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Modulation of the contractility of micropatterned myocardial cells with nanoscale forces using atomic force microscopy

Neerajha Nagarajan[1], Varun Vyas[2], Bryan D Huey[2,3], and Pinar Zorlutuna[1,4]

Abstract

The ability to modulate cardiomyocyte contractility is important for bioengineering applications ranging from heart disease treatments to biorobotics. In this study, we examined the changes in contraction frequency of neonatal rat cardiomyocytes upon single-cell-level nanoscale mechanical stimulation using atomic force microscopy. To measure the response of same density of cells, they were micropatterned into micropatches of fixed geometry. To examine the effect of the substrate stiffness on the behavior of cells, they were cultured on a stiffer and a softer surface, glass and poly (dimethylsiloxane), respectively. Upon periodic cyclic stimulation of 300 nN at 5 Hz, a significant reduction in the rate of synchronous contraction of the cell patches on poly(dimethylsiloxane) substrates was observed with respect to their spontaneous beat rate, while the cell patches on glass substrates maintained or increased their contraction rate after the stimulation. On the other hand, single cells mostly maintained their contraction rate and could only withstand a lower magnitude of forces compared to micropatterned cell patches. This study reveals that the contraction behavior of cardiomyocytes can be modulated mechanically through cyclic nanomechanical stimulation, and the degree and mode of this modulation depend on the cell connectivity and substrate mechanical properties.

Keywords

Cardiomyocytes, atomic force microscopy, mechanical stimulation, micropatterning, cardiovascular diseases

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Introduction

Earlier studies on cardiomyocyte contractility mostly focused on electrophysiological characterization of the membrane potential of the cells for understanding the action potential generation and variation in electrical signal propagation upon electrical or biochemical stimulus.[1] However, cardiomyocytes show excitatory responses to stimulation solely by mechanical forces through their stretch-activated ion channels and can fire action potentials upon mechanical stimulation through a pathway known as mechanoelectric feedback (MEF).[2] As such, changes in the mechanical microenvironment of a cell can affect the generation of electric potentials in the heart.[3] For example,

[1] Bioengineering Graduate Program, University of Notre Dame, Notre Dame, IN, USA
[2] Institute of Materials Science, University of Connecticut, Storrs, CT, USA
[3] Department of Materials Science and Engineering, University of Connecticut, Storrs, CT, USA
[4] Department of Aerospace and Mechanical Engineering, University of Notre Dame, Notre Dame, IN, USA

Corresponding Author:
Pinar Zorlutuna, Department of Aerospace and Mechanical Engineering and Bioengineering Graduate Program, University of Notre Dame, Notre Dame, IN 46556, USA.
Email: Pinar.Zorlutuna1@nd.edu

myofibroblasts secrete higher amounts of extracellular matrix (ECM) after heart tissue damage such as myocardial infarction, which results in the formation of stiff scar tissues within the heart muscle.[4,5] Since the mechanical characteristics of this newly formed tissue are different from the healthy muscle tissue, the contraction force and membrane potential conduction velocity are affected. Impairment in MEF can also result in other heart conditions such as cardiac arrhythmia, myocardial fibrillation, and heart failure.[6–8] Therefore, understanding the spatial and temporal variation in mechanical properties of cardiomyocytes in response to mechanical stimuli is important for developing novel cardiac therapies as well as for creating physiologically realistic disease models.[6,9] The knowledge gained would also help developing novel bioengineered machines such as cardiac cell powered biorobots.[10–12] These devices are driven by the spontaneous contractions of the seeded muscle cells, and their functionality can be controlled by tuning the substrate properties such as stiffness or introducing topographical features to control cell alignment.[13]

In addition to electrophysiological studies, there have been studies examining the changes in mechanics of cardiomyocyte contractility, such as the contractile forces generated by these cells and their contraction rate, upon electrical and chemical stimulation.[14,15] More recently, the contractile response of cardiomyocytes to mechanical stimulation has started to be investigated. The effect of the magnitude, duration, and the focal point (point of stimulation) of the mechanical stimulation has been shown to be important parameters that play a role in determining the cell response to such stimuli.[16–18] However, most of these studies were conducted either on single cells that are stimulated at the single-cell level[19,20] or on groups of cells (or tissue segments) that are stimulated at the macroscale (e.g. all cells stimulated at once by stretching the substrate on which they are cultured on).[21] Therefore, it remains a question how networks of connected cells or multicellular tissues respond to single-cell-level mechanical stimulation, initiated from one of the cells within the network. In addition, most of the in vitro studies investigating contractile response of the cardiomyocytes to mechanical stimulation stretch the cells to mimic the tensile forces experienced in vivo. Although myocardial cells predominantly exert tensile forces on each other during rhythmic contractions, there are studies showing that the compression of these cells can also trigger MEF.[22] This phenomenon is commonly observed during cardiopulmonary resuscitation (CPR). However, the effect of compressive mechanical stimulation has not been investigated much. Here, we present a novel approach implementing atomic force microscopy (AFM) for single-cell-level stimulation of defined cardiomyocyte populations confined within, to examine the changes in the synchronous beating of the cell network through localized applications of nanoscale compressive forces.

AFM is one very precise way of generating such single-cell-level and nanoscale mechanical stimulation. Over the last decade, AFM has been increasingly used for biological applications, especially for high-resolution imaging and investigating mechanical characteristics of cells.[23,24] A unique advantage of AFM is its high force sensitivity, spatial resolution, and operational capability in a liquid environment, which makes it ideal for studying biological samples.[9,23,25] With the aid of AFM, studies have established the variation in mechanical properties of the cardiomyocytes such as changes in cell stiffness with age,[19] cardiac remodeling in response to mechanical stretch,[26,27] and influence of substrate mechanics on the differentiation of cardiomyocytes from pluripotent stem cells.[28]

In this study, we examined the effect of single-cell-level cyclic mechanical stimulation in vitro via AFM on the beating frequency of a defined-sized myocardial cell network. Toward this end, we used a colloidal probe of 10 μm diameter, which is the average size of a neonatal cardiomyocyte, to mimic a neighboring cardiomyocyte in the cell network.[29,30] A colloidal probe was chosen in order to avoid disruption of the cells during indentation. The probe exerted localized cyclic nanoscale mechanical stimulation on primary neonatal cardiomyocytes, either cultured as micropatterned cell patches (300 by 600 μm) or as sparsely distributed single cells, each cultured on stiff (i.e. glass, Young's modulus >10 GPa) or compliant (i.e. poly(dimethylsiloxane) (PDMS), Young's modulus approximately 300 kPa[31]) substrates. Although the size of the AFM colloidal probe used for the mechanical stimulation of the cells is in the micrometer range, the force applied to the cells during the mechanical stimulation is at the nanoscale, in the range of 100–900 nN, hence imitating nanoscale-cell-level forces. Our results show that upon single-cell-level stimulation of patches of synchronously beating cardiomyocytes, the synchronous beating frequency of the cell network on the PDMS substrates reduces significantly in comparison to their spontaneous contraction rate prior to nanomechanical stimulation, while the beating frequency remains steady or increases for the ones cultured on glass substrates. The degree of the modulation depends on the substrate stiffness and the cell density. Finally, quiescent nonbeating single cells on the unpatterned samples were initiated to express rhythmic contractions upon mechanical stimulation, essentially achieving "nano-CPR."

