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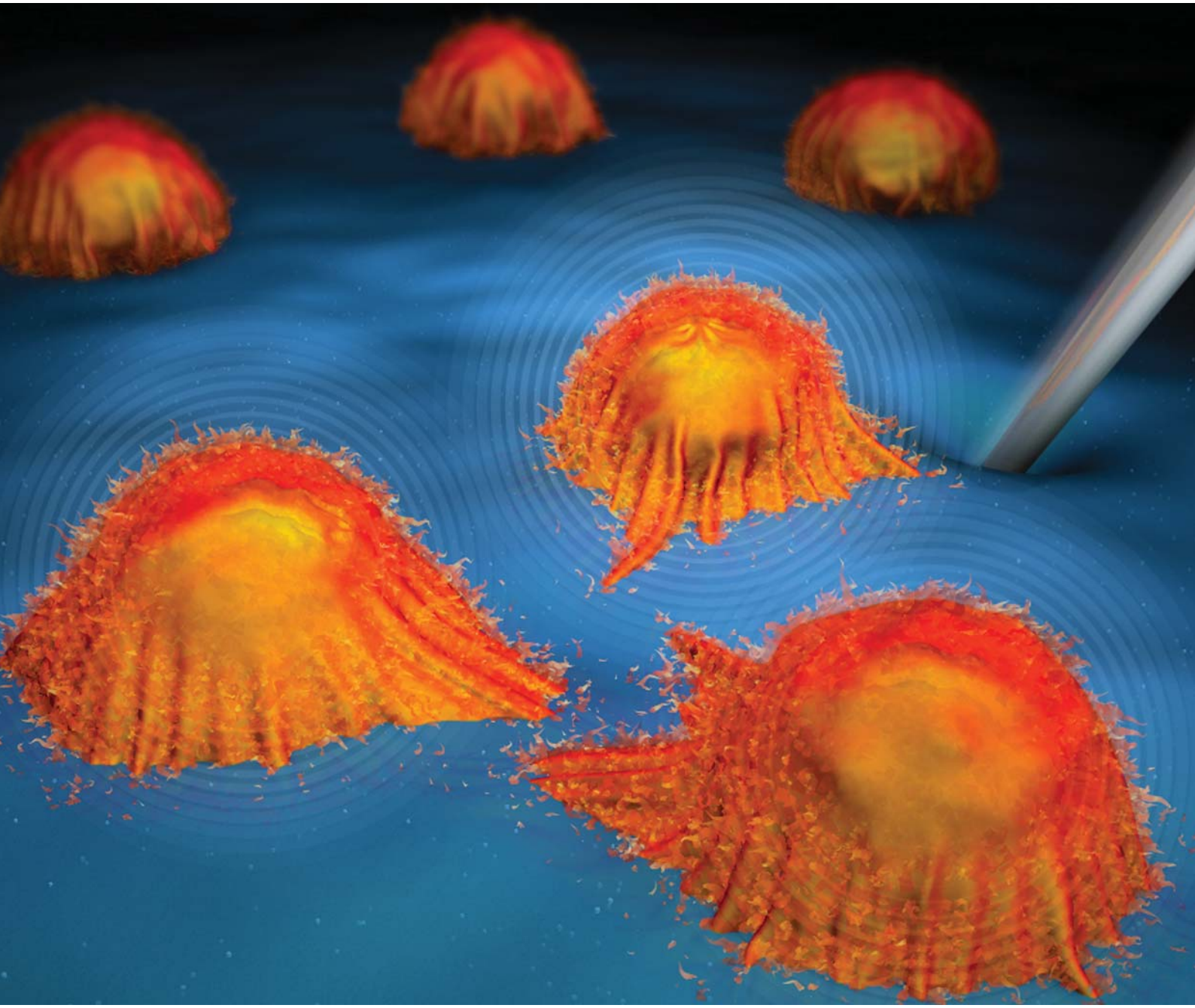

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PAPER

How far cardiac cells can see each other mechanically†

Xin Tang,[ad] Piyush Bajaj,[bd] Rashid Bashir[bcd] and Taher A. Saif*[ad]

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We ask the question whether cardiac cells, separated by a soft solid medium, can interact with one another mechanically, and if so, then how does the interaction depend on cell–cell separation, and the stiffness of the medium. First, we show that cardiac cells can be stimulated by a mechanical signal alone. We culture primary chicken embryonic cardiomyocytes on a 2D soft substrate. A mechanical probe is used to apply local cyclic stretch on the substrate near quiescent cells (cells not beating). Within 10 cycles of stretch, the cells begin to beat, and continue to do so for hours after the stimulation, while control non-beating cells remain quiescent. Next, we show that a beating cardiac cell (instead of the probe) can stimulate a neighbor. Our 2D culture of cardiomyocytes on a deformable soft substrate (0.5–1 kPa) shows that closer the cells are as a pair, higher is the probability of both of them beating over longer time. This probability is much higher than that of both cells beating as a pair when they are cultured on a harder substrate (47 kPa), or the probability of a single cell beating on the same soft substrate (0.5–1 kPa). The cell–cell interaction, namely, the stretch induced by one contractile cell onto another is modeled using the principles of continuum mechanics. The close correspondence between the predicted cell–cell interaction and the experimental observations suggests that cardiac cells are mechanically coupled through the deformable substrate, and that the coupling decreases with increasing distance between them and the substrate stiffness. The quantitative analysis and the experimental results provide a biophysical basis for the understanding of cell–cell interaction in cardiac tissue through the deformation field of the *in vivo* soft microenvironment.

