Mechanical communication through the ECM is frequency-dependent due to cell sensitivity to mechanical signal shape

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Abstract

Cells communicate mechanically by sensing and responding to mechanical deformations generated by their neighbors in the extracellular matrix (ECM). Cell-generated deformations propagates through the ECM and therefore mechanical communication depends on the mechanical properties of the matrix. By directly measuring the mechanical coupling between a cardiac cell and an artificial 'mechanical cell' and monitoring the dependence of beat-to-beat variability on mechanical coupling, we can quantify the sensitivity of cardiac cells to mechanical signals. Here we show that due to the dynamic viscoelastic properties of collagen hydrogels (a major component of the cardiac ECM), the shape of the mechanical signal changes in a frequency-dependent manner as it propagates through the gel, resulting in mechanical communication that depends on beating frequency. Moreover, we show that cardiac cell's sensitivity to the shape of the mechanical signal results from its responsiveness to the loading rate, with an optimal loading rate for efficient mechanical communication. The dependence of signal shape on ECM viscoelasticity and the existence of an optimal loading rate suggest that there may be ideal viscoelastic properties for mechanical communication between cardiac cells.

Introduction

It was recently established that cells are able to communicate mechanically by responding to mechanical deformations generated by their neighbors(1–6). It is a challenging task to measure and define mechanical communication between cells. An additional challenge is to separate the mechanical component of intercellular communication from other modes of communication, such as a change in the amount or type of secreted chemo-attractants. Often, mechanical communication is defined as migration towards a contractile cell or frequency of cell-cell contact and mechanical coupling is qualitatively estimated by imaging matrix fiber alignment spanning cell pairs(7, 8). In cardiac cells, we have shown previously that mechanical communication is essential for synchronized beating. We have previously shown that spontaneously beating cardiac cells can be paced using a 'mechanical cell', which consist of a tungsten probe that mimics the deformations generated by a neighboring beating cell (1). There is no physical contact between the cell and the probe, and the interaction therefore is purely mechanical and mediated through propagation of deformations in the matrix. In addition, we have measured mechanical coupling directly and showed that beat-to-beat variability decays exponentially with the level of mechanical coupling(3).

The ECM is highly non-linear viscoelastic material and therefore exhibits both elastic and viscous responses in a frequency dependent manner. In high frequencies it behaves as a pure elastic material while in lower frequencies molecular relaxation processes occur within the network and influence material response. Several recent studies have demonstrated the importance of ECM viscoelasticity in determining cell response(9–15). Intermediate viscosity was shown to maximize cell spreading(10) and fast stress relaxation results in robust cell migration on soft substrates(16).

Mechanical cell-cell communication is mediated by the extracellular matrix (ECM) and due to its viscoelasticity, cell-generated deformations will be filtered via the viscoelastic medium and therefore amplified, dissipated, or delayed in a frequency-dependent manner. As a result, the local mechanical signal generated by beating cardiac cells is expected to change its shape, phase, and amplitude in a way that depends on the beating frequency. Using collagen I, the major component of the cardiac ECM, we show that mechanical communication on collagen is frequency-dependent as a result of the change in the mechanical signal shape while it propagates through the matrix. We further show that cell sensitivity to signal shape results from its sensitivity to the loading rate of the mechanical signal with an optimal loading rate of 1um/sec at a beating frequency of 1Hz.

Results and discussion

<u>Mechanical communication on ECM is frequency-dependent</u>

Primary cardiomyocytes were isolated from neonatal rat hearts and cultured on either polyacrylamide gels or collagen I hydrogels. Hydrogels made of collagen I, a native component of the cardiac ECM, were used to explore the role of the dynamic viscoelastic properties of the ECM. We used glycated collagen matrices (17) with collagen concentration of 5mg/ml and ribose concentration of 250mM, to obtain Young's modulus of ~1 kPa, comparable to the Young's modulus of our polyacrylamide substrate. Substrate stiffness in this range was shown to support optimal cardiac cell beating (18). We recently introduced the experimental setup (1, 3, 19) which is shown schematically in Figure 1a. The 'mechanical cell' is a home-built mechanical device which consists of a tungsten probe mounted on a piezo stage with x-y-z actuators. Driven by the actuators, the probe cyclically pulls the substrate towards and away from the cardiac cell. The mechanical probe deforms the substrate such as to mimic the mechanical deformations generated by a neighboring beating cardiac cell. There is no physical contact between the cell and the probe and the interaction therefore is solely mechanical and mediated through propagation of deformations in the underlying substrate.