Materials

Four-inch silicon wafers were purchased from University Wafers (Boston, Massachussets, USA), and the photoresist, SU8-2100, and propylene glycol mono-methyl ether acetate (PEGMEA) were obtained from MicroChem Corp (Newton, Massachussets, USA). PDMS elastomer was purchased as a kit (base and curing agent) from Dow Corning (Midland, Michigan, USA). Fibronectin (FN) from bovine plasma, Pluronic-F127, tyrode's salt solution with sodium

bicarbonate, penicillin-G sodium salt, triton® X-100, goat serum, 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) powder, and Dulbecco's phosphate buffer were purchased from Sigma-Aldrich (St Louis, Missouri, USA). Trypsin and Hanks' balanced salt solution (HBSS) without calcium or magnesium were obtained from Gibco (Grand Island, New York, USA). Dulbecco's modified Eagle's media (DMEM) with 4500 mg/L glucose, 4.0 mM L-glutamine, and 110 mg/L sodium pyruvate and fetal bovine serum (FBS) were obtained from Thermo Fisher Scientific (Kalamazoo, Michigan, USA). Alexa-488-tagged fibrinogen from human plasma, calcium fluo-4, acetoxymethy, cell permeant, proLong® gold antifade reagent, goat anti-rabbit IgG (H + L) secondary antibody, alexa fluor 594 conjugate, goat anti-mouse IgG (H + L) secondary antibody, and alexa fluor 488 conjugate were obtained from Molecular Probes (Waltham, Massachusetts, USA). Alexa fluor® 594 phalloidin and primary antibodies, rabbit monoclonal vimentin, rabbit monoclonal antibody connexin-43, and mouse monoclonal cardiac troponin-I were obtained from Abcam (Cambridge, Massachusetts, USA). Collagenase type-II was purchased from Worthington Biochemical Corporation (Lakewood, New Jersey, USA), and polyethylene glycol (PEG)-grafted poly-L-lysine (PLL) was obtained from surface solutions (Dübendorf, Switzerland). The 25-mm borosilicate glass coverslips were purchased from Chemglass Life Sciences (Vineland, New Jersey, USA), and paraformaldehyde, 16% solution EM grade, was obtained from Electron Microscopy Sciences (Hatfield, Pennsylvania, USA). Mylar masks were fabricated by Advanced Reproductions Corp. (North Andover, Massachusetts, USA).

Methods

Cell micropatterning

Cardiomyocyte cell patches of rectangular geometry (600 by 300 μm) were created using microcontact printing. Briefly, silicon wafers were coated with SU8-2100 photoresist, exposed to ultraviolet light through the designed transparency mask and developed using PEGMEA. PDMS base and curing agent were mixed in 10:1 ratio, poured over the patterned silicon wafers, degassed, and cured. FN was microcontact printed onto plain glass coverslips or PDMS-coated glass coverslips using these elastomeric stamps. Each stamp was coated with FN solution (50 μg/mL), dried using nitrogen gas, and gently brought into contact with the substrate. PDMS-coated glass substrates were prepared by spin coating PDMS (prepared as 10:1 mixture of the base to the curing agent) at 1000 r/min for 30 s to obtain a thickness of approximately 200 μm. In order to prevent nonspecific cell attachment, the unpatterned regions on the glass coverslips were coated with PLL-g-PEG (1 mg/mL) and the ones on the PDMS substrates were coated with 2% Pluronic F127 solution.

Cell isolation and culture

All experiments were conducted in accordance with the Institutional Animal Care and Use Committee of the University of Notre Dame. The cardiomyocyte isolation was carried out following previously established protocol. Briefly, the hearts were excised from 2-day-old neonatal Sprague Dawley rat pups, diced into small parts, incubated overnight in trypsin (0.05% w/v in HBSS) followed by 0.1% collagenase type-2 treatment and enriched for cardiomyocytes through 2-h pre-plating.[32] The isolated cardiomyocytes were seeded on to FN micropatterned glass and PDMS surfaces, and FN-coated plain glass coverslips, and maintained under standard cell culture conditions in DMEM supplemented with 10% FBS and 5% penicillin up to 5 days. Seeding density for the unpatterned plain glass samples was 0.5×10^5 cells/mL in order to obtain single cells and for the micropatterned cultures it was 2.5×10^6 cells/mL in order to obtain confluent myocardial cell networks.

Cell characterization

Phenotype of the isolated cells was characterized by double immunostaining as described previously.[33] Briefly, cell culture samples from day 1 and day 5 were fixed in 4% paraformaldehyde, permeabilized using triton X-100, and blocked with goat serum. Samples were immunostained sequentially using a cardiomyocyte marker (cardiac troponin-I) and a fibroblast marker (vimentin). Cell nuclei were counterstained with DAPI. For phenotypic characterization, the ratio of cardiomyocytes to cardiac fibroblasts was quantified in all samples, micropatterned, and nonpatterned single cell cardiomyocytes using ImageJ software. Fixed cells were also immunostained sequentially with cardiomyocyte marker (cardiac troponin-I) and gap junction maker (connexin-43). To understand the orientation of cytoskeletal filaments and quantify cell spreading area, fixed cells were stained for actin with alexa fluor 594 phalloidin and counterstained with DAPI. For quantification of cell spreading area, four images were captured from two different sample for each time point, and area of 20 different cells was marked and measured using ImageJ software. A histogram was then plotted to illustrate the distribution of cell spreading area (Online Supplementary Figure 2).

Cell functionality and connectivity of the cells were characterized by fast calcium flux imaging. Briefly, cardiomyocytes were loaded with fluo-4-AM ester (3 μM in 1% pluronic-F127 in Tyrode's salt solution) and incubated at 37°C for 30 min. Propagation of calcium flux was imaged with an Andor spin disk confocal microscope (Northern Ireland) at a capture rate of 31.4 fps.

Nanomechanical stimulation

Nanoscale mechanical stimulation was conducted using an Asylum Research MFP-3D AFM with integrated inverted

optics (Nikon TE-2000; Japan) and a liquid sample holder cell. In order to mimic a neighboring beating cell, a colloidal probe of 10 μm in diameter was used for indentation measurements and nanomechanical stimulation of the cardiomyocytes. The measurements were performed in Tyrode's solution using a temperature-controlled liquid chamber, implementing AC (standard or "i-drive") or contact mode for imaging and mechanical loading, respectively. All measurements were performed on the day 5 of the cell cultures and within an hour of placing the samples in the AFM system. In all measurements, the cells were spontaneously beating at a rate of 0.5–1 Hz before AFM measurements, except for the nano-CPR experiments where cells that are not initially beating were mechanically stimulated with the AFM probe to start contracting.

Before the start of the stimulations, the colloidal probe was held with a constant force, at a fixed point along the *Z*-axis in contact with the cell surface, which allowed quantitative recording of normal contractile displacements of the cell via the AFM feedback loop. This confirmed that the cell used to initiate the mechanical stimulation was viable and spontaneously contracting cardiomyocyte. The response of the cells to nanomechanical stimulation was quantified by counting the cell contraction and thereby the frequency, based on the bright-field video recordings. The AFM probe was positioned on individual cells that are identified as cardiomyocytes through simultaneous bright-field optical microscopy.

Micropatterned myocardial cell networks, as well as nonpatterned single cardiomyocyte cells, were mechanically stimulated using the colloidal probe through localized cyclic probing; the applied mechanical loads ranged from 100 nN to 900 nN. Single nonpatterned cardiomyocytes were stimulated at 100, 400, and 700 nN at 3 Hz frequency ($n = 10$). The micropatterned cell networks were stimulated with 300 nN at 5 Hz frequency ($n = 10$). The samples were recorded optically with light microscopy simultaneous to the AFM perturbations for 90 s (the 30 s of initial spontaneous beating, 30 s during cyclic mechanical stimulation by the AFM probe, and 30 s following the stimulation) and the beat rate was quantified. The variation in indentation depth of the cell membrane by the probe was quantified for a range of applied forces from 100 nN to 900 nN.

Statistical analysis of the measured data was carried out using the *t*-test for paired two sample means, in Microsoft Excel 2010.

Results

Cell micropatterning and characterization

In order to keep a constant cell density and obtain synchronously beating networks of cardiomyocytes for the nanomechanical stimulation studies, we confined the cardiac cells on fibronectin (FN) micropatterned substrates. Figure 1 shows the rectangular FN micropatterns (Figure 1(a)) and confluent attachment of cells within these patterns (Figure 1(c)). The cells remained confluent and confined within the pattern until the AFM experiments on day 5. FN adsorption on both substrates was comparable, as seen from the quantification of their fluorescence intensity for day 1 and day 5 using ImageJ software [1.48v, Wayne Rasband, NIH] (Figure 1(b)). This ensured that the composition of the substrate directly in contact with the cells was kept the same for both substrates, and the factor influencing cell's response was stiffness of the substrate.