Introduction

Mechanical microenvironment and mechanical forces, both intracellular and extracellular, influence a wide range of cell functionality, including cell locomotion,[1,2] growth,[3,4] proliferation,[5,6] apoptosis,[7,8] and differentiation.[7,9,10] Cells show dramatic sensitivity to the substrate stiffness in 2D cultures.[11] For example, naive mesenchymal stem cells (NMSCs) differentiate to neurons, muscle cells and osteoblasts when cultured on 1, 20 and 40 kPa polyacrylamide gel substrates, respectively.[9] Cells "feel" the stiffness of the environment by generating force on the substrate and by deforming it.[12] In turn, intracellular forces develop which cause conformational changes in stretchable force sensing molecules, resulting in gene expression and signaling cascades.[13–15] Thus stiffness information is transduced to cell functionality, such as, increased cell stiffness, higher intracellular forces, and stress fiber organization with the higher substrate stiffness.[6,11,16,17]

The effect of substrate stiffness on cardiac cells bears particular physiological significance. Myocardial infarction results in scar tissues and stiffening of the microenvironment.[18–20] The effects of such stiffening on cardiac cells have been explored in[21] using 2D cell culture *in vitro*. It was found that cardiac cells beat on a soft substrate for much longer times compared to the cells on a hard substrate. However, cardiac cells on hard substrates bridged by fibroblasts can beat for longer times than those without fibroblasts connection,[22] possibly due to softer microenvironment rendered by the fibroblasts. Fibroblasts of cardiac origin are also known to be capable of synchronizing electrical activity of multicellular cardiac tissue over extended distances (~600 μm).[23] The time scale of such communication is within minutes.[24,25] Such communication might be due to mechanical force coupling between the cardiac cells through the fibroblasts, besides the coupled bio-chemical pathways.

Mechanical stimulations, *e.g.* the stretch or shear loading, result in the excitatory electrophysiological response in cardiac cells, known as mechano-electric feedback (MEF).[26–29] For example, mechanical stretch is known to cause trans-membrane cationic currents, altered action potential,[30,31] and activation of

[a]*Department of Mechanical Science and Engineering, College of Engineering, University of Illinois at Urbana-Champaign, Urbana, Illinois, USA 61801. E-mail: saif@illinois.edu*
[b]*Department of Bioengineering, College of Engineering, University of Illinois at Urbana-Champaign, Urbana, Illinois, USA 61801*
[c]*Department of Electrical and Computer Engineering, College of Engineering, University of Illinois at Urbana-Champaign, Urbana, Illinois, USA 61801*
[d]*Micro and Nanotechnology Laboratory, College of Engineering, University of Illinois at Urbana-Champaign, Urbana, Illinois, USA 61801*
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Cl^- channels.[32] Shear fluid pulses on a monolayer of neonatal rat ventricular cells, induced by localized fluid jets, evoke action potentials that propagate across the monolayer, and reentrant arrhythmia.[33] Although to date the mechanism of MEF remains elusive, mounting evidence indicates that the mechanosensitive ion channels (MSCs), mostly Cl^- or K^+ selective, are mediators of MEF,[27,28,34–36] MSCs are found in a wide variety of cardiac cells, including chick,[37–39] rat,[35,40] frog,[41] guinea pig[42,43] and human.[44–46]

Stretch activation of cardiac cells raises the possibility that a beating cell on a soft substrate may induce stretch on its distant neighbor by deforming the flexible substrate. The neighbors thus can "see" each other mechanically through the substrate. In this paper, we explore this possibility, and ask two questions: (1) can a quiescent cardiac cell (cell not beating) on a soft substrate be actuated (set to beating) by a local mechanical stretch of the substrate by an inert probe. The probe is physically distant from the cell so that it represents the stretch induced by another cardiac cell. The inert probe also serves the purpose of delivering solely the mechanical signal, without any bio-chemical cues, to the quiescent cardiac cells. We find, indeed, the probe can cause the quiescent cell to beat in contrast to the control cell that remains quiescent; and (2) how far two neighboring cardiac cells can mechanically influence each other on a soft substrate. We seek this question by culturing cardiac cells on a soft substrate without any fibroblast, and by noting the beating patterns of pairs of cells. We find, closer the cells are, longer they beat as a pair, *i.e.*, the cells keep each other awoke (beating) for a longer time. With increasing stiffness of the substrate, this mechanical interaction decreases. This distance dependence or the range of "seeing each other" can be well predicted by the theory of linear elasticity and finite element analysis (FEM). The study suggests that *in vivo* cardiac cells could be mechanically coupled to each other in addition to their electrical and chemical coupling, and that the cells interact with each other through the deformation field of the *in vivo* soft tissue. The softness of the tissue not only determines their functionality but also their range of interaction, both of which might be essential for the normal functioning of the heart.

Results

Local substrate stretching can excite the quiescent cardiomyocytes

Primary chicken cardiomyocytes, extracted from embryonic chicken hearts, were cultured on inert soft 2D polyacrylamide (PA) substrates (stiffness $E = 1.05 \pm 0.17$ kPa, calibrated by Atomic Force Microscopy; see Materials and methods). The density of cells plated on 2D substrates was strategically controlled as 50 000 to 100 000 cells per cm^2, counted by the standard hemocytometer. This range of cell density results in the spatial separation of the individual plated cells to be 0–200 μm from one another. Note that the primary cell samples, extracted from chicken hearts, consist of a mix of both cardiomyocytes and cells from the connecting tissues, such as vascular smooth muscle cells, fibroblasts, and endothelial cells, *etc.* Since most connecting tissue cells cannot grow on a very soft substrate, *e.g.* at 1 kPa, due to the lack of cell traction necessary for cell spreading, these cells undergo apoptosis,[47] resulting in a physical separation between the cardiac myocytes. Unlike the fibroblasts, the cardiac myocytes survive on mechanical soft substrates with high viability. During culture, some of the cardiac myocytes beat, while others occasionally beat or keep quiescent for most of the culture time. A cell was determined as quiescent if it was found not beating by continuous video observation for 4 minutes prior to any mechanical stimulation. Based on our experience, the cardiac cells which do not beat for these 4 minutes remain quiescent, and do not spontaneously start beating (at least within the duration of video observation, often for an hour).