We have recently shown that cardiac cells can be paced mechanically using a 'mechanical cell'(1) and that phase entrainment depends on the level of mechanical coupling (3). Quantitatively, beat-to-beat variability of a cardiac cell (ξ) decays exponentially with the strength of mechanical coupling (χ) to either a 'mechanical cell' or a neighboring cardiac cell according to $\xi = ae^{-k(\omega)\chi}$ where k is the exponential decay constant (Figure 1, Videos S1-S2 and (3)). The inverse value of the decay constant is the value of mechanical coupling required for the beat-to-beat variability to decay by a factor of 1/e. Additional details on the experimental assay and the measurements of beat-to-beat variability and mechanical coupling can be found in the supplementary information and in previous work (3).

In the general case, the exponential decay constant, k, which corresponds to the sensitivity of the cell to mechanical coupling, depends on the beating frequency of the cardiac cell. However, as shown in Figure 2d, on linear elastic materials such as polyacrylamide, the beat-to-beat variability exponential decay does not depend on frequency and equals 5.5±0.23, implying that on linear elastic materials, cell sensitivity to mechanical coupling does not depend on cell beating frequency. However, for ECM materials such as collagen, the behavior is qualitatively

different. As shown in Figure 2b,e, the decay constant on collagen, and therefore mechanical communication efficiency, is frequency-dependent and increases monotonically with the beating frequency.

Cells are sensitive to the shape of the mechanical signal

The nonlinearity and viscoelasticity of collagen are expected to change the shape of the oscillating mechanical signal as it propagates through the matrix. Indeed, a clear frequency-dependent change in the shape of the propagating strain was observed on collagen hydrogels (Figure 3a-b). To test whether the change in shape of the mechanical signal is the origin of the frequency-dependent cell response, we repeated the experiment with a different protocol. As we have shown previously, since polyacrylamide gels are linear elastic gels, there is no apparent change in the shape of the signal while it propagates through the gel. A sinusoidal form applied locally in the gel, will keep its form and still be sinusoidal next to the cell (schematically shown in Figure 2a). We repeat the experiment, but this time for any given beating cell (say in frequency ω_1), we apply on polyacrylamide an oscillatory signal that has the shape that is observed on collagen in frequency ω_1 next to the cell (after propagating within the gel; schematically shown in Figure 2b-c). For example, for a cell beating at 0.3Hz, the 'mechanical cell' generates on the polyacrylamide gel a signal with the shape of the red curve in Figure 2c instead of a sinusoidal signal.

As clearly shown in Figure 2e, we are able to reproduce the frequency-dependent cell response by mimicking on polyacrylamide the frequency-dependent signal shape observed on collagen. This implies that the frequency-dependent shape of the mechanical signal by itself can explain the change in cell sensitivity to mechanical coupling at different frequencies.

Collagen fibers are unable to resist compressive strain and are known to buckle under forces generated by cells if compressive and reorient along tensile forces(8, 20). Collagen buckling is indeed observed in our experiments as shown in Figure 3a and Video S2. The change in shape of the mechanical signal as it propagates through collagen matrices can be understood in the framework of collagen fiber buckling as demonstrated using a simplified 1D toy model. Consider a simple 1D system consists of two buckled beams (which represent two adjacent collagen fibers) connected in series by a joint and supported by a fixed support in point C and two roller supports in points A and B. Each of the rollers are connected to a spring (which represents environment elasticity), see Figure 3d. A sinusoidal displacement is applied at point A (q_A) and the resultant displacement of point B is calculated (q_B). This simplified model can

reproduce qualitatively the change in shape and its dependence on frequency as shown in Figure 3d-f and discussed in the supplementary information.

Mechanical communication is most efficient at an optimal strain rate

As shown in Figure 2e, mechanical coupling sensitivity depends on signal shape and signal shape by itself can reproduce the effect of having a viscoelastic material as a substrate. Different signal shapes are associated with different loading rates and it was recently shown that cell response is sensitive to the rate of applied force for fibroblast spreading under cyclic strain(21). To directly test the effect of loading rate on cell sensitivity to mechanical coupling and its ability to communicate mechanically efficiently, we repeated the experiments on polyacrylamide with a set of trapezoid signals. The loading rate is not constant for a sinusoidal signal whereas trapezoid signals have constant slopes and therefore a well-defined loading rate. All of the trapezoid strain profiles, have the same frequency but different slopes that correspond to different loading rates.

As done in the previous set of experiments, we gradually increase the trapezoid-shaped oscillations amplitude of the probe at a frequency identical to the beating frequency of the cell and monitor beat-to-beat variability as a function of the strength of mechanical coupling. We conducted these experiments on cardiac cells that beat at 1Hz using 1Hz trapezoid signals on polyacrylamide substrates. When increasing the amplitude of the probe, we keep the loading rate of the trapezoid signal constant by changing the stall times between rise-and-fall times.

As clearly shown in Figure 4a, the decay constant of the beat-to-beat variability with mechanical coupling, i.e., the sensitivity of cells to mechanical coupling, has a non-monotonic dependence on the loading rate with a maximal value at a loading rate of ~1um/sec. Therefore, mechanical communication between cardiac cells beating at 1Hz is most efficient if the signal loading rate is ~1um/sec.

