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Mechanosensing of substrate thickness

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Abstract

Structure and function of the adherent cell depend in a crucial way on its microenvironment, including the stiffness of its substrate. It is often asserted that substrate thickness (as opposed to stiffness) plays a negligible role and therefore may be considered semi-infinite. This assertion has been recently challenged, but the characteristic length scale to consider in this regard is poorly understood. We show here that this characteristic length scale is the lateral cell size. As substrate thickness approaches the lateral dimension of the cell, the apparent stiffness of the substrate is amplified to levels much greater than the intrinsic stiffness of the substrate. This change in apparent stiffness is readily sensed by the cell, leading to changes in cell spreading area, stiffness, and contractile forces. In contrast to these responses that occur over the length of the cell, mechanosensing around an isolated point force is influenced greatly by intrinsic substrate stiffness but to a negligible extent by substrate thickness. We conclude that mechanosensing of substrate thickness is dominated in large part by traction forces spread over the lateral dimension of the cell.

I. INTRODUCTION

The stiffness of the cellular microenvironment alters cell phenotype, its pattern of migration, cytoskeletal remodeling, and gene expression and can even direct stem cell fate [1–8]. For the cell adherent upon a flat elastic substrate, it has been widely assumed that substrate depth can be considered as being essentially semi-infinite if substrate displacements in the surface plane are small in comparison to the substrate depth [9]. In that case, the shear modulus of the substrate material would be sufficient to define the effective stiffness sensed by the cell. To the contrary, Butler *et al* [10] argued on theoretical grounds that the semi-infinite approximation necessarily fails when substrate thickness falls below some characteristic thickness, h_0 . This idea has been pursued by others experimentally and has been the subject of much recent investigation [11–17]. These studies show that for $h < h_0$ cellular behaviors are strongly influenced by the presence of the rigid foundation below the substrate. However, there is no consensus on what factors set the value of h_0 , which ranges in those studies from several microns [12,14,16,17] to as much as 60 μm [13]. It has been argued, further, that these discrepancies arise from differences in focal adhesion (FA) size [12], cell lateral dimension [11], cell height [14], or stiffness of the substrate [14]. While it is now generally agreed the semi-infinite approximation fails, the length scale below which that approximation fails remains unclear.

Here, we show by theory that h_0 approximates the lateral size of the cell. We then confirm this prediction in the human fetal lung (HFL) fibroblast using measurements of cell size and

spreading, cell elastic modulus, and cell traction forces as a function of substrate thickness. We argue, further, that discrepancies in h_0 reported previously in the literature arise from different measures of cellular response.

II. THEORY

Traction calculations

We have previously given the solution to the recovery of tractions given a measured displacement field on the surface of a substrate of finite thickness [18]. Here, we give an alternative but equivalent formulation that reveals the symmetry properties of the solution. Specifically, we consider an elastic slab defined on $-\infty < (x, y) < \infty$, with uniform thickness h ($0 \leq z \leq h$). For boundary conditions, the displacements at $z = h$ are given by measurement; the displacements at $z = 0$ are zero, and we assume negligible normal traction at the cell/gel interface. We work in Fourier space with respect to $\vec{r} = (x, y)^T$ by writing all functions as $f(\vec{r}, z) = \sum_{\vec{q}} e^{i\vec{q} \cdot \vec{r}} \hat{f}(\vec{q}, z)$ and where the two-dimensional wave vector is $\vec{q} = (\alpha, \beta)^T$. We thus have the problem of recovering (in Fourier space) the tractions $\hat{\vec{T}}$ given the displacements (at $z = h$) $\hat{\vec{u}}$. The solution for this is given by Trepat *et al.* [18], but the formulas there do not reveal the simple structure of the relationship between the tractions and the displacements. To this end, we note that the tractions are a vector field, which in turn is a function of two independent vectors, $\hat{\vec{u}} = [\hat{u}_0 \ \hat{v}_0]^T$ and $\vec{q}$. The requirement that the tractions be homogeneous linear functions of $\hat{\vec{u}}$ then demands that $\hat{\vec{T}}$ be a linear combination of $\hat{\vec{u}}$ and $(\vec{q} \cdot \hat{\vec{u}})\vec{q} = (\vec{q}\vec{q}^T)\hat{\vec{u}}$; thus, on symmetry arguments alone, it follows that the tractions must be of the form $\hat{\vec{T}} = f_1(q)\hat{\vec{u}} + f_2(q)(\vec{q}\vec{q}^T)\hat{\vec{u}}$ or

