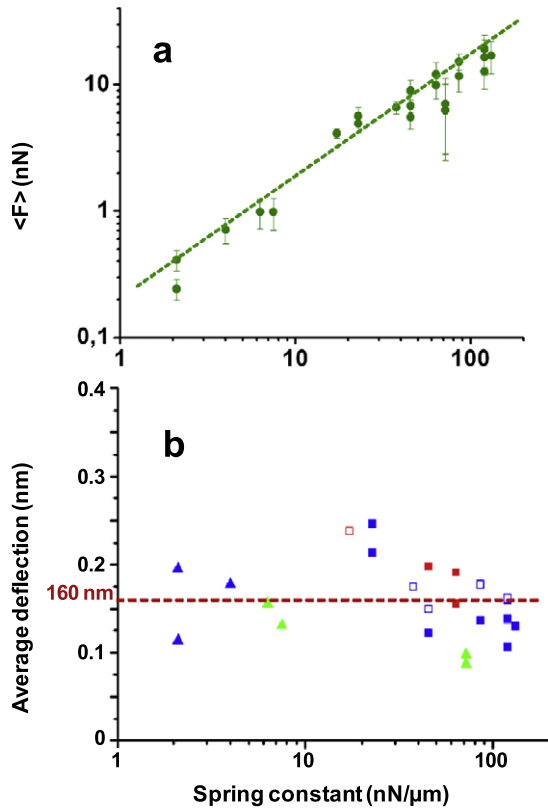
As described in our previous studies [42, 20], we have plotted the mean force  $\langle F \rangle$  as a function of the spring constant of the pillars,  $k$  (figure 7(d)). We have shown that the traction forces increase with the substrate stiffness, as expected according to other studies [31].

Our quantitative results also demonstrate that a linear relation correlates the forces exerted by cells and the substrate rigidity (figure 8(a)) [42]. Consequently, the deflection of the elastic, micron-scale pillars on which cells are plated, is independent of the rigidity of the pillars. The mean displacement of the pillars thus remains constant over the two decades of the micro-posts flexibility values and is found to be  $\sim 160 \text{ nm}$  (figure 8(b)). Interestingly, the maximal displacement of the pillars, which corresponds to the tail of the histograms and thus the largest forces [42], is also found to remain constant over the same range of rigidities (not shown). Hence our work suggests that the mechanosensitive activity of epithelial cells is limited by the deformations of the substrate.

However, whether the cell mechanosensitivity is controlled by the stress in the extracellular matrix or by the de-

formation remains an open question. Indeed, recent theoretical models, based on the activation of a mechanosensor located within focal adhesions (FAs) by the stress [35, 34], predict the dynamical evolution of cell/matrix adhesions as a function of the stiffness of the matrix. In the regime corresponding to our experiments, FAs reach a saturation size proportional to Young’s modulus of the ECM. Consequently, the total force that each adhesion transmits to the substrate is also proportional to the matrix rigidity, as observed in our experiments. This theoretical model can explain some trends observed in our experiments. However, the regulation of the mechanosensitivity of epithelial cells could be also mediated by the deformation of larger structures, such as stress fibers that could contract up to a given distance, instead of a purely local mechanism based on force transduction through focal adhesion mechanosensors.

Here a direct comparison of the traction forces exerted on continuous soft gels and micropillars with comparable Young moduli would be helpful to determine the influence of the local mechanical properties of the ECM on the transmission of cellular forces. We estimate the equivalent Young modulus,  $E_{\text{eff}}$ , of a micropillar surface by  $E_{\text{eff}} = \frac{9k}{4\pi a}$ , where we assume that  $a$  roughly corresponds to either the size of FAs or the radius of the pillars [20]. This assumption is consistent with the use of pillars of 1 and  $2 \mu\text{m}$  diameter in our study. By varying the spring constants from 1 to  $200 \text{ nN } \mu\text{m}^{-1}$  and choosing  $a$  as the radius of the pillars, we obtain a range of  $E_{\text{eff}}$  varying



**Figure 8.** (a) Log–log plot of the average traction force exerted by epithelial cells as a function of the spring constant of the pillars (adapted from [42]). (b) Average deformation of the pillars as a function of their spring constant. The different colors correspond to different densities of pillars (ratio of the post surface over the total surface): 10% (green), 22% (blue) and 40% (red). Triangle and square symbols respectively correspond to pillars of 1 and 2 μm in diameter. Hollow and filled symbols respectively correspond to experiments done with either an overall fibronectin coating of the substrate (images acquired by bright field microscopy) or micro-contact printing of fluorescent fibronectin on the top of the pillars.

from 1.5 to 150 kPa. Interestingly, this estimation will help to compare our results on epithelial cells with the ones obtained in previous experiments on continuous substrates, as previously done for single cells [31, 49].