After seeding the cells on micropatterns, we examined the cell phenotype in order to assess the functionality and phenotypic distribution of the isolated cardiac cells on day 1 and day 5. Heart wall tissue is heterogeneous; the isolated cell population consists of nonmyocytes (mostly cardiac fibroblasts), along with the cardiomyocytes, the ratio of which has been shown elsewhere to be important for contractility. As seen in Figure 1(d-e) and Online supplementary Figure 1, cardiac troponin-I exhibits bold healthy striations in the cardiomyocytes, while the fibroblast cytoskeleton is clearly visible as revealed by vimentin staining. Quantification of these immunostaining samples showed that cardiomyocytes constitute about 60% of the cultured cells on day 1 and about 57% on day 5, consistent with the literature (Figure 1(f)).[34]

Cells were stained for actin filaments with Alexa fluor 594-tagged phalloidin, to examine the cytoskeletal structure of cardiomyocytes seeded on a glass substrate (Figure 2(a), left) and PDMS substrate (Figure 2(a), right). Although the total actin concentration on both substrates is comparable (Figure 2(a), bottom panel), a difference in their morphology develops, especially by the fifth day in culture, as seen from the high magnification images (Figure 2(a), middle panel). While the cells seeded on the stiffer glass substrates exhibit a spread-out structure, with bold striations and a higher number of stress fibers, the cells seeded on the PDMS substrate possess bundled cytoskeletal filaments with no visible striations. The quantification of cell spreading area showed that the cells cultured on glass and PDMS substrates had comparable spreading area on the first day of the culture, $1540 \pm 526.3\ \mu m^2$ and $1400 \pm 608.2\ \mu m^2$, respectively. However, after 5 days in culture, the cells on glass substrate attained an average spread area of $6000 \pm 1590\ \mu m^2$, while the cells on the PDMS attained an average spread area of $2400 \pm 835\ \mu m^2$ (Online Supplementary Figure 2). In addition, the amount of connexin-43 gap junctions on cells cultured on PDMS was significantly lower than those on the glass, even after 5 days in culture (Figure 2(b), bottom panel). Gap junctions were clearly visible at the boundaries of the cells cultured on glass substrates on the fifth day in culture (Figure 2(b), left), while they do not appear to be well formed and not clearly visible in the cells cultured on PDMS substrates (Figure 2(b), right). Figure 2(b), top panel, shows the developed gap junction on micropatterned cells cultured on glass at higher magnification (left) and PDMS (right).

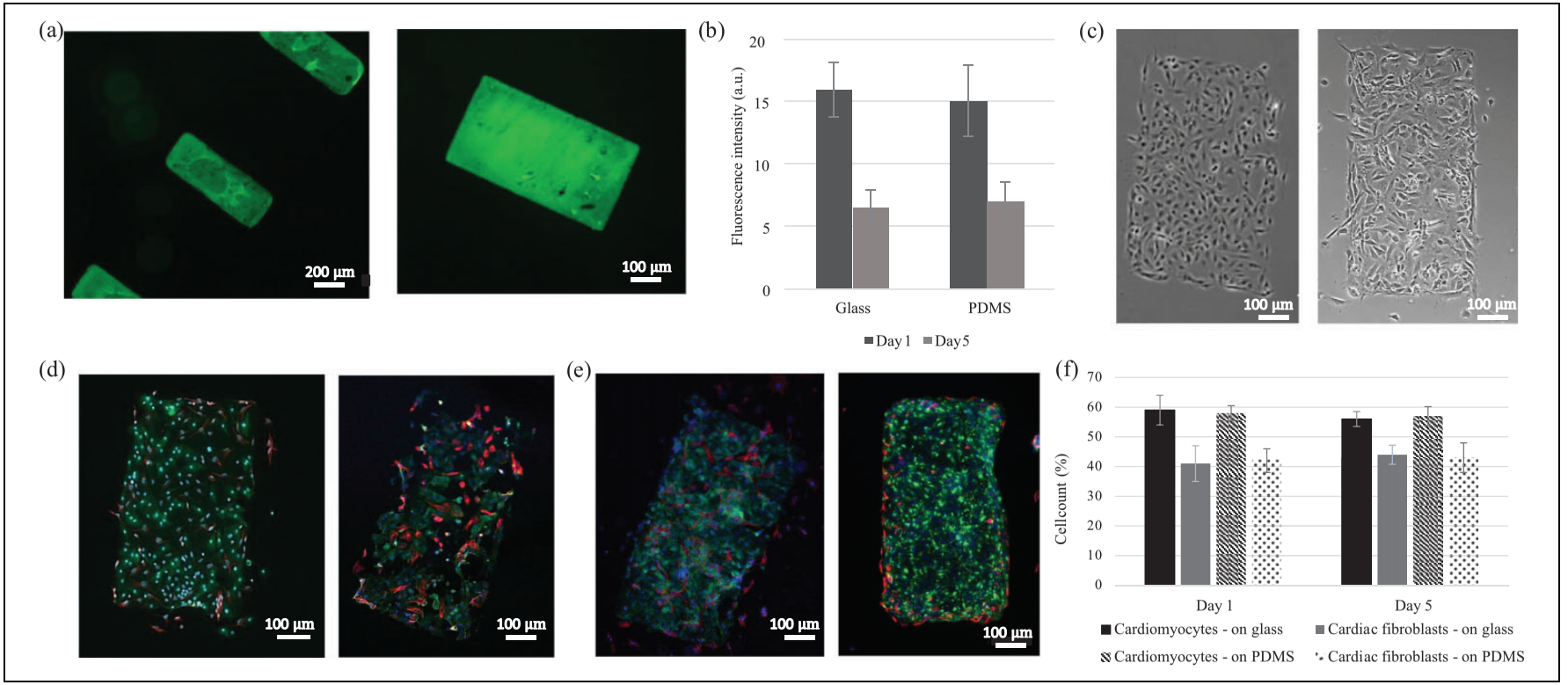

Figure 1. Fibronectin patterning and phenotypical characterization of the myocardial cells: (a) fluorescent image of fibronectin micropatterns; (b) quantification of fibronectin adsorption on glass and PDMS ($n = 6$; (e), right); (c) bright-field images of the myocardial cells on these patterns on day 1 (left) and on day 5 (right); bouble immunostaining of the myocardial cells for cardiac marker troponin-I (green) and fibroblast marker vimentin (red) for cells on glass (left) and PDMS (right), as on day 1 (d) and on day 5 (e). The cell nuclei are countered stained with DAPI (blue); (f) quantification of the distribution of the cell phenotype in single-cell culture and in the micropatterned cell patches ($n = 3$). PDMS: poly(dimethylsiloxane); DAPI: 4′,6-diamidino-2-phenylindole, dihydrochloride.