In order to include the result obtained with oscillatory signals on polyacrylamide and collagen substrate at 1Hz as reported in Figure 2, we use the maximal loading rate of the mechanical signals shown in Figure 2a-c (since the loading rate is not constant). These points fall on the same curve. Since the decay constant is not an intuitive measure, we show the decrease in beat-to-beat variability as a function of loading rate for a given mechanical coupling of 0.1 (Figure 3b). For cells contracting at 5% strain, mechanical coupling value of χ=0.1 on 3kPa PA corresponds to a distance of 80 μm (along the axis perpendicular to the contraction axis, i.e,

distance of 4-8 cell diameters). As clearly shown in the Figure 4b, while the beat-to-beat variability is reduced by nearly 80% in the optimal loading rate of 1 um/sec, only 20% of the beat-to-beat variability is reduced in the lowest or highest loading rates tested (1.73 μm/sec and 0.36 μm/sec respectively).

Conclusions

Mechanical communication between cells is mediated by mechanical deformations which propagates through the ECM. Here we show that as the mechanical signal propagates through the ECM, its shape and consequently the associated loading rate is changing, thereby influencing mechanical communication. Modifications to the viscoelasticity of the mechanical environment (for example, by changing the level of enzymatic crosslinking in the ECM or matrix composition), will cause a change in the mechanical signal shape. The existence of an optimal loading rate for mechanical communication, therefore, suggests that there are optimal viscoelastic properties for mechanical communication.

Understanding the dependence of mechanical communication on the dynamic viscoelastic properties of the environment will allow us to tune ECM mechanics in order to achieve synchronized behavior of interacting cells or repair mechanical communication to restore normal function.