In order to apply stretch on a quiescent single cardiomyocyte by deforming the substrate, a rigid tungsten probe was mounted on a piezo stage with x–y–z actuators (Fig. 1). It contacted the gel surface and exerted a small compressive force on the gel near a cardiac cell. Fig. 1a shows such a probe at 45 μm away from a quiescent cardiac cell. Driven by the actuator, the probe cyclically pulled the soft substrate in a pulsatile fashion with the varied frequency, from 20 to 45 cycles per minute (ESI, Fig. S2†). As the soft and flexible substrate was cyclically pulled, the cell adhered to the substrate near the probe was also cyclically stretched. In Fig. 1b, the cell stretch was about 6%. This simple but effective method exerted mechanical force onto the cardiac cell while avoiding the direct contact between the rigid probe and the cells. Also, the tungsten probe was not bio-chemically functionalized. Thus, only mechanical signal was propagated towards the cell through the substrate. Throughout the experiment, the beating activities of the cardiomyocytes were monitored by an inverted phase-contrast microscope and time-lapse video recording.

The probe was slightly tilted with the vertical (Fig. 1a). Thus, as it was moved away from the cell, the angle of probe increased due to its compliance. Consequently, the contact force between the probe and the substrate decreased, and the probe may slide on the substrate. As the probe was moved towards the cell, the contact force increased which prevented the sliding. In order to monitor the actual mechanical stimulation on the cell, and for later analysis (next section), we monitored the position of a marker on the soft culture substrate (point A in Fig. 1b). The marker moved when the probe deformed the substrate without sliding. During sliding, the probe continued to move leaving the marker stationary. Thus the mechanical stimulation on the cell can be quantified from the motion of the marker. The relation between the motion of the probe without sliding and that of point A was obtained as follows: the probe was initially placed at about 45 μm away from a cardiac cell. It was then moved towards the cell. The corresponding motion of A along the direction of motion of the probe (*i.e.*, a component of its motion) was monitored. The probe was then moved back to the original configuration when A returned to its origin. The motions of the probe and that of A show a linear dependence (ESI, Fig. S1†). The linearity is due to the linear elasticity of the gel.[48–50] The continuity of the line is due to no slippage between the probe and the substrate. The slope of the line is about 0.31–0.34, which indicates that the probe displacement was three times that of the marker A. This relation will be used in the analysis later.

Fig. 2a shows the displacement of marker A and the corresponding activation scenario of the cell as a function of time. Note that the amplitude of A decreases with time due to the

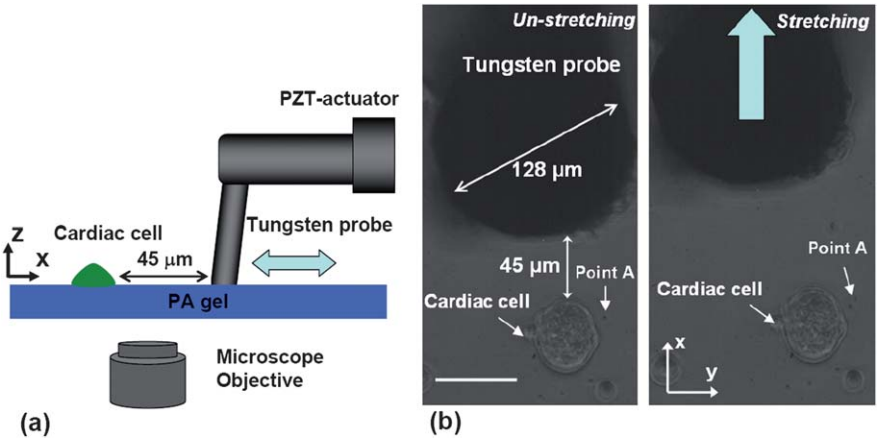

Fig. 1 Awaking a quiescent cardiac cell by a distant probe. The probe stretches the cell by deforming the soft PA substrate. (a) Schematic of a probe at 45 μm away from a quiescent cardiac cell on a soft substrate. The microscope objective is underneath the Petri dish containing the soft substrate and cells to monitor the entire cell excitation process. (b) Phase-contrast images of a chicken cardiomyocyte adhered to a 1 kPa gel substrate functionalized by laminin ECM. The images also show the probe tip in contact with the substrate. The probe applies a small compressive force on the substrate and is then moved horizontally away and towards the cell. This induces a deformation of the substrate. The cell thus gets stretched and compressed in a pulsatile fashion. The probe may slide during motion. The marker, A, on the surface of the substrate moves when the probe is not sliding. Thus the motion of A represents the actual deformation of the substrate. Scale bar: 50 μm.

increasing sliding between the probe and the substrate. The maximum displacement of A is about 4.5 μm at the seventh stretching cycle when the cell was stretched by 6.52% (Fig. 2b and c). After seven cycles of such stretches (about 20 seconds), the quiescent cell started to beat, and its self-contraction and stretching were much larger compared to those imposed by the probe (Fig. 2c). The cell continued to beat with the beating period of 15–25 seconds for hours after the mechanical stimulation is stopped. This period is much longer than the period of beating during stimulation. Note that the cardiac cell was initially quiescent, at least for 4 minutes prior to the stimulation. The control experiments were also performed: the quiescent cardiac cells, chosen based on the same rules and cultured under the same condition, were continuously video recorded for 8 minutes in the absence of mechanical probe stretching. We found, they remained quiescent throughout entire 8 minute observation. Thus the beating of the stretched cell was due to mechanical stimulation of the probe, and was not a spontaneous activity of the cell.