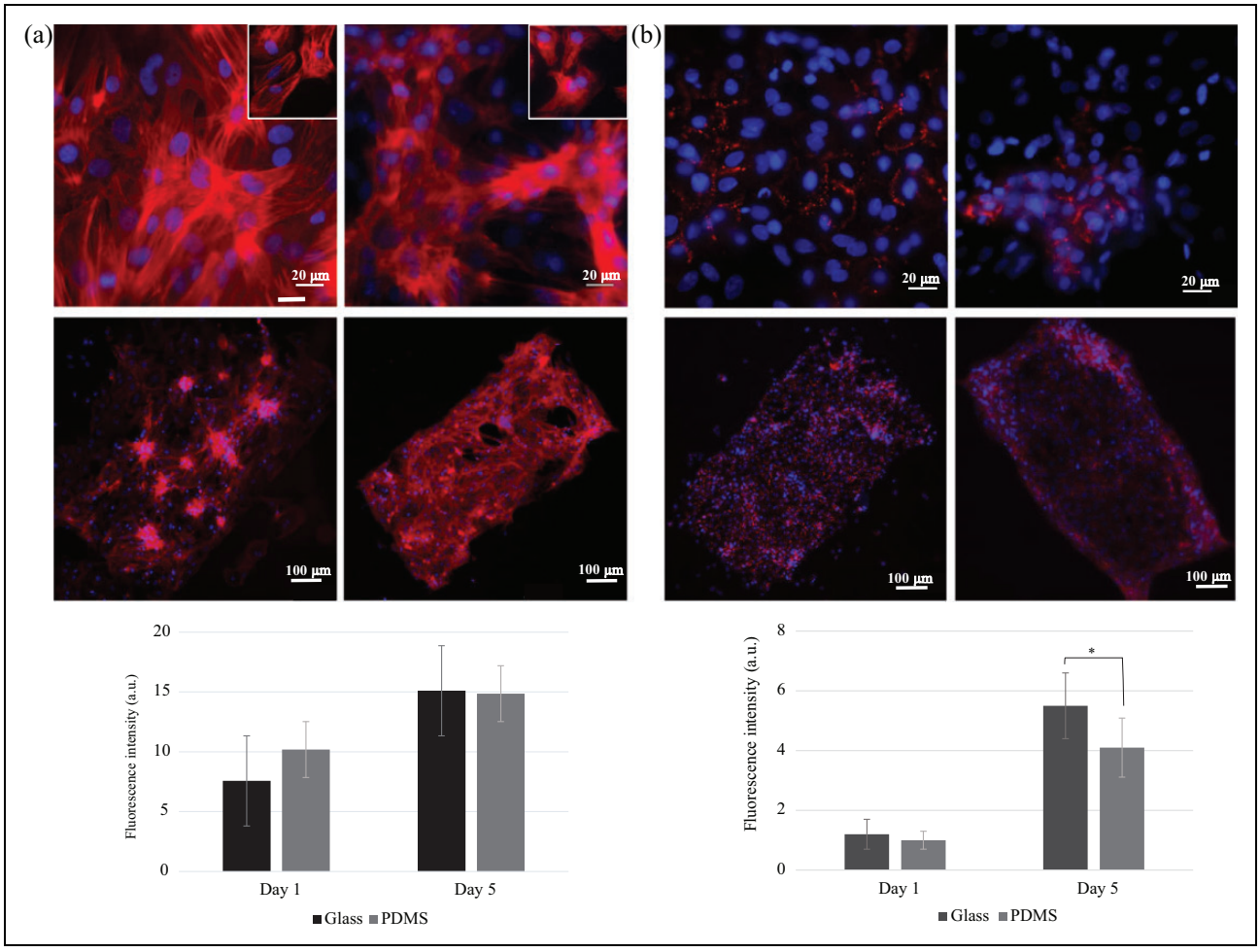

Figure 2. (a) Phalloidin staining of the actin filaments (red) and (b) double immunostaining of connexin-43 gap junctions (red) of the micropatterned cells cultured on glass (left) and on PDMS (right) on day 5, and their respective fluorescence intensity quantifications ($n = 3$), bottom panel. Cell nuclei are countered stained with DAPI (blue) for all samples. PDMS: poly(dimethylsiloxane); DAPI: 4′,6-diamidino-2-phenylindole, dihydrochloride.

Calcium flux analysis

To examine the spontaneous and synchronous contraction and the network connectivity of the cardiomyocytes in the micropatterned cell patches,[21,22] calcium ion conduction (or the calcium flux) was analyzed using a spinning disk confocal microscope (Figure 3; Online Supplementary Movies 1 and 2). The analysis showed that micropatterned cardiomyocyte networks exhibited synchronous propagation of calcium ions across the entire patch (Figure 3(b) and (c); Online Supplementary Movie 2), which confirmed the interconnectivity across the cells. On the other hand, although spontaneously beating with observable intercellular calcium waves (Figure 3(a); Online Supplementary Movie 1), single cardiomyocytes exhibited nonsynchronous calcium flux and contraction behavior.

Nanomechanical stimulation

In this study, AFM was used to stimulate cardiomyocytes that are cultured either as isolated single cells or as micropatterned cell networks, at single-cell level with nanoscale forces in order to quantify the responses of the perturbed as well as the surrounding cells to the localized mechanical loading via simultaneous bright-field optical imaging (Figures 4 and 5; Online Supplementary Movies 3 and 4). All samples exhibited spontaneous contractions of about 1 Hz before the AFM indentations, and cells remained healthy, continuing to beat spontaneously after mounting the sample in the AFM's liquid sample holder. According to representative alternating current (AC) amplitude mode AFM images of the cultured cells (Figure 4(a), right), the morphology appears as expected for healthy cells, exhibiting spreading onto the substrate extending more than 20 µm beyond the prominent cell body and cytoskeletal structures.

Figure 4(b) demonstrates the typical degree of cell membrane indentation as a function of the force applied by the colloidal probe. A contact force of 5 nN[35,36] is used to establish the origin of the displacement of the probe apex into the cell (vertical dotted line in the figure). The cell deformation ranged from 1.56 µm to 2.98 µm for applied

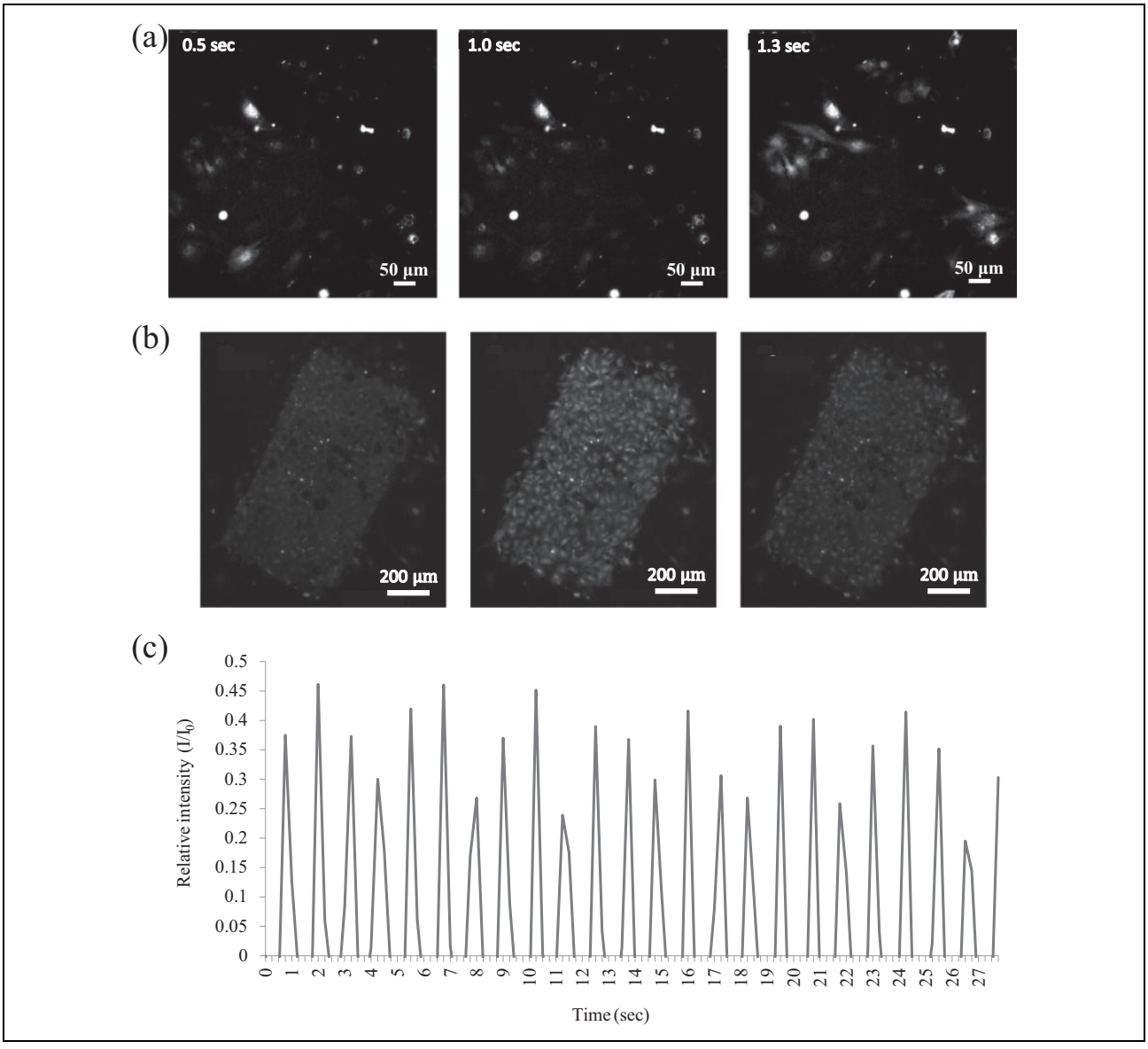

Figure 3. Analysis of the cell connectivity in micropatterned myocardial cell networks. Time lapse images of calcium flux in spontaneously beating single cells (a) and micropatterned cell patches (b). Calcium flux propagation over time for 30 s in the cell patches (c). Also refer to Online Supplementary Movies 1 and 2.

loads of 100–900 nN, respectively, which corresponds to periodic indentations of 25%–50% of the normally 5–7 µm thick cells cultured on glass or PDMS. While substantial indentations in the cell membrane can impair the cytoskeletal machinery of the cells for the range of forces applied in this study, the cell functionality did not change appreciably following periodic loading as evidenced by continued cell contractions, indentation profiles, and general cell morphology.