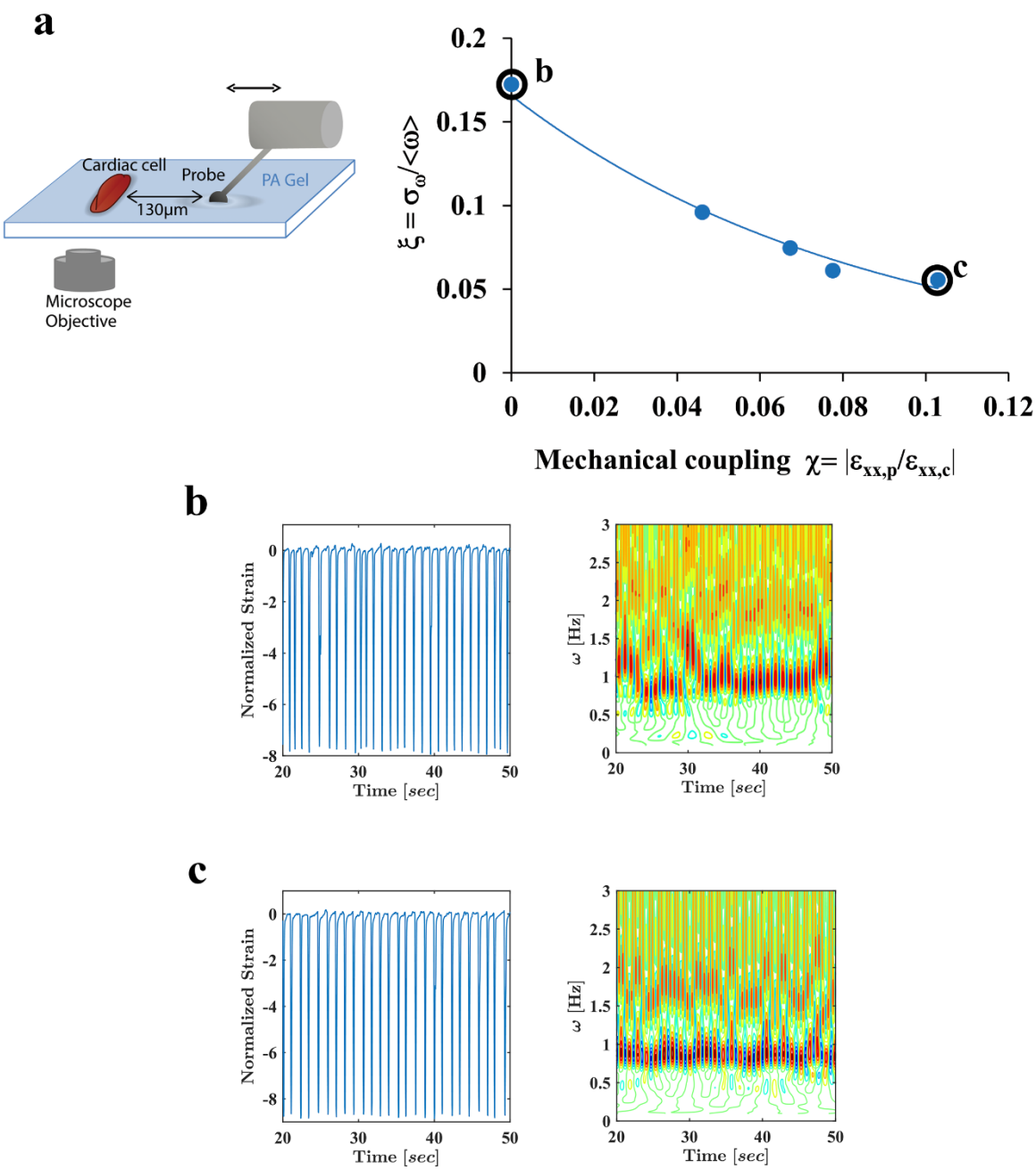

Figure 1: Beat-to-beat variability is reduced exponentially with increased mechanical coupling to a 'mechanical cell': a: *left:* A schematic representation of the 'mechanical cell' setup. A tungsten probe applies an oscillatory stretch which mimics the deformations generated by a beating cardiac cell. *Right:* The dependence of beating variability, for a representative isolated beating cardiac cell, on the strength of mechanical coupling to a 'mechanical cell'. Beating noise (ξ) decays exponentially with the strength of mechanical coupling (χ) to a 'mechanical cell' according to $\xi = ae^{-k\chi}$ **b:** The normalized strain (*left*) and frequency (*right*) as a function of time without mechanical coupling, (the point marked in (a) by (b)). **c:** The normalized strain (*left*) and frequency (*right*) as a function of time for a mechanical coupling of χ=0.103. (This point is marked in (a) by (c)).