$$\hat{\vec{T}} = M\hat{\vec{u}}, \quad M = f_1(q) \begin{bmatrix} 1 & 0 \\ 0 & 1 \end{bmatrix} + f_2(q) \begin{bmatrix} \alpha^2 & \alpha\beta \\ \alpha\beta & \beta^2 \end{bmatrix}, \quad (1)$$

where $f_1(q)$ and $f_2(q)$ are functions of the scalar $q = |\vec{q}|$, given by [18]

$$f_1(q) = E \frac{cq}{2(1+\sigma)s}, \quad (2)$$

$$f_2(q) = E \frac{1}{2(1-\sigma^2)qs} \times \frac{(3-4\sigma)\sigma sc^2 - (1-\sigma)cqh + (1-2\sigma)^2 s + s(qh)^2}{(3-4\sigma)sc + qh}, \quad (3)$$

where E and σ are Young's modulus and Poisson's ratio, and for convenience, we define $c = \cosh qh$ and $s = \sinh qh$. In the form presented here, it is clear that one needs to evaluate only one term in M , e.g., the M_{11} element; the term independent of α yields $f_1(q)$ and the term proportional to α^2 yields $f_2(q)$.

Equation (1) is the solution to the inverse problem of recovering tractions from a displacement field in a form where the explicit vectorial nature of the solution is transparent. It follows that the forward problem must have the same general form since the displacements, being vectors, are homogeneous linear functions of the tractions. Thus, we have

$$\vec{u} = M^{-1} \vec{T}, \quad M^{-1} = g_1(q) \begin{bmatrix} 1 & 0 \\ 0 & 1 \end{bmatrix} + g_2(q) \begin{bmatrix} \alpha^2 & \alpha\beta \\ \alpha\beta & \beta^2 \end{bmatrix}, \quad (4)$$

where $g_1 = 1/f_1$ and $g_2 = -f_2/f_1(f_1 + q^2 f_2)$.

The formulas for an incompressible substrate are found by setting $\sigma=1/2$, for which $g_1 = 3s/Ecq$ and $g_2 = -3[sc^2 - cq h + 2s(qh)^2]/2Ecq^3[c^2 + (qh)^2]$. There is no singularity in this limit; unlike finite element methods, incompressibility poses no difficulty in this implementation.

For long wavelengths ($qh \rightarrow 0$) we get the solution for simple shear: $\widehat{u} = (h/\mu) \widehat{T}$ in Fourier space or $\vec{u} = (h/\mu) \vec{T}$ in real space, where $\mu = E/2(1+\sigma)$ is the shear modulus. In contrast, for short wavelengths ($qh \rightarrow \infty$), we recover the Boussinesq solution,

$$\widehat{T} = E \frac{q}{2(1+\sigma)} \begin{bmatrix} 1 & 0 \\ 0 & 1 \end{bmatrix} \widehat{u} + E \frac{\sigma}{2(1-\sigma^2)q} \begin{bmatrix} \alpha^2 & \alpha\beta \\ \alpha\beta & \beta^2 \end{bmatrix} \widehat{u}. \quad (5)$$

This solution is the inverse of the classical Boussinesq forward solution, which gives displacements as functions of tractions. It is equivalent to the matrix inversion of the Fourier-transformed forward solution given in Eq. (3) of Butler *et al.* [10] (As others have noted [19], there is a typographical error in that work; the off diagonal elements in Eq. (3) of Butler *et al.* [10] should have negative signs.)

III. METHODS

A. Finite element calculations

We calculate the displacement field resulting from an isolated point force using an in-house three-dimensional finite element program, where the model substrate is discretized with eight noded hexahedral elements with smaller elements close to the point force. Boundary conditions are zero displacement of the bottom surface, the top surface is free of tractions except at a node where a unit force F_x is applied, and lateral surfaces far from the source have zero normal displacements. We have confirmed that the displacement field is largely independent of mesh density and unaffected by proximity of lateral boundaries. Numerical values of displacements are affected in a very small region around the force due to its singular nature, but the regime where displacements decay as $1/r$ is faithfully captured.