Examining the response of isolated cardiomyocytes to cyclic nanoscale stimulations, Figure 4(c) presents the normalized average contractile (''beat'') frequency observed over a 1.5-min period for 100, 400, and 700 nN peak loads applied at a rate of 3 Hz. These results are separated into three time periods of 30 s each (pre, during, and immediately after the periodic perturbations) and normalized to the beat frequency before perturbation (''pre-poke'') for comparison of the effect of different culture and substrate stiffness conditions. The isolated single cells either continued to maintain the same spontaneous beating frequency for the lower magnitude of applied force (100–300 nN) or occasionally became quiescent leading to relatively high standard deviations of the beat rates. On average, the cyclic stimulation did not elicit any significant change in the beat rate for the single-cell samples beyond the increased rate during poking at the highest load (700 nN). Figure 4(d) explores a variation of these experiments, except originally quiescent single cells were periodically indented at 5 Hz with 100 nN for 30 s. In numerous cases, this initiated continuous periodic cell beating that continued after the

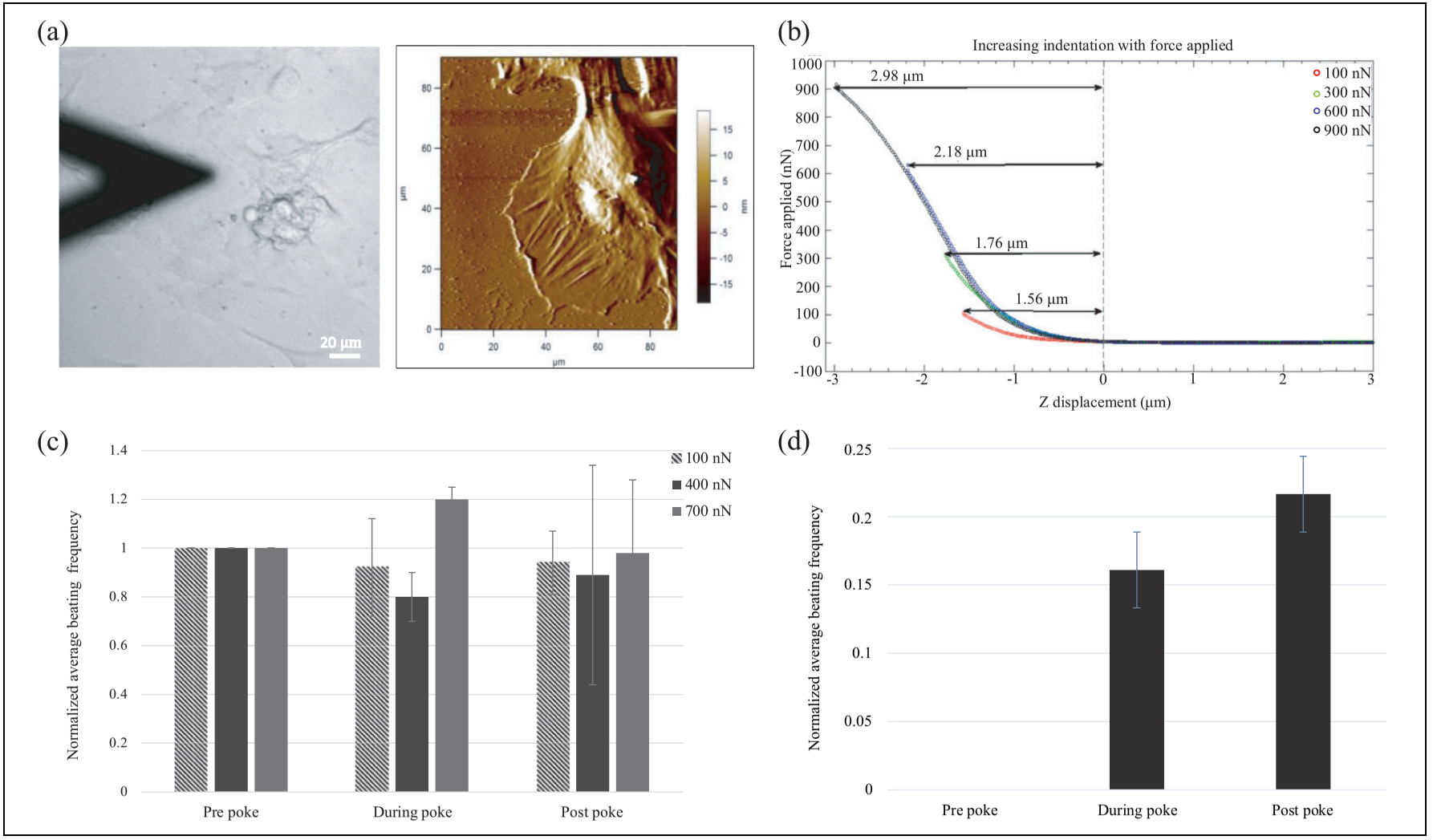

Figure 4. Nanomechanical stimulation of isolated single cardiomyocytes: (a) AFM probe on the surface of a single cell (left) and the AC-amplitude image of a representative cardiomyocyte using a commercial silicon nitride probe with a conical tip and apex of <50 nm radius of curvature (right); (b) variation in indentation depth with respect to the applied force; (c) response of single cardiomyocytes to varying forces of mechanical stimulation with a colloidal probe (10 µm diameter): 100, 400, and 700 nN repeated for 30 s at a rate of 3 Hz ($n = 10$); (d) jump-start response ("nano-CPR") of initially quiescent micropatterned cells to stimulation of 100 nN at 5 Hz for 30 s using a colloidal probe (10 µm diameter, $n = 7$). AFM: atomic force microscopy; CPR: cardiopulmonary resuscitation; AC: alternating current.

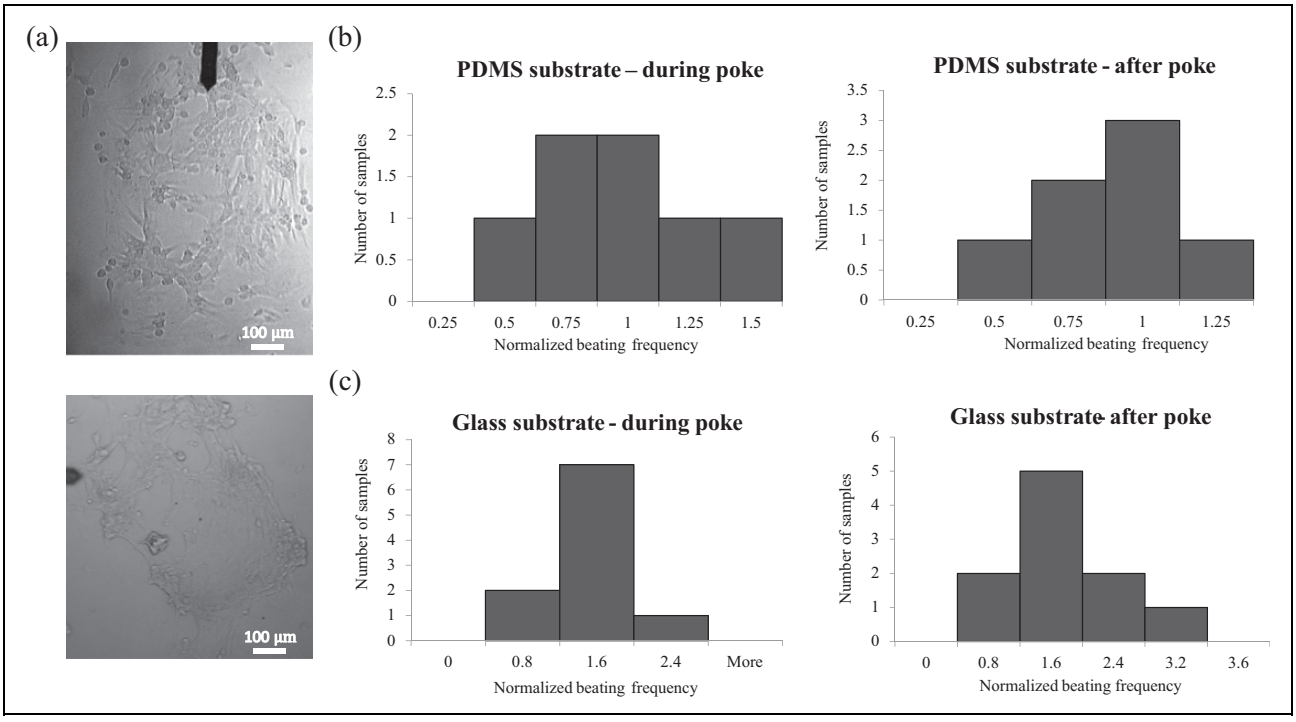

Figure 5. Nanomechanical stimulation of the myocardial cell networks with 300 nN at 5 Hz using a colloidal probe (10 μm diameter, $n = 10$): (a) localized mechanical stimulation of a single cell within the micropatterned cell network with the colloidal AFM probe on softer (PDMS), top, and on stiffer (glass) substrates, bottom; modulation of the beat rate of cells cultured (b) on softer (PDMS and (c) on the stiffer (glass) substrates. Also refer to Online Supplementary Movies 3 and 4. AFM: atomic force microscopy; PDMS: poly(dimethylsiloxane).

stimulation. Such AFM-induced "jump-starting" of cells exhibiting no prior spontaneous contractions proves that the single cells do sense and respond to the mechanical stimulation.