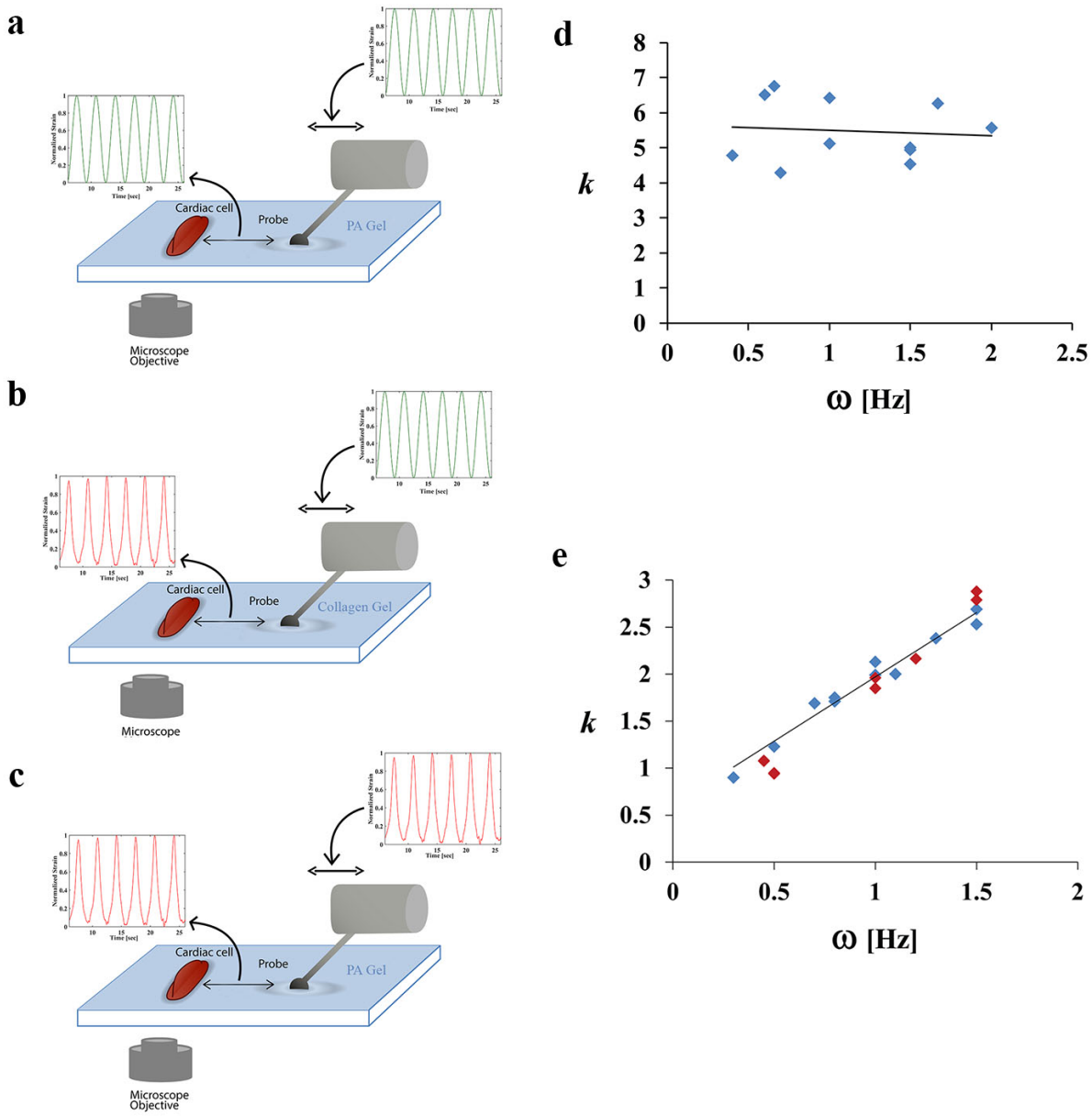

Figure 2: Mechanical communication is frequency-dependent on ECM as a result of cell sensitivity to the mechanical signal shape: a-c: Schematic illustration of the experimental protocols. An isolated cardiac cell interacts mechanically with a 'mechanical cell' on Polyacrylamide (PA) (a,c) or on collagen (b). The frequency of 'mechanical cell' oscillations equals to the average frequency of the beating cardiac cell to separate the effect of pacing a cardiac cell from that of beat-to-beat variability reduction. A sinusoidal strain signal is applied by the mechanical cell and is measured next to the cell after it propagates through the substrate. On PA substrates (a) the propagated signal has the same shape as the applied signal, however on collagen, the propagated signal changes its shape (b). In (c), the mechanical cell applies on the substrate the signal shape which is observed next to the cell in (b). In each experiment (c), the shape used is taken from an experiment (b) where the mechanical cell oscillates in the same frequency as the beating cell in (c). Beat-to-beat variability (ξ) decays exponentially with the strength of mechanical coupling (χ) to a 'mechanical cell' according to $\xi = ae^{-k(\omega)\chi}$ (see Figure 1). **d:** The decay constant, *k*, is plotted as a function of frequency for isolated cardiac cells interacting with a 'mechanical cell' applying a sinusoidal mechanical signal on polyacrylamide (a,d) or collagen (b,e-

blue diamonds). **e:** The decay constant, k, is plotted as a function of frequency for isolated cardiac cells interacting on polyacrylamide using the frequency-dependent shape observed on collagen (c,e-red diamonds). Notice that the decay constant, *k* does not depend on frequency on polyacrylamide, while there is a strong dependence on frequency on collagen that can be reproduced by mimicking on polyacrylamide the frequency-dependent signal shape observed on collagen.