B. Polyacrylamide gel substrate of uniform thickness

The compliant gel substrate was made by placing a mixture of acrylamide and bisacrylamide on a piece of pre-treated glass with hydrophobic surface (SurfasilTM, Siliconizing Fluid, Thermo Scientific, Waltham, MA). We chose concentrations that yielded gels with shear moduli of 400 Pa (5.5% acrylamide and 0.05% bis-acrylamide, both from Bio-Rad Laboratories, Hercules, CA) and 600 Pa (5.5% of acrylamide and 0.076% bis-acrylamide) [7]. The polyacrylamide prepolymer mixture was then covered with a square cover slip functionalized with 0.4% bind-silane (γ -methacryloxypropyltrimethoxysilane; Acros Organic, Belgium) that allows for covalent binding between polyacrylamide gel and glass. The thickness of the polyacrylamide gel was controlled by adding a known volume of acrylamide and bis-acrylamide mixture with small fluorescent beads (200 nm; Invitrogen, Eugene, OR) and measured by fluorescent microscopy equipped with a z-directional micrometer. This was done by focusing the microscope from the glass surface up to the gel surface and measuring the travel of the objective lens. The prepolymer was placed in a nitrogen chamber for 20 min to polymerize. After the acrylamide mixture polymerized, the

polymer was removed from the bottom glass. The polymerized gel was then functionalized for cell attachment with sulfosuccinimidyl-6-[4-azido-2-nitrophenylamino] hexanoate (Pierce, Rockford, IL) in 0.1 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Invitrogen, Eugene, OR) and exposed to UV light for 5 min. Following repeated washes with phosphate buffered saline (PBS), type I collagen (100 $\mu\text{g/ml}$, PureColTM; INAMED Biomaterials) was added over the gel surface. The gels were then incubated at 4 ° C overnight. On the following day, the gels were washed thoroughly with PBS and cell culture medium was added for 24 h.

C. Polyacrylamide gel substrate of variable thickness

To capture cell thickness effects in a single experimental preparation, we devised wedge-shaped gels [Fig. 3(b)]. The thickness of the wedge-shaped gel varied over the gel length, spanning a range from significantly less than the lateral dimensions of the cell (the “thin” region) to thicknesses much greater than the cell lateral dimensions (the “thick” region). These gels were prepared in a manner similar to that described above but with one important modification. During gel polymerization, a piece of the functionalized cover slip was laid over the prepolymer with one edge resting on a 1-mm-thick glass [Fig. 3(b)].

D. Cell culture

HFL fibroblasts (passage: 6–12; ATCC, Manassas, VA) were maintained in Dulbecco’s modified Eagle medium (DMEM) (Cellgro, Mediatech, Inc., Manassas, VA) with 10% FBS (fetal bovine serum) and incubated at 37 ° C in 5% CO₂.

E. Measurements of cell spreading area

On the day of experiment, the cells were plated on the collagen-coated polyacrylamide gel with seeding density 25 cells/mm². The cells were then fixed in 4% formaldehyde (Sigma-Aldrich, St. Louis, MO) after 3.5 h. Total gel area covered by the cells was measured from phase contrast images. The cell number was obtained by counting the nuclei stained with 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (Sigma-Aldrich, St. Louis, MO). The cellular F-actin was stained using rhodamine phalloidin (Sigma-Aldrich, St. Louis, MO).

F. Measurements of the cell elastic shear modulus

The shear modulus of HFL fibroblasts was measured using optical magnetic twisting cytometry (OMTC) [20]. OMTC is a microrheometer in which a predefined specific torque is applied to cells using 4 μm in diameter magnetic beads. The beads were coated with a peptide containing Arg-Gly-Asp (RGD) ligands, so that the beads preferentially attached to the cells via integrin receptors and in this manner, probe cytoskeletal stiffness [20,21].