Unlike isolated single cells, micropatterned cell patches exhibit spontaneous synchronized beat rates throughout the network of cells. Moreover, the beat rate of the synchronously beating cardiomyocytes can be modulated by stimulation with 300 nN at 5 Hz, with results correlating to the substrate they cultured on (Figure 5). For cells cultured on a PDMS substrate with an initial normalized beat rate of 1 Hz, the beat rate of the cells either decreased or stayed the same for the majority of the tested samples (Figure 5(b) and Online Supplementary Movie 3) after nanomechanical stimulation. Only two of seven samples showed a slight increase in their beating rate during the stimulation and only one of those maintained it poststimulation. Interestingly, the cells cultured on stiffer glass substrates either maintained their spontaneous rate of contraction or showed an increase in the beating rate during and after the stimulation period, except for two samples which exhibited lower rate of contraction (Figure 5(c); Online Supplementary Movie 4). Paired t-test showed no significant difference between the rate of contraction before and after stimulation for cells seeded and cultured on glass substrate ($p = 0.055$), while the decrease in the rate of contraction after the stimulation was significant for cells cultured on PDMS substrate ($p = 0.044$).

While micropatterned cells required a minimum of 300 nN applied force at 5 Hz to observe any change in their beating rate (Figure 5), stimulation of spontaneously beating single cells at 3 and 5 Hz did not result in a different response (data not shown for brevity). Interestingly, although the spontaneously beating single cells did not show distinct changes in their response to stimulation at 3 or 5 Hz and continued to maintain their spontaneous beating rate, stimulation at 5 Hz was required to jump-start the quiescent cells (Figure 4(d)).

Discussion

AFM measurements have proven to be one of the most efficient technique for obtaining information at the single-cell level and also precise quantitative information on cell and substrate mechanical properties.[9,23,37,38] There are several studies in the literature showing changes in cell behavior upon solely mechanical stimulation, and many other techniques besides AFM have been used to study the mechano-sensitive responses of cardiomyocytes. Some of these studies include single-cell approaches such as microposts[39] and dynamic axial stretch with carbon glass fibers[40] or glass micropippetes[20] and macroscale stimulation with ultrasound,[41,42] microbeads,[8,43] or using microfluidic channels[44] that mimic hemodynamic cues.

For example, Nguyen et al. developed a microfluidic cell culture system to replicate hemodynamic mechanical

cues experienced by ventricular cardiomyocytes.[44] They observed reductions in gene and protein expression of contractile proteins of the cardiomyocytes after isolation from the tissue, which was recovered when the cells were cultured using the biomimetic microfluidic cell culture system. Moreover, these cells had stronger contractility and beat rates compared to a standard cell culture. The micromechanical milieu also plays a huge role in early embryonic development, pluripotency, and differentiation efficiency. Geuss et al. used paramagnetic beads for mechanical stimulation of embryonic bodies (EBs) through magnetic forces. The EBs embedded with magnetic beads were subjected to periodic mechanical stimulation, which resulted in a higher number of differentiated cardiomyocytes and also enhanced the contraction strength of these differentiated cells.[43] On the single-cell level, PDMS micropost arrays have been used for quantification of contractile forces generated by cardiomyocytes where the displacement of the microposts can be used to quantify the contraction force exerted by the cells attached to the posts.[41,45,46]

Micropatterning techniques have been used to control the localization and shape of cells to provide better control over the cell microenvironment in cell culture studies. The type of ECM molecule used for patterning the cells,[47–49] substrate stiffness,[50–52] and the geometry of the micropatterns have all been shown to influence the cell behavior and function.[53] For example, in a recent study investigating the effect of cell shape on the rate of smooth muscle cell contraction, FN micropatterns of different geometries were patterned. It was observed that smooth muscle cells grown in elongated shapes had lower contractile strengths but exhibited a greater percentage increase in contraction upon biochemical stimulation. Similarly, in vitro studies of cardiomyocytes have shown that a number of functional properties such as calcium dynamics, contractility, and voltage-gated ion currents are influenced by the cell shape and the mechanical microenvironment.[54,55]

In this work, we investigated the response of cardiomyocyte cell networks cultured as micropatterned cell patches to localized, single-cell-level external nanomechanical stimulation via an AFM probe. It should be noted that here the nanostimulation refers to the measure of applied force on the cells and not to the size of the probe used, which has dimensions on the order of a few micrometers and is thus similar to that of average contact areas between adjacent cells. Beyond examining the mechanical response of a single cell to single-cell-level stimulation,[6,40,56] or whole tissue sections[22,57] or engineered tissue constructs[58–60] to macroscale stimulation,[39] this novel micropatterned cell patch approach combined with state-of-the-art simultaneous AFM probing and optical imaging implemented here enabled us to study the effect of single-cell-level stimulation on a defined population of cell networks that was kept constant for each trial. First, we proved the connectivity of the micropatterned myocardial cell network, which we show to consist of 60% cardiomyocytes and 40% cardiac fibroblasts, using calcium flux analysis. Calcium flux across cardiomyocytes network is a crucial indicator of cardiac cell contractility and connectivity.[7,61] The uptake and release of calcium ions can be directly correlated with the contraction (systole) and relaxation (diastole) of a cardiomyocyte, and stretching of the cardiomyocyte membrane results in cytoskeletal remodeling with microtube modulation, which results in increased rates of calcium waves.[7]

We observed synchronous beating behavior and beat rate modulation response of these myocardial cell networks to nanoscale mechanical stimulation, which did not occur in isolated single cells. Although the single cells were able to respond to the mechanical stimulation in an on-and-off manner, including the quiescent cells, that began to beat upon mechanical stimulation (nano-CPR), their beat rate essentially remained the same regardless of the magnitude and frequency of the mechanical stimulation. On the other hand, micropatterned cell patches responded to the mechanical stimulation with a modified beating rate after the stimulation. The degree and mode (an increase or decrease) of this modification depended on the stiffness of the substrate on which the cells were cultured. The increase in beat response can be attributed to the presence of cell–cell interactions and cell–cell junctions such as the connexins, within the cell network of the micropatterned cell patches.[9,62] In the confluent cell micropatterns, contraction of a single cell induces a series of mechanical forces in the neighboring cell(s) and subsequently initiates a continuous beat response in all of the cells within the patch. These results are consistent with the literature where isolated single cells had a lower strength of contraction compared to cells with at least one neighbor that had stronger contractions for a longer time period.[20] For the quiescent cells, the indentation could have functioned as the mechanical trigger to open stretch-activated ion channels on the cells, thereby reinitiating the contractions.