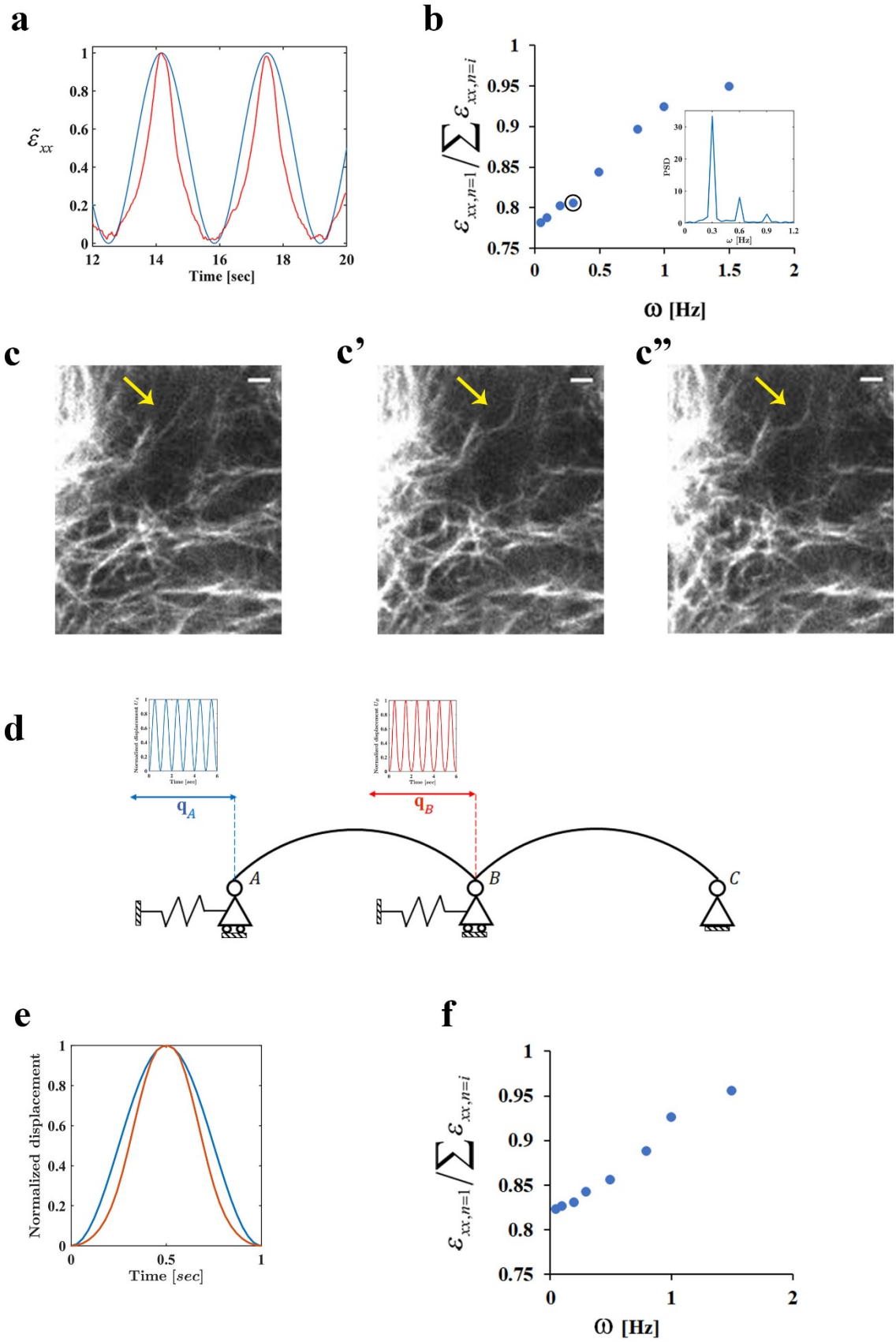

Figure 3: Buckling of collagen fibers can explain the change in shape of propagated mechanical signal: a: Representative normalized strain signal at 0.3Hz at a distance of d=84um from the mechanical

probe (red curve) and next to the probe (blue curve). **b:** The weight of the first harmonic relative to the harmonic content as a function of frequency. The circled point corresponds to the signal presented in the red curve in (a) (at a distance of 84um from the mechanical probe. The Fourier transform of the signal is shown in the subset. **c-c":** Representative movie frames of labeled collagen fibers locally deformed by the probe at three time points: beginning of probe movement (**c**, time zero, no deformation), after 1.5 seconds (**c'**) and after 1.8 seconds (**c"**). The yellow arrow is pointing at a representative fiber which undergoes buckling. Scale bar is: 5μm **d:** A simplified 1D toy model: Two buckled beams (which represent two collagen fibers) are connected in series by a joint and supported by a fixed support in point C and two roller supports in points A and B. Each of the rollers is connected to the environment with a spring. A sinusoidal displacement is applied on point A (q_A in a) and the resulted displacement of point B is calculated (q_B in b). **e:** The normalized displacement for one cycle of displacement is plotted for point A (blue curve) and for point B (red curve). **f:** The weight of the first harmonic relative to the harmonic content as a function of frequency as obtained in the simulation.