HFL fibroblast cells were plated on collagen-coated gels at a seeding density of 30 cells/mm² in DMEM solution for 20 h. Next the cells were incubated with RGD coated beads (1.7×10^5 beads/well) for 20 min. The wells were then washed twice to remove any unbound beads. The well was placed on the microscope stage in a custom designed chamber [20]. Using magnetizing coils affixed within this chamber, the microbeads were magnetized and then twisted with a known oscillatory magnetic torque. The displacements of the beads were recorded by a charge-coupled device camera. The ratio of specific torque amplitude to displacement amplitude was used to compute elastic modulus of the cell.

G. Measurements of cell traction forces

We measured cell traction forces as described previously [10,18,22]. HFL fibroblast cells were plated sparsely (2 cells/mm²) on collagen-coated polyacrylamide gel (607 Pa), with

200 nm embedded fluorescent beads. A phase contrast image and an image of fluorescent beads immediately underneath the cells were taken 4 h after plating. The cells were then detached from the gels (trypsin: 2.5%, Cellgro, Mediatech, Inc., Manassas, VA) and a second image of those same fluorescent beads was obtained. The displacement map was obtained by cross correlating the pair of fluorescent bead images. From the displacements, we computed tractions as described in Sec. II. From the traction field, we computed a single scalar measure of cell contractility called cell net contractile moment [10].

H. Statistical analysis

Statistical significance ($p < 0.05$) was assessed using unpaired t-test assuming unequal variance.

IV. RESULTS

A. Theory

We quantified the effect of finite gel thickness through consideration of an increase in apparent stiffness from the stiffness of gel itself, which we call the stiffness amplification factor, S . We defined S by the ratio of the actual traction obtained from Eq. (1) for a given displacement in cell-gel plane to the traction which was calculated using Eq. (5) for the same displacement. We denote the latter as the Boussinesq traction. We find a striking dependence of this relationship on gel thickness h [Fig. 1]. As the gel thickness decreases below the lateral spread of the tractions ($qh \ll 1$, or, equivalently, when $r \gg h$ in real space), the tractions required per unit displacement rise sharply compared to those required in a gel of infinite depth [Fig. 1]. Regardless of orientation, when qh is large (or, equivalently, when $r \ll h$ in real space), the solution approaches that for a semi-infinite substrate. When qh is small ($r \gg h$), however, we find a significant increase in the stiffness amplification factor. This stiffening effect is not at all small; for example, when qh is 0.1, a value which is well within the physiological range, the apparent or effective stiffness is greater than the gel modulus by about tenfold. Note that for displacements in the x direction, the increased stiffness displays a plateau type feature near the transition $qh = 1$ between simple shear $qh \ll 1$ and semi-infinite thickness $qh \gg 1$.

B. Numerical simulation

Stiffening effect of finite substrate is absent in the case of an isolated point force. For example, a substrate with thickness h if subjected to force F_x generates displacement field u_x that initially ($r \ll h$) decays along the direction of force at the rate as the fields on semi-infinite substrate ($u_x h E / F_x \propto x / h$) [Fig. 2]. Hence, an isolated point force has the same response on finite substrate and semi-infinite substrate until $r \ll h$. The displacements, however, decay progressively faster, $\Delta(u_x h E / F_x) / \Delta(x/h) \gg 1$, as $r \gg h$. If the isolated force is not singular but distributed over a finite region d , then it follows from Fourier analysis that the stiffening effect will be present if the substrate has $h > d$. This is in agreement with recent studies of critical thickness [12].

C. Experiments

To test the theoretical predictions described above, we first cultured cells on gels with various thicknesses. We found that when the gel thickness was less than 200 μm , cell spreading area increased sharply [solid line in Fig. 3(a)]. The same phenomenon was seen in the wedge-shaped gel [dotted line in Fig. 3(a)]. There were no significant differences between the projected areas of cells plated on substrates of uniform thickness and those plated on wedges in regions of comparable thickness [Fig. 3(a)]. Similarly, F-actin staining showed differences in cell spreading area in thin (50 μm) and thick gels (400 μm) [Figs. 3(c) and 3(d)].