Out of the fifteen quiescent cells that were stimulated by the probe, 8 were found to beat. The quiescent-to-beating activation time was cell-specific but all were within tens of seconds. The

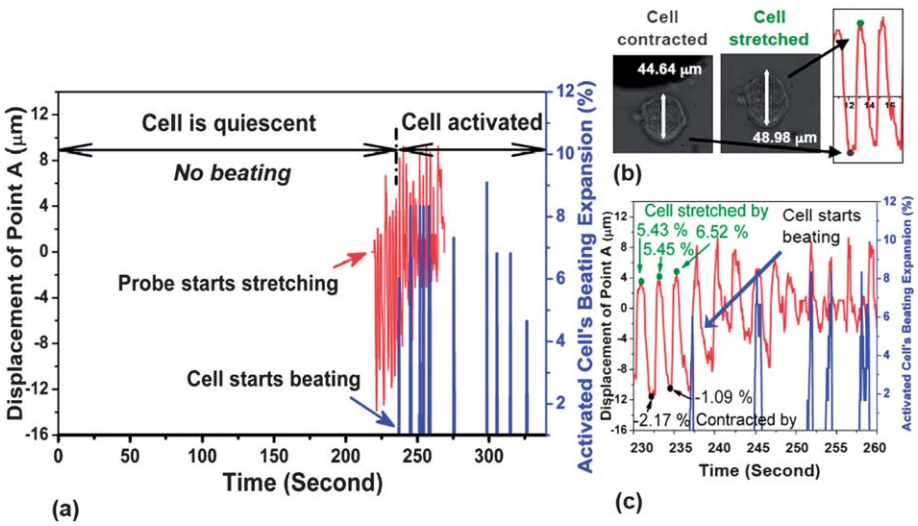

Fig. 2 (a) Displacement of point A on the substrate in response to the motion of the probe as a function of time. This displacement represents the deformation of the substrate. Its amplitude varies with time due to the sliding between the probe and the substrate. The blue line shows the contraction–expansion strain along the vertical diameter (indicated by the white double-head arrow in (b)) of the cell after it begins to beat. Before the stimulation by the probe, the cells were confirmed as quiescent (no beating) based on 3–4 minutes of observation (see Results section). (b) Cell shape at the maximum and minimum substrate stretches. (c) Strains in the cell due to deformation of the substrate before it begins to beat. The representative maximum stretching and contraction of the soft substrate are labeled.

average stretch applied by the probe on the cells was 5.89 ± 0.62% of their original size. After activation, the period of beating of the cardiac cells was also cell-specific, varying within 15–55 seconds (ESI, Fig. S2†). In all cases, the post-stimulation beating period was much longer than the period during stimulation. These results indicate that chicken cardiomyocytes are sensitive to mechanical stimulation and that they can commit to contraction–relaxation cycles by mechanical cue alone.

In the excitation experiments, the cell culture dishes were removed from the 37 °C incubator to the microscope stage at room temperature of 25 °C. In order to minimize a drop in temperature of the medium, the dish was placed on a hot plate at temperature 37 °C on the microscope stage. Each stimulation experiment takes less than 4–7 minutes. To ascertain whether the change of beating frequency during and after the application of stimulation was not biased by the drop in the culture medium temperature, we continuously monitored the frequency of naturally beating cardiomyocytes with time of exposure to room temperature as the control. It is found that within 7 minutes, the beating frequency dropped by 11.1 ± 9.8%. This drop due to the temperature change is 5–6 folds less than the drop in frequency due to the removal of mechanical stimulation. This suggests that the frequency drop after the removal of mechanical stimulation was not due to decrease in temperature.

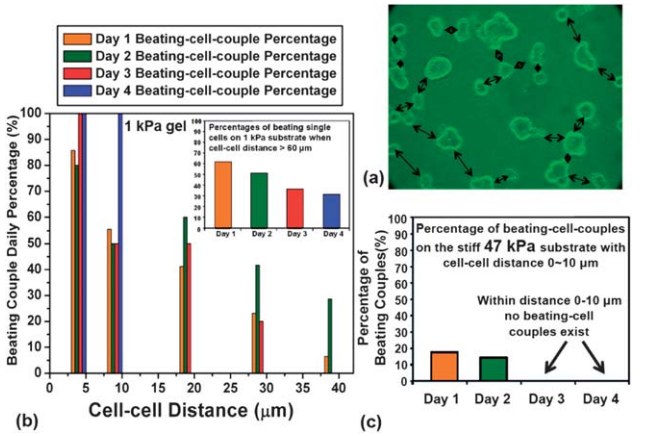

Fig. 3 (a) Determination of cell pairs by measuring the distance between the nearest neighbors. No fibroblasts were present to connect the cardiac cells. (b) On 1 kPa gel, cell pairs are grouped by distances between neighbors, such as a group consists of pairs with partners 0–5 μm, 5–10 μm, 10–20 μm apart, *etc.* $n = 246$. Percentages of pairs within each group with both cells beating are plotted for day 1–4. Higher percentages of pairs with close-by cells beat for longer times, possibly by stimulating each other. (Inset) Percentages of single cells beating *vs.* time. These cells have no partners within 60 μm. (c) On 47 kPa gel, percentage of beating pairs (both cells of the pair beating) within mutual distance 0–10 μm is shown on day 1, 2, 3 and 4, respectively. $n = 52$.