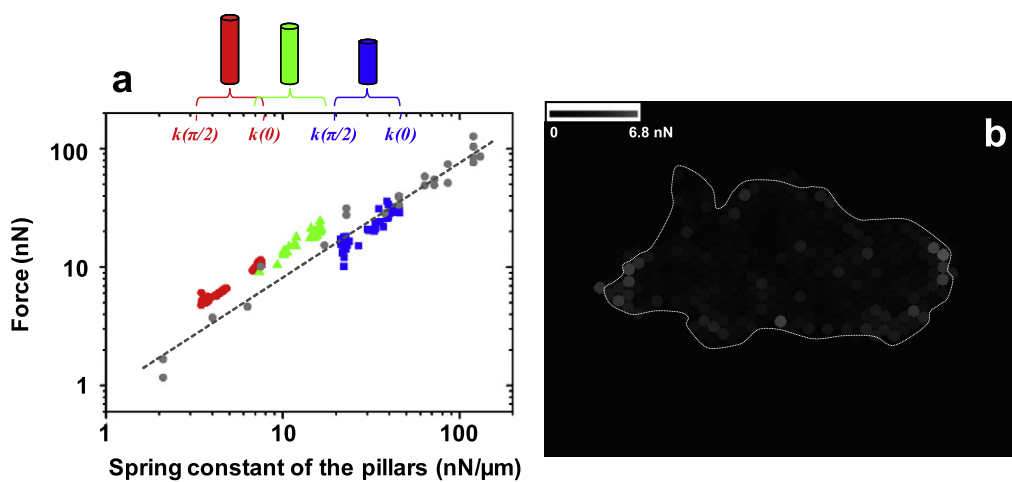
Finally, our previous study [43] has shown that microfabricated substrates exhibiting anisotropic rigidity can induce the growth of epithelial cell sheets in the stiffest direction of the substrate. We have used ellipsoidal microposts whose stiffness is around four times larger in the major direction of the ellipse (minor axis ( $b \sim 1 \mu\text{m}$ ) half the major one ( $a \sim 2 \mu\text{m}$ )). The spring constant is given by the following relation:

$$k(\theta) = \frac{3\pi E ab}{4 L^3}(a^2 \cos^2 \theta + b^2 \sin^2 \theta) \quad (2)$$

where  $E$  and  $\theta$  are respectively Young’s modulus and the angle of the deflection with respect to the major axis.

To explain the behavior of epithelial cells on such substrates, we have performed different experiments on anisotropic pillars with variable heights and analyzed the distribution of the largest traction forces as a function of  $k(\theta)$ . In agreement with our results on cylindrical pillars, these data demonstrate that the maximal forces exerted by cells respond to the local rigidity of the substrate,  $k(\theta)$ , in a similar way: it shows a linear increase of the forces with the stiffness (figure 9(a)). Furthermore, we have observed that regions of high traction stress and large cellular deformations within the sheets of cells are concentrated at the edges, in particular at the two poles of the islands along their long axis (figure 9(b)). One can hypothesize that the large cell deformations at the poles (data not shown, [43]) could correspond to leading cells that exert stronger traction forces and could be responsible for pulling the others during tissue growth, as described in various biological situations [29, 18, 37].

Altogether, these data can partially explain the preferential orientation of epithelial cell sheets along the stiffest direction



**Figure 9.** (a) Relationship between the force and spring constant for substrates composed of anisotropic micropillars. Each colored point represents the maximal force detected for one image as a function of  $k(\theta)$  corresponding to the orientation of the force. The different colors (red ●, green ▲ and blue ■) correspond to 3 different heights of pillars (6.1, 4.7 and 3.3 μm), respectively. The intervals represented on the upper part of the graph correspond to the range of stiffness, from  $k(\pi/2)$  to  $k(0)$ , of a given substrate. The gray dots are the values of the maximal forces for cylindrical pillars. (b) Gray scale map of the cumulated average magnitude of the forces applied over a 1 h period by a typical cellular island (adapted from [43]).



of the substrate. First, we have observed that the stress is maximal at the periphery of the cell islands. Then, since the force–deformation relation is given by  $F = k \cdot \Delta x$ , the largest forces exerted by cells are oriented in the more rigid direction for a constant deformation. As a consequence, it would be expected that the cell sheets would elongate with time along the direction of maximal stiffness. In this context, future experiments should study the dynamics of force transmission and, in particular, the growth of focal adhesions, on anisotropic substrates that should depend on the local stiffness as theoretically predicted [6].

Interestingly, the recent study by Trepat *et al* [47] characterizing the growth of epithelial cell sheets on flexible polyacrylamide gels shows that substrate stiffness does not strongly affect the growth rate of the cell colonies and the mechanical activity of the edge. These differences between continuous and discrete substrates may be attributed to the local elastic response of the micropillar system, which does not allow the propagation of deformations through the substrate [39]. Further experiments on epithelial cell growth that would directly compare both techniques would be helpful to get a deeper understanding of the response of continuous cell cultures to substrate stiffness.

## 5. Conclusion

In this paper we have studied experimentally the traction forces exerted by epithelial cell groups. We have shown that micropillar arrays are a versatile tool to modify the mechanical properties of the substrate. It appears that epithelial cells act collectively in the transmission of forces within the group and exert large traction forces mostly localized at the periphery. In particular, we have characterized the response of epithelial cells to substrate stiffness. It appears that the traction forces of epithelial cells varied linearly with the rigidity of the substrate over about two decades. While the intracellular signaling mechanisms remain to be clarified, these results suggest that cells probe the softness of its environment by controlling the amount of deformation. A local change of the rigidity induces a growth of cells in the stiffest direction of the substrate.

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