To study the effect of gel thickness on the cell stiffness, we measured the elastic modulus of the cells using OMTC. Compared to the cells on glass substrate, the cells on gel substrate were significantly softer [Fig. 4]. When we compared cells across the wedge, we found that cells on the gels thinner than $200\ \mu\text{m}$ were significantly stiffer than the cells on the gels thicker than $300\ \mu\text{m}$. To study the effect of gel thickness on the cell contractility, we used traction force microscopy. Similarly, cellular contractility quantified by the strain energy [10] increased sharply with gel thicknesses less than $100\ \mu\text{m}$ [Fig. 5].

V. DISCUSSION

A. Discrepancies concerning mechanosensing and substrate thickness

Several groups [12–14] have investigated the role of substrate thickness, h , by defining a characteristic thickness, h_0 , beyond which the biological outcomes are insensitive to h . However, the estimates of h_0 differ widely from a few microns [12,14,16,17] to as much as $60\ \mu\text{m}$ [13]. Maloney *et al.* [12] used distortion and displacement of adhesion site as parameters and proposed that h_0 is primarily related to the size of FAs, in which case substrate thicknesses matters only when h_0 is on the order of the FA size (a few microns). A similar idea was proposed by Sen *et al.* [14], who considered the average deformation of a substrate with Young's modulus of less than 8 kPa for different cell types. In contrast, Merkel *et al.* [13] considered the displacement field of a point force on a substrate with finite thickness and investigated how closely the displacement matches that of a semi-infinite substrate. For cardiac fibroblasts plated on substrates with Young's modulus of 16–30 kPa, they concluded that finite-thickness effects become relevant below $60\ \mu\text{m}$. These different findings result from diverse operational definitions of “mechanosensing” and make comparisons difficult. Furthermore, experiments done with relatively stiff substrates [13,14] might have resulted in negligible changes in response to changes in substrate thickness.

B. Extent of cell sensing its environment depends on lateral dimension of cell

From the theory described above, when substrate thickness, h , is much larger than the lateral spread of tractions (i.e., $qh \gg 1$ in Fourier space), the displacement field approaches the Boussinesq solution. In contrast, when h is smaller than the lateral spread of the tractions, the corresponding displacement field is quantitatively smaller, which from the displacement point of view is equivalent to that of a stiffer substrate. In our experiments, we measured the specific outcomes of the cells on gels with different thicknesses. We found that the cells plated on the thinner substrate have larger projected area, higher elastic modulus, and exert larger traction forces than the ones plated on thicker substrate. What we also found was that the length scale of lateral dimension of cells on thick substrates was around $100\ \mu\text{m}$ (Fig. 3). As gel thickness increases beyond this length scale, the mean projected cell area and traction forces exerted by the cell decreased to a plateau. These findings imply that cellular mechanisms exist to sense substrate stiffness, which reside not just locally in the vicinity of focal adhesions [23] but rather across the entire cell body. However, the actual mechanism by which cells transduce the ratio of forces exerted to cellular deformation remains an open question.

C. No thickness effect on local mechanosensing around focal adhesions

Displacements secondary to pointlike tractions, e.g., associated with localized focal adhesions, behave differently in the near and far fields. The transition between these two regimes occurs near $r = h$, where r is the lateral distance from the source. In particular, the in-plane displacements, u , near a pointlike source fall off like $1/r$ (Fig. 2). In contrast, far from the source, u falls off much faster, and the asymptotic behavior of $u(r)$, $r \rightarrow \infty$ remains an open question. It follows that mechanosensing of displacements near a focal adhesion is

unlikely to be affected by substrate thickness unless it is very thin, consistent with the arguments of Maloney *et al.*[12].

D. Experimental limitations

Despite the fact that the use of chemically inert polyacrylamide gels enables the decoupling of mechanical and biochemical changes in extracellular matrix [3], there exist several potential confounding factors. In particular, both autocrine and paracrine signals may be affected by substrate thickness through changes in local concentrations of secreted factors within the gel. Isolating these effects remains open, but we minimized possible chemical signaling as well as mechanical sensing from neighboring cells [24] by using sparse cell densities to ensure cell-cell distances larger than at least one cell diameter.