Neighboring cardiac cells stimulate each other mechanically

In the previous experiment, we showed that an inert probe can excite a quiescent cardiac cell to beat by applying mechanical stimulation through the substrate. This suggests the possibility that a beating cell might also stimulate a neighbor by stretching and releasing the soft substrate. Such stimulation may 'awake' a quiescent neighbor, which in turn may stimulate the neighbor that awoke it in the first place. Thus the beating of the pair may become coupled, and they may continue to beat for a longer time compared to a cell too far from any neighbor. The coupled beating will depend on the distance between the neighbors—farther the neighbors are, less is the mutual stimulation. Here, we will explore the spatial distance between two cells within which they might stimulate each other through the soft mechanical substrate.

To examine whether the mutual stimulation between two close-by cells is due to mechanical interaction alone, in contrast to the possibility that chemical factors released by close-by cardiac cells may also play a signal-transmitting role in communication, we studied the cell-beating patterns on 2 different substrates with low (1 kPa) and high (47 kPa) stiffness. The exact stiffness of substrates were calibrated and confirmed by AFM (see ESI, Fig. S4†). If the mutual stimulation is primarily chemical, then the degree of stimulation will depend on the distance between the cells alone, irrespective of the substrate stiffness. If the stimulation is primarily mechanical through a deformable substrate, then stiffer the substrate, lesser is the interaction for a given distance between the cells, and the cells in a pair may not beat together for long time.

We performed video imaging of cardiac cells plated on a same soft substrate (1 kPa) at 246 different locations on day 1–4 of plating (Fig. 3a). For each cell in the view-field, we identified the nearest neighbors, and grouped the cells as pairs with the neighbors at distances 0–5 μm, 5–10 μm, 10–20 μm, 20–30 μm, and 30–40 μm apart. Cells that did not have any cell neighbor within 60 μm were considered as single. We recorded the fraction of pairs within each given group (for example, pairs with 10–20 μm apart partners) with both cells beating (Fig. 3b). Similarly, the fractions of single cells that were beating during day 1–4 were determined (Fig. 3b, inset). Fig. 3b shows that the cells with their closet neighbors at *i.e.* 0–10 μm beated (together with the neighbor) for longer times, as hypothesized. For example, on day 4, 100% of the pairs with partners within 0–5 μm beated as couples. The fraction is 50% when partners are 5–10 μm apart. Single cells lack any mechanical stimulation from each other, and hence lower fraction of them beat over a long time. On day 1, 2, 3, and 4, the percentages of single beating cells are 62.5%, 50%, 34% and 18% respectively.

On the stiffer (47 kPa) substrate, the cells' beating patterns at 52 different locations were monitored. Since the stiff substrate is 47-fold more rigid than the soft one, the mechanical deformation of the substrate by cellular traction is negligible beyond 10 μm (verified by both theoretical prediction and finite element analysis; see details in the next section and Fig. 6), hence we only studied the beating pattern of cells within 0–10 μm apart. We found, the percentage of beating-cell-couples within 0–10 μm was 17% on day 1 and 14% on day 2, dramatically lower than those on 1 kPa gels. On day 3 and 4, there were no beating-couples within this effective distance range. The data suggest that the chemical factors, if any, released by cardiac cells at small mutual distance, are not sufficient to sustain the long lifetime of beating-couples. These results, together with the finding that an inert probe can stimulate a quiescent cell through the deformable substrate, imply that mechanical stimulation, either from a probe or from a neighboring cell, regulates the beating scenario of

cardiac cells. In order to interpret these mutual mechanical interactions between cells in light of the stimulation of quiescent cells by the probe, we carry out the following analysis.

Range of mechanical interaction on soft substrates

Consider a force, F, applied at a point on the surface (with elastic shear modulus G and Poisson ratio ν) of an elastic half-space. For the linear-elastic, homogeneous and isotropic material, the elastic modulus, $E = 2G(1 + \nu)$. The displacement, u, of a point on the surface of the substrate along the direction of the force at a distance x from the force is given by $u = \frac{F}{2G\pi x}$.[51] Similarly, a couple with two forces, F, acting at a distance Δ (Fig. 4) from one another produces a displacement u at a distance x from the dipole as,

$$u = \frac{F}{2G\pi}\left(\frac{1}{x} - \frac{1}{x+\Delta}\right) = \frac{F}{2G\pi}\frac{\Delta}{x(x+\Delta)} \quad (1)$$

Now, consider a segment of length Δ on the surface at a distance x from the dipole. Then the increase in length of the segment, $d\Delta$, due to the dipole is:

$$d\Delta = \frac{F}{2G\pi}\left(\frac{1}{x} - \frac{1}{x+\Delta}\right) = \frac{F}{2G\pi}\left(\frac{1}{(x+\Delta)} - \frac{1}{x+2\Delta}\right) = \frac{F}{G\pi}\frac{\Delta^2}{x(x+\Delta)(x+2\Delta)} \quad (2)$$

If Δ represents the size of a cell and F the dipole force it generates, then $d\Delta$ represents a mechanical stimulation by the cell on a substrate at a distance x away. The net stretching ratio, ε, of the substrate due to the cell is:

$$\varepsilon = \frac{d\Delta}{\Delta} = \frac{F}{G\pi}\frac{\Delta}{x(x+\Delta)(x+2\Delta)} \quad (3)$$