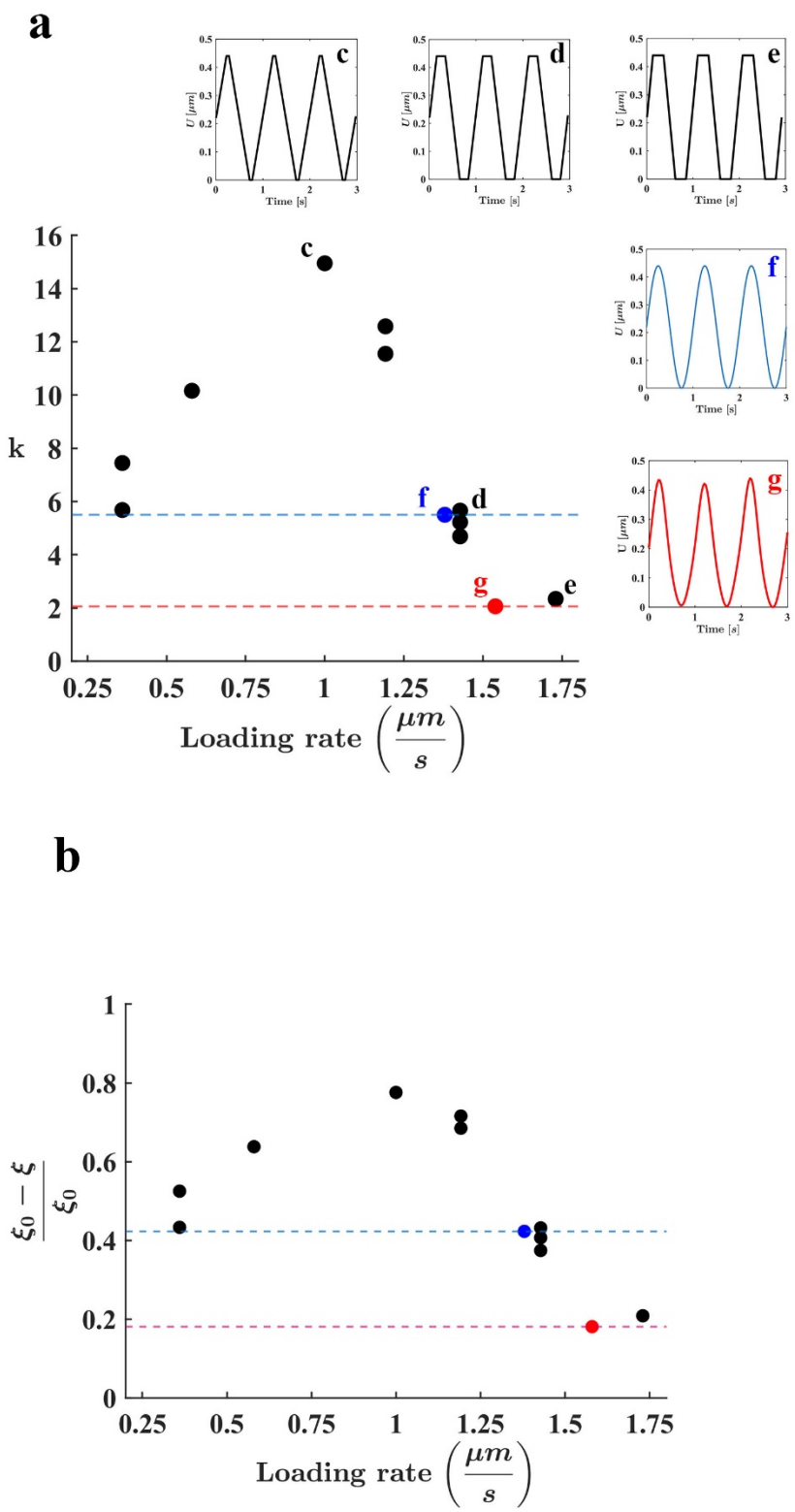

Figure 4: Mechanical communication is loading-rate dependent: a: The decay constant, *k*, is plotted as a function of the loading rate of the mechanical signal for cells interacting with a trapezoid oscillatory signal (●, black circles), an oscillatory sinusoidal signal (●, blue circle) and with an oscillatory signal shape observed on collagen next to the cell at 1Hz (●, red circle). All signals are oscillatory signals

applied on polyacrylamide at a frequency of 1Hz. For the non-trapezoid signals, the loading rate is defined as the maximal loading rate, i.e., the maximal derivative of the signal. **c-g:** the mechanical signals generated next to the cells in the points marked by **c-g** in the main graph respectively. k=5.5 and maximal loading rate=1.37 um/s for polyacrylamide; and k=2.1 and maximal loading rate=1.58 um/s for collagen. **b:** The change in beat-to-beat variability (ξ_0-ξ) relative to the initial beat-to-beat variability (ξ_0) as a function of the mechanical signal loading rate for mechanical coupling of χ=0.1.

Materials and Methods

All laboratory procedures conform to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health. Animal usage was approved by the Animal Care and Use Committee of the Technion, Israel Institute of Technology.

Cell culture: Neonatal cardiomyocytes were isolated from zero-day-old Sprague Dawley (SD) rat pups using the Neonatal Cardiomyocyte Isolation System (Worthington, NJ) according to the manufacturer's instructions as described before(1). Briefly, hearts were rinsed with ice-cold HBSS and trypsinized for 16 hours at 4°C. Following trypsinization, hearts were incubated with trypsin inhibitor at 37°C, and then with collagenase solution for 30 minutes. Cells were triturated, pelleted and resuspended in L15 media (Worthington, NJ). Cells were then subjected to Percoll gradient for separation of cardiomyocytes from non-myocytes. Isolated cardiomyocytes were resuspended with culture media: F10 media (Sigma-Aaldrich, MO) supplemented with 5% fetal calf serum (Biological-Industries, Israel), 5% donor horse serum (Life-technologies, CA), penicillin 10u/ml, streptomycin 0.1mg/ml, 5-Bromo-2'-deoxyuridine (5-BrdU) 0.05mg/ml and $CaCL_2$ 1mM (Signa-Aldrich, MO). Cultured cardiomyocytes were grown for at least 72 hours and up to 6 days.Approximately $8x10^5$ cells were plated on 30mm glass bottom plates (Greiner, Austria) covered with either collagen or matrigel patterned polyacrylamide gels.