E. Technical advantages of using wedge substrates

We compared results in cells plated on a wedge-shaped gel with those plated on gels of uniform but different thicknesses. We found no significant difference between the projected areas of the cells on flat gels and to corresponding thickness regions on the wedge-shaped gels. We conclude that the wedge-shaped substrate behaves as a surrogate for a panel of uniform thickness gels. This leads to a practical advantage of the wedge-shaped substrate: experiments can be performed on single preparations with the wedge, spanning a large range of thicknesses, without the need for multiple well and multiple thickness substrate preparations.

F. Implications for cell monolayers

While some studies show that adhesion, communication, and scattering of cell groups are dependent on substrate stiffness [24–27], others [7,18] show that cells are insensitive to substrate stiffness once they form cell-cell contracts. The findings reported here may reconcile this apparent discrepancy. In the former group of studies, cells are cultured as small islands on substrates with a substrate thickness on the order of the island's size. Therefore, the apparent stiffness might closely match the intrinsic gel stiffness. In the latter group, cells are cultured as large colonies (several millimeters in size) on substrates of $\sim 100\ \mu\text{m}$ in thickness, and the apparent stiffness may be much greater than the intrinsic stiffness since the cells would be collectively responding to the essentially infinite stiffness of the bottom glass.

VI. CONCLUDING REMARKS

In summary, we found the following: (1) the human fetal lung fibroblast cell senses a rigid boundary even at a distance and adjusts its stiffness, contractile forces, and spreading area in response and (2) this sensing becomes negligible when substrate thickness is larger than a characteristic thickness given by the lateral dimension of the cell. We conclude that both intrinsic stiffness and thickness of the substrate are independent contributors to the cell's microenvironment. The cell senses its microenvironment at different length scales: local forces at a focal adhesion can sense the intrinsic stiffness of the substrate, whereas tractions spread over the lateral dimensions of the cell can sense depths. It follows that cells can mechanosense both intrinsic microenvironmental stiffness and substrate thickness independently; this may contribute to the wide variety of phenotypic responses that cells have at their disposal.

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FIG. 1.

Effect of substrate thickness, h , on the apparent stiffness of the gel; q is the magnitude of the Fourier wave number (effectively the inverse of lateral cell dimension). The stiffness amplification factor, S , is the ratio of tractions obtained from a finite-thickness formulation [Eq. (1)] to that obtained from the Boussinesq solution [Eq. (5)]. The black line depicts stiffness amplification calculated from the traction components in the same orientation as the displacement wave vector (e.g., the x component of tractions when the displacements vary sinusoidally in the x direction). The gray line depicts stiffness amplification for tractions orthogonal to the displacement wave vector (e.g., the x component of tractions when the displacements vary sinusoidally in the y direction). The dotted lines are the asymptotic behaviors for thin substrates; these correspond to simple shear between two parallel plates.

FIG. 2.

Decay of displacements, u_x , away from the point force, F_x , along the x axis. Close to the point force ($x/h \ll 1$) the displacements on a finite-thickness substrate decay with the same rate as the displacements on a semi-infinite substrate (line without markers). Beyond $x/h = 1$, these displacements decay significantly faster. The nondimensionalized displacement $u_x h E / F_x$ is solely a function of x/h , with E as the intrinsic stiffness of gel.

FIG. 3.

Cell spreading of HFL fibroblast cells on different thicknesses of polyacrylamide gel. (a) Average cell area as a function of substrate thickness, with cells plated either on wells of uniform thickness or on a wedge. Error bars are standard deviations on more than 400 cells. (b) Schematic diagram of wedge-shaped gel making protocol. (c) F-actin staining of HFL cells on thin region (50 μm) of wedge and (d) F-actin staining of HFL cells on thick region (400 μm) of wedge (scale bar= 100 μm).

FIG. 4.

Elastic modulus, g' (mean $\pm$ SE), measured at 0.75 Hz of HFL fibroblast cells on different thicknesses of polyacrylamide gel. Zero thickness corresponds to measurements done with cells plated on glass. Error bars are standard deviations; $n = 65-164$ beads attached on a similar number of cells.

FIG. 5.

(a) Net contractile moment and (b) net strain energy of cells plated on gels with different thicknesses. Computations done by unconstrained FTTC. Note the sharp increase in contractility and strain energy of the cells on 50 μm thickness gel. Error bars are standard errors, $n = 5 - 10$ cells.