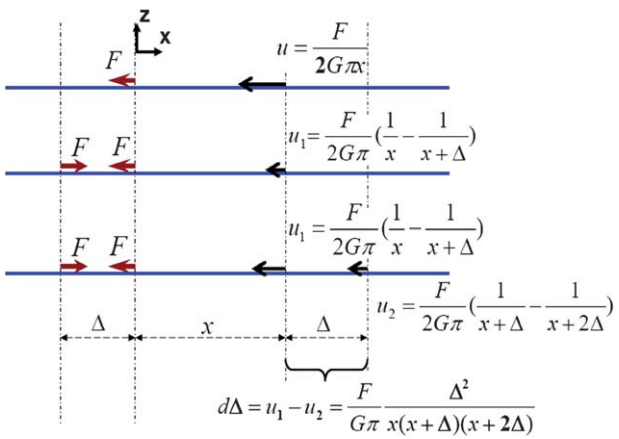

Fig. 4 Schematic of a force dipole model on an elastic half-space. The horizontal displacement of a point on the surface along the direction of the dipole is given by u. $d\Delta$ is the stretch of a segment of length Δ on the substrate due to force dipole. If a cell of length Δ is attached to the substrate, then it resists the stretch $d\Delta$. However, together with the substrate, it stretches by a lesser amount. Thus $d\Delta$ represents the stimulation on the cell by the substrate.

Thus, the stimulation decays rapidly as $\frac{\Delta}{x(x+\Delta)(x+2\Delta)}$. Here $d\Delta$ is the stretch of the free surface from the original length Δ. If a cell is attached to the surface at the same location, it will resist the substrate deformation. The cell will also get stretched depending on the relative stiffness between the cell and the substrate. In order to obtain the stretch of the cell, we need the stiffness of the cell. We estimate the stiffness of the cardiac cell from the numerical simulation of the experiment with the probe and the single cell as follows.

We model (ESI, Fig. S3†) the substrate as an isotropic elastic film with modulus of 1 kPa (measured by AFM, see Materials and methods in the ESI†) attached to a rigid substrate (glass). The film spans an area of 500 μm × 500 μm. It is 70 μm thick. The vertical sides of the film and the bottom surface are fixed, *i.e.*, restrained from motion. The cell is considered as a homogeneous, isotropic, linear elastic solid with a diameter of 40 μm and thickness of 4 μm, similar to the geometry of the cell in Fig. 1. The cell is attached to the substrate uniformly along the interface. The probe is modeled as a rigid cylinder with diameter 128 μm and is attached to the substrate (*i.e.*, no sliding possible). It is 45 μm away from the cell.

In the experiment with the probe and the cardiac cell, the maximum displacement of the probe without sliding was about 10.82 ± 1.21 μm. The displacement of marker A is 3.61 ± 0.41 μm (Fig. 1b). The corresponding stretch of the cell was found to be about 5.82% ± 0.65% of the initial size. In the simulated experiment, we apply 11 μm displacements on the probe. The corresponding stretch of the cell is obtained from finite element analysis. For an arbitrary elastic modulus of the cell material, the experimental and the simulated stretches of the cell do not match. Thus, we vary the cell material modulus until the experimental and the simulated stretches match. This modulus of the simulated cell is found to be $E_{\text{cardiac-cell}} = 3.5$ kPa and 3 kPa when the stretch is 5.61% and 5.92% respectively, both close to the experimental value of 5.82% ± 0.65% (Fig. 5a). This modulus range (3–3.5 kPa) is in excellent agreement with the experimental cardiac myocytes stiffness data reported earlier, *e.g.* 2.5–3.3 kPa[52] or as 2.2–5.4 kPa.[53] In the following, we use the cell elastic modulus as 3.5 kPa. Note that it is the stiffness of the cell along

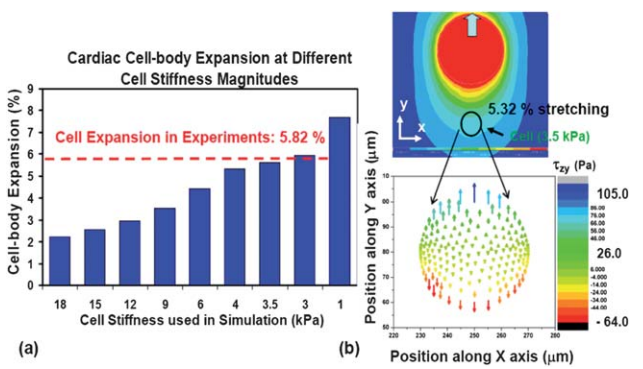

Fig. 5 Results of the cell stretch experiment simulated by the finite element analysis (see also Fig. 7). (a) Cell stretch due to probe actuation is computed for various elastic moduli of the cell material. The cell with modulus of 3–3.5 kPa results in a stretch that is comparable to the experimentally measured value of 5.8%. (b) The traction between the cell and the substrate is shown when then cell modulus is 3.5 kPa.

the substrate that prevents the free stretch of the substrate during probe displacement. This stiffness results from the combination of the cell thickness (4 μm) and the elastic modulus (3.5 kPa). For the simulated cell, finite element analysis provides the traction between the cell and the substrate. The maximum shear stress propagated to the cell due to probe stretch was 77.2 Pa, when the cell was stretched by 5.32% (Fig. 5b). The resulting cellular force dipole is 200 nN. We can now analyze cell–cell mechanical interaction on the same soft substrate.