Mechanical modulation of cardiomyocyte beating behavior has separately also been shown using acoustic ultrasound waves for inducing cardiac pacing.[41,42] In a recent study by Livneh et al., the authors used spatiotemporally controlled, biphasic high-intensity focused ultrasound (HIFU) insonation pattern to induce successfully pacing in rat hearts.[42] Here, HIFU insonation (positive pressure) and refraction (negative pressure) were applied sequentially, with controlled intermittent delay periods, to achieve pacing. Similarly, Fleishman et al. successfully paced neonatal rat ventricular cardiomyocytes using periodic pulses of 2.5 MHz ultrasound at 300 ms intervals.[41]

Substrate stiffness also plays an influential role in the rate and amplitude of contraction and cell functioning.[51] In this study, we compared the effect of a compliant versus a stiffer substrate, PDMS and glass, on the beating response of cardiomyocytes to nanoscale mechanical stimulation and observed that cells responded with a decrease in their

beat rate when they are on softer surfaces, while they maintained or increased their beat rate when they are cultured on stiffer substrates. The increase in beat rate can be attributed to improved cytoskeletal organization of cells seeded on stiffer substrates.[31,50,52] Consistent with the literature, our results showed a distinct difference in the organization of actin filaments in the cells cultured on the glass substrate compared to the PDMS substrate.[31] After 5 days in culture, the cells seeded on glass had developed stronger stress fibers and had a larger cell spreading area compared to ones on PDMS substrates. On the other hand, the cells seeded on PDMS substrate appeared to have fused cytoskeletal filaments and a bundled appearance with adjacent cells. In addition, although the cell spreading area was comparable for both substrates after 24 h of seeding, the cells seeded on glass attained a larger area of spreading (an average area of about 6000 μm^2) compared to the cells cultured on PDMS (an average area of about 2400 μm^2) by the fifth day in culture. The difference in cell spreading area over time could have been due to poor development of actin stress fibers over time in cells cultured on the softer substrate.[63,64] As seen in a study by Rodriguez et al., neonatal cardiomyocytes seeded on flexible post arrays of higher stiffness had better myofibril structure with larger sarcomere length, Z-bandwidth, and higher calcium levels as compared to similar cells seeded on posts of lower stiffness.[65] Similarly, a study by Jacot et al., in which neonatal rat cardiomyocytes were cultured on polyacrylamide gels of varying elastic moduli, showed that cells cultured on compliant substrates exhibit poorly developed cytoskeletons, while on stiffer substrates the cytoskeleton was isotropic with more stress fibers.[50] In this study, although the seeding density was identical on both substrates, and we observe the similar amount of cardiomyocytes to cardiac fibroblasts in addition to the comparable development of actin filaments on both types of substrate, we also observe a significant difference in expression of gap junction marker, connexin-43, between the two substrates. Cells on PDMS substrates formed a fewer number of gap junctions compared to cells on glass substrates. The comparably lower expression of connexin-43 between cells cultured on PDMS substrate could explain the difference in the beating response of the cells to the mechanical stimulation, as a lower expression of connexins would indicate poor electromechanical coupling of adjacent cells. Other studies have also shown that the mechanical properties of the culture substrates can impact the spontaneous contractile activity of neonatal cardiomyocytes[31,32] and protein expression.[66] For example, a recent study by Boudreau-Beland et al. showed that cells cultured on softer PDMS substrates with lower stiffness had greater spatial and temporal instability of spontaneous rate activation owing to increased number of activation sites as compared to the cells cultured on glass.[31] However, they also observed significantly decreased expression of connexin-43 as compared to the cells cultured on a glass substrate which explained the increased instability they observed. We will further investigate this interesting finding in our future studies.

In conclusion, the mechanical microenvironment plays a critical role in cardiac function from influencing the rate of contraction to conduction velocity. Experiments carried out in this study lay an essential foundation for studying the effect of single-cell-level mechanical stimuli in modulating the synchronous beating behavior of myocardial cell networks. Understanding the contractility of myocardial cell networks at a single-cell level is important for applications ranging from novel myocardial therapies to biorobotics.

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Supplemental material

The online appendices/data supplements/etc are available at http://nab.sagepub.com/supplemental.

References

1. Kohl P, Camelliti P, Burton FL, et al. Electrical coupling of fibroblasts and myocytes: relevance for cardiac propagation. *J Electrocardiol* 2005; 38: 45–50.
2. Kamkin A, Kiseleva I, Wagner K-D, et al. Mechano-Electric feedback in the heart: evidence from intracellular microelectrode recordings on multicellular preparations and single cells from healthy and diseased tissue. In: Kamkin A and Kiseleva I (eds) *Mechanosensitivity in Cells and Tissues*. Academia, 2005, pp. 169–174.
3. Thompson SA, Blazeski A, Copeland CR, et al. Acute slowing of cardiac conduction in response to myofibroblast coupling to cardiomyocytes through N-cadherin. *J Mol Cell Cardiol* 2014; 68: 29–37.
4. Berry MF, Engler AJ, Woo YJ, et al. Mesenchymal stem cell injection after myocardial infarction improves myocardial compliance. *Am J Physiol Heart Circ Physiol* 2006; 290: H2196–H2203.
5. Hiesinger W, Brukman MJ, McCormick RC, et al. Myocardial tissue elastic properties determined by atomic force microscopy after stromal cell-derived factor 1α angiogenic therapy for acute myocardial infarction in a murine model. *J Thorac Cardiovasc Surg* 2012; 143: 962–966.
6. Aguirre AD, Vinegoni C, Sebas M, et al. Intravital imaging of cardiac function at the single-cell level. *Proc Natl Acad Sci U S A* 2014; 111: 11257–11262.
7. Seo K, Inagaki M and Fukano H. Relevance of cardiomyocyte mechano-electric coupling to stretch-induced

arrhythmias: optical voltage/calcium measurement in mechanically stimulated cells, tissues and organs. *Prog Biophys Mol Biol* 2014; 115: 129–139.
8. Barac DY, Reisner Y, Silberman M, et al. Mechanical load induced by glass microspheres releases angiogenic factors from neonatal rat ventricular myocytes cultures and causes arrhythmias. *J Cell Mol Med* 2008; 12: 2037–2051.
9. Azeloglu EU and Costa KD. Cross-bridge cycling gives rise to spatiotemporal heterogeneity of dynamic subcellular mechanics in cardiac myocytes probed with atomic force microscopy. *Am J Physiol Heart Circ Physiol* 2010; 298: H853–H860.
10. Williams BJ, Anand SV, Rajagopalan J, et al. A self-propelled biohybrid swimmer at low Reynolds number. *Nat Commun* 2014; 5: 3081.
11. Cvetkovic C, Raman R, Chan V, et al. Three-dimensionally printed biological machines powered by skeletal muscle. *Proc Natl Acad Sci U S A* 2014; 111: 10125–10130.
12. Holley MT, Nagarajan N, Danielson C, et al. Development and characterization of muscle-based actuators for self-stabilizing swimming biorobots. *Lab Chip* 2016; 16: 3473–3484. DOI:10.1039/C6LC00681G
13. Kim J, Park J, Yang S, et al. Establishment of a fabrication method for a long-term actuated hybrid cell robot. *Lab Chip* 2007; 7: 1504–1508.
14. Tandon N, Marsano A and Maidhof R. Optimization of electrical stimulation parameters for cardiac tissue engineering. *J Tissue Eng Regen Med* 2011; 5: e115–e125.
15. Tandon N, Cannizzaro C, Chao PH, et al. Electrical stimulation systems for cardiac tissue engineering. *Nat Protoc* 2009; 4: 155–173.
16. Chang W-T, Yu D, Lai Y-C, et al. Characterization of the mechanodynamic response of cardiomyocytes with atomic force microscopy. *Anal Chem* 2013; 85: 1395–1400.
17. Liu Y, Feng J and Shi L. In situ mechanical analysis of cardiomyocytes at nano scales. *Nanoscale* 2012; 4: 99–102.
18. Sheehy SP, Grosberg A and Parker KK. The contribution of cellular mechanotransduction to cardiomyocyte form and function. *Biomech Model Mechanobiol* 2012; 11: 1227–1239.
19. Lieber SC, Aubry N, Pain J, et al. Aging increases stiffness of cardiac myocytes measured by atomic force microscopy nanoindentation. *Am J Physiol Heart Circ Physiol* 2004; 287: H645–H651.
20. Tang X, Bajaj P, Bashir R, et al. How far cardiac cells can see each other mechanically. *Soft Matter* 2011; 7: 6151–6158.
21. Jacot JG, Martin JC, and Hunt DL. Mechanobiology of cardiomyocyte development. *J Biomech* 2010; 43: 93.
22. Shachar M, Benishti N, and Cohen S. Effects of mechanical stimulation induced by compression and medium perfusion on cardiac tissue engineering. *Biotechnol Prog* 2012; 28: 1551–1559.
23. Yang J. AFM as a high-resolution imaging tool and a molecular bond force probe. *Cell Biochem Biophys* 2004; 41: 435–450.
24. Gaitas A, Malhotra R, Li T, et al. A device for rapid and quantitative measurement of cardiac myocyte contractility. *Rev Sci Instrum* 2015; 86: 34302.
25. Liu J, Sun N, Bruce MA, et al. Atomic force mechanobiology of pluripotent stem cell-derived cardiomyocytes. *PLoS ONE* 2012; 7: e37559.
26. Jeong SI, Kwon JH, Lim JI, et al. Mechano-active tissue engineering of vascular smooth muscle using pulsatile perfusion bioreactors and elastic PLCL scaffolds. *Biomaterials* 2005; 26: 1405–1411.
27. Zhuang J, Yamada KA, Saffitz JE, et al. Pulsatile stretch remodels cell-to-cell communication in cultured myocytes. *Circ Res* 2000; 87: 316–322.
28. Hazeltine LB, Simmons CS, Salick MR, et al. Effects of substrate mechanics on contractility of cardiomyocytes generated from human pluripotent stem cells. *Int J Cell Biol* 2012; 2012: e508294.
29. Korhonen T, Hänninen SL, and Tavi P. Model of excitation–contraction coupling of rat neonatal ventricular myocytes. *Biophys J* 2009; 96: 1189–1209.
30. Levkau B, Schäfers M, Wohlschlaeger J, et al. Survivin determines cardiac function by controlling total cardiomyocyte number. *Circulation* 2008; 117: 1583–1593.
31. Boudreau-Béland J, Duverger JE, Petitjean E, et al. Spatiotemporal stability of neonatal rat cardiomyocyte monolayers spontaneous activity is dependent on the culture substrate. *PLoS One* 2015; 10(6): e0127977.
32. Ehler E, Moore-Morris T, and Lange S. Isolation and culture of neonatal mouse cardiomyocytes. *J Vis Exp* 2013. DOI:10.3791/50154
33. Shin SR, Jung SM, Zalabany M, et al. Carbon-nanotube-embedded hydrogel sheets for engineering cardiac constructs and bioactuators. *ACS Nano* 2013; 7: 2369–2380.
34. Fu J, Gao J, Pi R, et al. An optimized protocol for culture of cardiomyocyte from neonatal rat. *Cytotechnology* 2005; 49: 109–116.
35. Kwon E-Y, Kim Y-T and Kim D-E. Investigation of penetration force of living cell using an atomic force microscope. *J Mech Sci Technol* 2009; 23: 1932–1938.
36. Hoh JH and Schoenenberger CA. Surface morphology and mechanical properties of MDCK monolayers by atomic force microscopy. *J Cell Sci* 1994; 107(Pt 5): 1105–1114.
37. Engler AJ, Rehfeldt F, Sen S, et al. Microtissue elasticity: measurements by atomic force microscopy and its influence on cell differentiation. *Methods Cell Biol* 2007; 83: 521–545.
38. Wang L, Chen T, Zhou X, et al. Atomic force microscopy observation of lipopolysaccharide-induced cardiomyocyte cytoskeleton reorganization. *Micron* 2013; 51: 48–53.
39. Ribeiro AJS, Zaleta-Rivera K, Ashley EA, et al. Stable, covalent attachment of laminin to microposts improves the contractility of mouse neonatal cardiomyocytes. *ACS Appl Mater Interfaces* 2014; 6: 15516–15526.
40. Nishimura S, Seo K, Nagasaki M, et al. Responses of single-ventricular myocytes to dynamic axial stretching. *Prog Biophys Mol Biol* 2008; 97: 282–297.
41. Fleischman A, Vecchio C, Sunny Y, et al. Ultrasound-induced modulation of cardiac rhythm in neonatal rat ventricular cardiomyocytes. *J Appl Physiol* 2015; 118(11): 1423–1428. DOI:10.1152/japplphysiol.00980.2014