PDMS stamp: microfabrication and soft lithography: The patterns were designed using CleWin 5 (PhoeniX Software, NL). Isolated pairs of rectangles were designed. Rectangles were either 20 x 50um or 20 x 100um. Each rectangle represents a place that will be occupied by a cardiac cell. The distance between two cells in each pair varies throughout the mask (e.g. 10, 20, 40, 80, 120um). Chrome masks were manufactured by Delta Mask (Delta Masks BV, NL). A Si master with geometric patterns was fabricated using standard photolithographic technique as previously described(22). PDMS prepolymer was obtained via mixing Silicone elastomer

and curing agent at 10:1 ratio (Dow Corning, Midland, MI) and degassing the mixture in a desiccator for 30 min. The prepolymer solution was poured over the Si master mold and cured in the oven at 80°C for 2 h. The elastomeric stamp was then peeled off carefully and cut in 1 × 1 cm^2 squares for micropatterning.

Intermediate patterned glass coverslips: Either Matrigel or laminin (Corning Inc, NY) was diluted 1:10 in L15 medium (Thermo Fisher). 160µl of the solution was added to the top of elastomeric microstamps, and incubated at 4°C overnight. Following incubation, the solution was aspirated, and the surface of the stamps was dried with a low stream of N_2 gas. The stamps were then used to micropattern clean glass coverslips, via microcontact printing. The stamp was brought into complete conformal contact with a glass coverslip substrate for 5min at room temperature. Small weights (38 g) were placed over the PDMS stamp to aid complete protein pattern transfer from PDMS to intermediate glass

Patterned polyacrylamide plates: Polyacrylamide (PA) gel coated plates were prepared following the protocol described previously(3). Briefly, 30mm glass bottom plates (Greiner, Austria) were activated using 2% 3-aminopropyltrimethoxysilane and 1% glutaraldehyde solution. Polyacrylamide/Bis-acrylamide 7.5/0.03% solution was mixed with 0.02% suspension of carboxylated dark red fluospheres (Life Technologies), 0.05% Ammonium Persulfate and 0.4% Temed (Bio-Rad, CA). 7.5 µl of mixed PA solution was applied on each activated plate and covered with, matrigel patterned glass coverslip, to form a thin film. The patterned matrigel on the coverslips was transferred to the surface of the polyacrylamide substrates during gelation. Films were left to polymerize for 30 min in room temperature. Following polymerization, polyacrylamide substrates were incubated in phosphate-buffered saline for at least 1 h and the top coverslips were carefully removed with a razor blade(23)

Preparation of collagen coated dishes: Corning's collagen I (HC) solution was glycated before gel formation. Collagen solution was mixed with ribose in 0.1% acetic acid to final concentration of 250mM ribose, and incubated for 5 days at $4°C$ as previously described[80]. Glycated collagen gel coated plates were prepared according to manufacturer protocol (Corning's coating procedure)[81]. 30mm glass bottom plates (Greiner, Austria) were activated using 2% 3-aminopropyltrimethoxysilane, and 1% glutaraldehyde solution. 25 μl of glycated collagen solution was applied on each activated plate and covered with the 18mm round glass coverslip, to form a thin film. Films were incubated in $37°C$ 1 hour gelation.

Labeling collagen fibers: Collagen fibers were labeled using NHS-Rhodamine in a mole ratio of 1:14, for 2 hours on ice. Non-reacted NHS-Rhodamine was removed by dialysis in 16.65M acetic acid

Spinning disc confocal microscopy: Imaging was done using a Nikon Ti-E inverted microscope equipped with two Evolve EMCCD cameras (Photomatrix), 4 laser lines (405,491,561,642), phase contrast and Yokagawa spinning disc confocal. The dish was maintained at 37 ºC with 5% CO_2/95% air using a cage incubator (Okolab). Imaging was done using 40x 0.95 NA (air) PlanApo Lambda objective (Nikon). Fluorospheres were excited using 642nm laser (Vortran) and Lifeact-RFP was excited with 561nm laser (Cobolt). Lifeact imaging was done using 100x 1.49 NA oil objective (Nikon).

Mechanical probe assay: A rigid Tungsten probe with 25um tip diameter (Signatone) was mounted on a three axes (X-Y-Z) micro-positioning stages (Thorlabs) installed on a custom made adaptor for the microscope stage. The probe's tip is immersed into the culture medium and slightly indents the PA substrate (2um indentation) 130um away from the target cell as previously described(1). By gradually increasing the amplitude of probe oscillations, the beating noise of a spontaneously beating cardiac cell can be monitored as a function of the strength of mechanical interaction with the mechanical probe. We let the cell interact with the probe for 10 minutes before recording the signal and changing the amplitude to allow the cell to reach stationary behavior. Probe oscillation frequency is chosen to be equal to cell frequency in order to separate the effect of pacing from that of noise reduction.

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