Cell–cell mechanical interaction

Consider a cell C1 attached to the substrate. It beats with an amplitude and generates a force on the substrate. It has a neighboring cell, C2, at a distance x away, also attached to the substrate. We want to estimate the stretch in C2 due to contraction of C1 for various values of x. C1 induces the stretch in C2 by stretching the substrate. We model the cells as discs of size 40 μm diameter, 4 μm thick with isotropic elastic modulus of 3.5 kPa. Boundary conditions of the substrate are similar to those used in simulating the probe experiment. The contraction of C1 is simulated by applying a force dipole on the substrate as shown in Fig. 6a. The magnitude of the force dipole is 200 nN (derived from the analysis of Fig. 5b), acting at 40 μm apart. This magnitude is not critical for the conclusions to be drawn in the following, since the stretch of C2 will be normalized by its stretch at $x = 5$ μm. The stretch of C2 due to the dipole is obtained from finite element analysis for various values of x. Fig. 6b shows the strain in C2, namely, stretch in C2 divided by its original size (40 μm) along the dipole as a function of x. Clearly, the effect of C1 on C2 decays with x rapidly. We best fit the cell strain with the basis function $\frac{\Delta}{x(x+\Delta)(x+2\Delta)}$ obtained from eqn (3), with Δ as a fitting parameter. We find $\Delta = 148$ μm for the best fit curve shown in Fig. 6b. Note that the large value of $\Delta = 148$ μm compared to the cell size of 40 μm essentially reflects the large elastic modulus of the cell (3.5 kPa) with respect to the substrate (1 kPa). Fig. 6c shows the histograms of Fig. 3b, which gives the probability of finding a pair of beating cells with members at a given distance away. Superimposed on the histogram is the curve of Fig. 6b, but the cell strains are normalized by the strain at 5 μm away from the force dipole. The close correspondence between the predicted cell–cell mechanical interaction (finite element analysis or the analytical prediction) and the histogram suggests that the close-by cells are more probable to stimulate each other mechanically. The farther apart the cells, the less the probability of their mutual stimulation. Thus the close-by cells are more likely to beat for longer time as a pair. This analysis, however, does not account for the time evolution of mutual stimulation. There must be some time required for a cell to be stimulated by a neighboring cell. Initially (soon after plating), the cells are likely to beat randomly without the effect of a neighbor, and the distance between them does yet not play a role. As time progresses, they become mechanically coupled through the soft substrate.

The strong distance-dependence of mechanical interaction between cells suggests that if a cell has two neighbors, one closer than the other (not all three necessarily on the same line), then the nearby one will have significantly higher influence than the farther one. Such is the case with the group of cells in Fig. 7 at day 3 of culture on a 1 kPa substrate. The nearby pair (cell C1 and C2), about 42 μm apart, beats in synchrony with frequency of 64 cycles per min, although their beating is phase lagged by about half a period, *i.e.*, when one of them contracts, the other relaxes. The cell, C3, which is farther apart, about 65 μm away from C1 and 89 μm away from C2, beats with a frequency of 70 cycles per min. They all contract by about 5% during their own individual beating. However, when C2 contracts during beating,

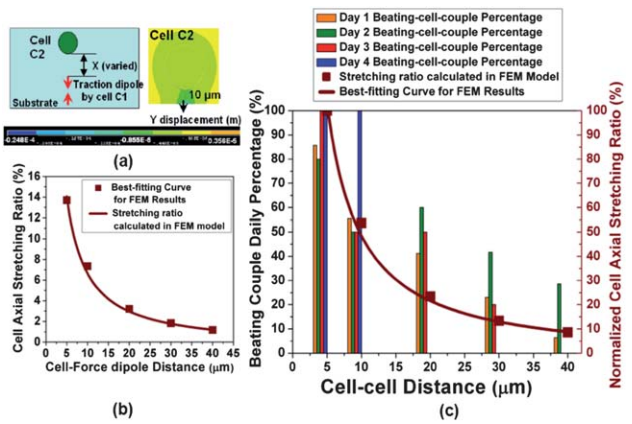

Fig. 6 How far the cells can see each other? (a) We simulate the cell–cell interaction by applying a force dipole (200 nN at a distance of 45 μm) on the substrate that mimics the contraction of a cardiac cell during beating. The corresponding stretch on a neighboring cell attached to the substrate is analyzed using finite element method. (b) The stretch is represented as a percentage of the original size as a function of the distance between the cells. The solid line is the best fit curve with the basis function $1/[x(x + \Delta)(x + 2\Delta)]$ from eqn (3). (c) Histogram of Fig. 5 is presented, together with the prediction from (b) normalized by the cell stretch ratio at 5 μm. This prediction accounts for mechanical interaction between two cells only (*i.e.*, stretch induced on one cell by the other).

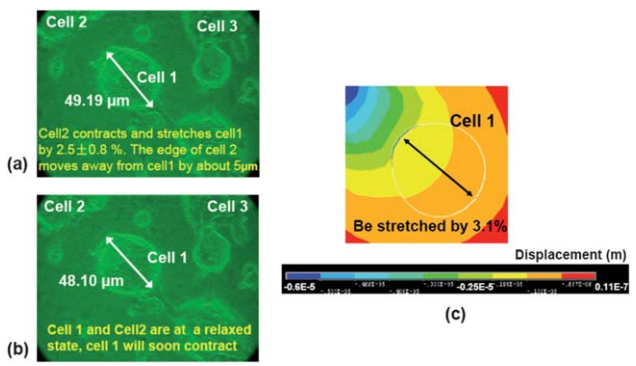

Fig. 7 Mechanical interaction between beating cardiac cells. (a) A group of beating cardiac cells on a soft (1 kPa) substrate. The nearby pair (cell 1 and cell 2), about 42 μm apart, beats with same frequency (64 cycles per min), although their beating is phase lagged by about half a period. The cell 3, which is farther apart, about 65 μm away from cell 1 and 89 μm away from cell 2, beats with a frequency of 70 cycles per min. Cell 2 is in contractile mode, it stretched cell 1 by about 2%; (b) Both cell 1 and 2 are at relaxed states. (c) Simulation of interaction between the cells using finite element analysis. A displacement of 5 μm is imposed on the boundary of cell 2, mimicking the experimental observation. Corresponding stretch in cell 1 is found to be 3.1%, close to the experimental stretch. The effect of contraction of cell 3 on cell 1 and 2 is negligible.