42. Livneh A, Kimmel E, Kohut AR, et al. Extracorporeal acute cardiac pacing by high intensity focused ultrasound. *Prog Biophys Mol Biol* 2014; 115: 140–153.
43. Geuss LR, Wu DC, Ramamoorthy D, et al. Paramagnetic beads and magnetically mediated strain enhance cardiomyogenesis in mouse embryoid bodies. *PLoS ONE* 2014; 9: e113982.
44. Nguyen M-D, Tinney JP, Ye F, et al. Effects of physiologic mechanical stimulation on embryonic chick cardiomyocytes using a microfluidic cardiac cell culture model. *Anal Chem* 2015; 87: 2107–2113.
45. Rodriguez ML, Graham BT, Pabon LM, et al. Measuring the contractile forces of human induced pluripotent stem cell-derived cardiomyocytes with arrays of microposts. *J Biomech Eng* 2014; 136: 051005.
46. Fu J, Wang YK, Yang MT, et al. Mechanical regulation of cell function with geometrically modulated elastomeric substrates. *Nat Methods* 2010; 7: 733–736.
47. Simmons CS, Petzold BC and Pruitt BL. Microsystems for biomimetic stimulation of cardiac cells. *Lab Chip* 2012; 12: 3235–3248.
48. Howard CM and Baudino TA. Dynamic cell–cell and cell–ECM interactions in the heart. *J Mol Cell Cardiol* 2014; 70: 19–26.
49. Curtis MW and Russell B. Micromechanical regulation in cardiac myocytes and fibroblasts: implications for tissue remodeling. *Pflüg Arch Eur J Physiol* 2011; 462: 105–117.
50. Jacot JG, McCulloch AD and Omens JH. Substrate stiffness affects the functional maturation of neonatal rat ventricular myocytes. *Biophys J* 2008; 95: 3479–3487.
51. Yeung T, Georges PC, Flanagan LA, et al. Effects of substrate stiffness on cell morphology, cytoskeletal structure, and adhesion. *Cell Motil Cytoskeleton* 2005; 60: 24–34.
52. Tallawi M, Rai R, Boccaccini AR, et al. Effect of substrate mechanics on cardiomyocyte maturation and growth. *Tissue Eng Part B Rev* 2015; 21: 157–165.
53. McCain ML and Parker KK. Mechanotransduction: the role of mechanical stress, myocyte shape, and cytoskeletal architecture on cardiac function. *Pflüg Arch Eur J Physiol* 2011; 462: 89–104.
54. Felix R, Calderón-Rivera A and Andrade A. Regulation of high-voltage-activated Ca^{2+} channel function, trafficking, and membrane stability by auxiliary subunits. *Wiley Interdiscip Rev Membr Transp Signal* 2013; 2: 207–220.
55. Ye GJC, Aratyn-Schaus Y, Nesmith AP, et al. The contractile strength of vascular smooth muscle myocytes is shape dependent. *Integr Biol* 2014; 6: 152–163.
56. Sugiura S, Nishimura S, Yasuda S, et al. Carbon fiber technique for the investigation of single-cell mechanics in intact cardiac myocytes. *Nat Protoc* 2006; 1: 1453–1457.
57. Miklas JW, Nunes SS, Sofla A, et al. Bioreactor for modulation of cardiac microtissue phenotype by combined static stretch and electrical stimulation. *Biofabrication* 2014; 6: 24113.
58. Fink C, Ergün S, Kralisch D, et al. Chronic stretch of engineered heart tissue induces hypertrophy and functional improvement. *FASEB J* 2000; 14: 669–679.
59. Zimmermann W-H, Schneiderbanger K, Schubert P, et al. Tissue engineering of a differentiated cardiac muscle construct. *Circ Res* 2002; 90: 223–230.
60. Morgan KY and Black LD 3rd. Investigation into the effects of varying frequency of mechanical stimulation in a cycle-by-cycle manner on engineered cardiac construct function. *J Tissue Eng Regen Med*. Epub ahead of print 11 June 2014. DOI:10.1002/term.1915
61. Bers DM. Calcium fluxes involved in control of cardiac myocyte contraction. *Circ Res* 2000; 87: 275–281.
62. Domke J, Parak WJ, George M, et al. Mapping the mechanical pulse of single cardiomyocytes with the atomic force microscope. *Eur Biophys J* 1999; 28: 179–186.
63. Galie PA, Khalid N, Carnahan KE, et al. Substrate stiffness affects sarcomere and costamere structure and electrophysiological function of isolated adult cardiomyocytes. *Cardiovasc Pathol* 2013; 22: 219–227.
64. Ochsner M, Textor M, Vogel V, et al. Dimensionality controls cytoskeleton assembly and metabolism of fibroblast cells in response to rigidity and shape. *PLoS ONE* 2020; 5: e9445.
65. Rodriguez AG, Han SJ, Regnier M, et al. Substrate stiffness increases twitch power of neonatal cardiomyocytes in correlation with changes in myofibril structure and intracellular calcium. *Biophys J* 2011; 101: 2455–2464.
66. Forte G, et al. Substrate stiffness modulates gene expression and phenotype in neonatal cardiomyocytes in vitro. *Tissue Eng A* 2012; 18: 1837–1848.