it causes 2.5 ± 0.8% strain in C1, while it displaces its edge (closest to cell 1) by about 5 μm (along a direction away from C1). C3 induces no noticeable stretch in C1 (ESI, Movie S1†). We observed this effect of proximity for several 3-cell systems. To test whether this mutual stretch is mechanical, we carry out the FEM analysis of the above 3-cell system to estimate the effect of one on the others. We simulate the contraction of the cell C2 by applying a displacement of 5 μm along the edge of the simulated cell 2. We find that the corresponding induced stretch in cell C1 is 3.1%, close to the experimental value of 2.5 ± 0.8%. The study suggests that the threshold induced strain needed for mechanical coupling might be less than 2.5%, *i.e.*, if one cell can induce 2.5% strain on another, then they may become mechanically coupled. They may keep each other 'awake', and they synchronize their beating. Similarly, when the cardiac cells are connected by connecting tissues over long distances, even 150 μm away, they become mechanically coupled, and they can synchronize their beating (ESI, Movie S2†).

Discussions and conclusions

It is recognized that the cardiomyocytes in various animals are capable of performing mechano-electric feedback (MEF).[27–29,54] Namely, the mechanical stimulus applied on cardiomyocytes results in the excitatory response.[30–33] The present study shows that beating cardiac cell can stretch its neighbors by deforming a soft medium in between. Thus they can stimulate each other mechanically through the substrate, possibly by invoking the MEF. The experiment with the inert tungsten probe that awakes a quiescent cell to beat by deforming the substrate clearly shows that such stimulation is mechanical. The underlying mechanism of mechanical stimulation through the substrate is likely to be the activation of stretch sensitive ion channels due to stretch, or equally likely, the activation of cell signaling pathways triggered by cells' stress sensor due to the mechanical deformation. Thus the coupling between the cells is both mechanical and biochemical. The latter follows the former. The exciting results are the stimulation can be mediated by mechanical deformation alone over astonishingly large distances.

The answer to the question of how far a cell can see its neighbors mechanically lies in the magnitude of its stretch induced by the neighbors. For a given type of cardiac cell, this distance depends on the stiffness of the medium that bridges the cells. For the case of cells on a 2D substrate, the strain of a cell due to the force dipole of a neighbor is well approximated by a function of the form $\frac{F}{G\pi}\frac{\Delta}{x(x+\Delta)(x+2\Delta)}$, where F is the force of the dipole, G is the shear modulus of the substrate, and Δ is a constant. Thus, as the elastic modulus of the substrate increases, the effect of a neighbor at a given distance away decreases. If there is a threshold strain, ε_{th}, needed for the cell to be stimulated, then

$$\varepsilon_{th} = \frac{F}{G\pi}\frac{\Delta}{x(x+\Delta)(x+2\Delta)} \quad (4)$$

gives the relation between G and x for the cells to be mechanically coupled. ESI, Fig. S5† shows the relation qualitatively for a given F and ε_{th}. Thus, for a pair of cardiac cell and a given substrate stiffness, there is a spacing, x_{th}, between the cells that is just enough for the cells to be mechanically coupled. If the distance is higher, $x > x_{th}$, the cells may become mechanically decoupled, and they may beat incoherently, or stop beating. Similarly, for a given x_{th}, if the substrate stiffness increases, the cells may not "see" each other anymore, and they cannot keep each other awake. This is exactly the case for cell pairs with partners 0–10 μm apart on 1 kPa and 47 kPa gel substrates (Fig. 3). On a stiff (47 kPa) substrate, the pairs beat with both partners for only 2 days, whereas on a soft (1 kPa) substrate most of such pairs beat (both cells of the pairs beating) after 4 days. However, on the same soft substrate, when the cells are far apart from one another (60 μm or higher), only 30% of the cells beat after 4 days. A recent experimental study shows that cultured quail embryonic cardiomyocytes (without fibroblasts) on soft substrates maintain beating for days, whereas those on a hard substrate that mimic post-infarct fibrotic scar tissue stop beating in two days.[21] Here, cell–cell mechanical interaction might have played a role in long term beating on the soft substrate. Thus, equal-distant cells on a soft substrate show much stronger interaction than those on a hard substrate, which, together with the finding that quiescent cells can be stimulated to beat by inert mechanical probes, suggest that cell–cell communication is more mechanical than chemical. Hence, beating of cardiac cells can be prolonged by mutual stimulation by neighbors due to mechanical coupling between them through the deformable substrate. As the substrate stiffness increases, the mutual coupling decreases, and the duration of mutual beating may decrease. Such might be the case after myocardial infarction when myocardium becomes mechanically stiffer due to the fibrotic rigidification.[18–20] Following infarction, the myocardial tissues gradually stop beating. In human heart failure, ventricular fibrillation often results in the damaged cardiac region with larger than micrometre scale. Such long cell–cell separation distance can diminish any mechanical stretching signal produced by beating cardiac cells.

In conclusion, our experiments and computational simulations suggest that cardiomyocytes can interact with one another remotely through the deformation of the soft substrate. The interaction originates from the mechanical stretch induced by one cell on the other. The deformable soft media around the cells transfer the deformation. Mechano electric effect in cardiac cells then possibly allows the stretch induced cell to be stimulated, which in turn interacts with the neighbor that stimulated it in the first place. Thus the cells become mechanically coupled. Closer the cells are, higher is the mechanical coupling. These cells keep each other beating for a long time. The range of interaction depends on the stiffness of the medium between the cells. For a given distance between the cells, stiffer the medium, less is the interaction which may result in the lack of synchrony and coordination between the cells. Such a mechanically decoupled cell may cease beating. These findings have implications in the understanding of myocardial infarction when cardiac tissues become stiff due to fibrotic scar formation.

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