Mechanics Regulates ATP-stimulated Collective Calcium Response in Fibroblast Cells

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SUPPLEMENTARY MATERIAL

Movies S1–S5
Movie Captions
Supplementary Materials and Methods
Figures S1–S12

MOVIE CAPTIONS

Movie S1. A fibroblast cell culture on PA gel ($E = 690$ Pa) stimulated by 40 μM ATP.

Movie S2. A fibroblast cell culture on PA gel ($E = 4500$ Pa) stimulated by 40 μM ATP.

Movie S3. A fibroblast cell culture on PA gel ($E = 36000$ Pa) stimulated by 40 μM ATP.

Movie S4. A high density fibroblast cell culture with fewer gap junctions excited by 40 μM ATP. The cell culture was treated with 50 μM palmitoleic adid for 30 minutes prior to ATP excitation.

Movie S5. Fibroblast cells encapsulated in hydrogel ($E = 85$ Pa) were stimulated by 80 μM ATP.

SUPPLEMENTARY MATERIALS AND METHODS

Determination of substrate Young's modulus

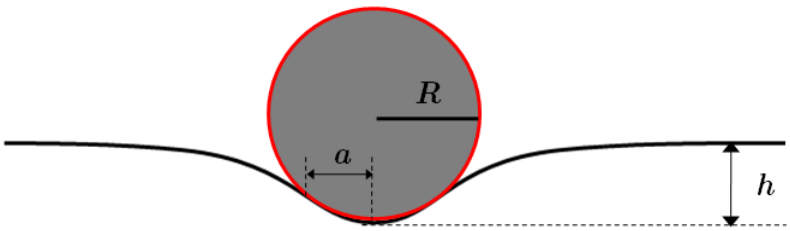

Figure S1. Schematic of setup to determine Young's modulus of gel substrates [46].

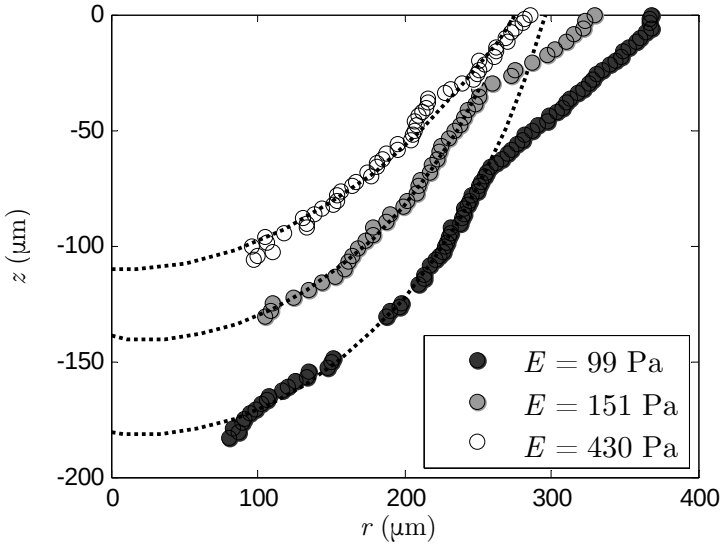

Figure S2. Substrate deformation for Extracel hydrogel.

Table S1. Composition of Extracel hydrogel used in 3D chemosensing experiments (all volumes in μL)

E (Pa)	Gelin	Glycosil	Crosslinker	DMEM	Cells	Total volume (μL)
85	16	16	8	16	4	60
154	16	21.33	10.67	8	4	60
333	16	26.67	13.33	0	4	60

Table S2. Recipe for 25% acrylamide stock solutions (all volumes in mL)

E (Pa)	40% Acrylamide	2% Bis-Acrylamide	DI H_2O	Total volume (mL)
690	3.13	1.25	0.63	5
4500	3.13	0.42	1.46	5
36000	3.13	0.83	1.04	5

Table S3. Composition of PA gel used in 2D chemosensing experiments (all volumes in μL)

E (Pa)	25% Acryl / Bis-A	DI H_2O	10% Ammonium Persulfate	TEMED	1 μm fluorescent beads	Total volume (μL)
690	60	431.75	2.50	0.75	5	500
4500	150	341.75	2.50	0.75	5	500
36000	210	281.75	2.50	0.75	5	500

Quantification of single cell response and oscillations.

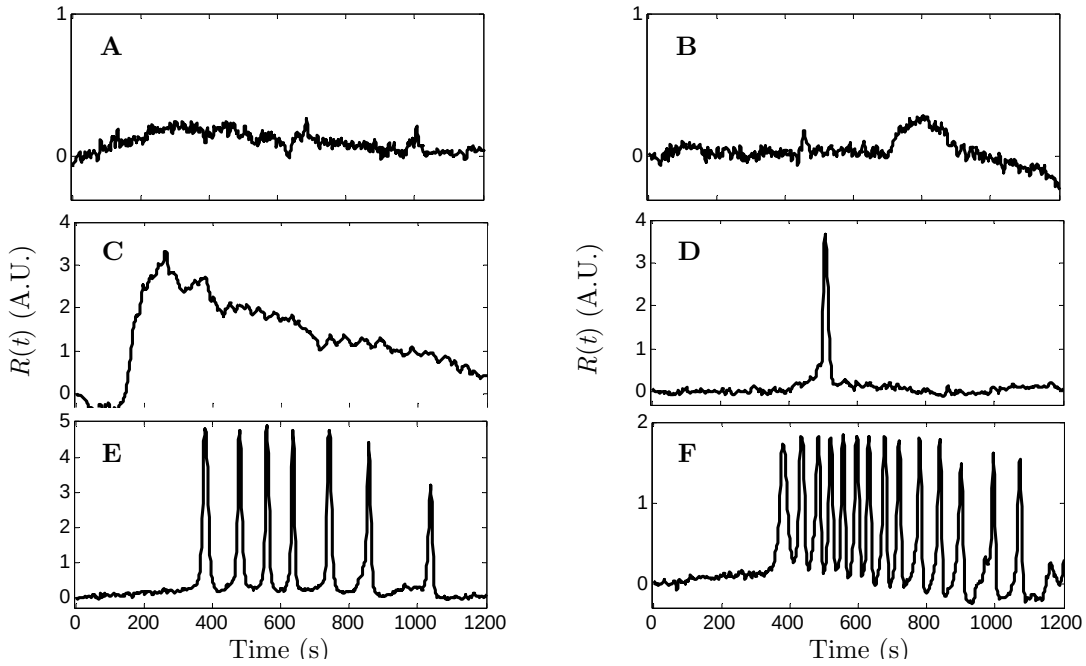

Figure S3. Quantification of a single cell's response: if at any point $R(t) \geq 0.5$, the cell is called a responsive cell. If a responsive cell has two or more peaks as determined by the peakfinder function in MATLAB, the cell is called a responsive, oscillating cell. (A–B) Examples of non-responsive cells. (C–D) Examples of responsive, non-oscillating cells. (E–F) Examples of responsive, oscillating cells. The cell in panel E has 7 peaks, thus 6 oscillations, and the cell on panel F has 15 peaks, thus 14 oscillations.

Independence of oscillation period on substrate stiffness

Fibroblast cell cultures on 2D PA substrates and in 3D hydrogel substrates exhibit Ca^{2+} oscillations during response to ATP. The dominant oscillation period for fibroblast cells in a dense culture mainly decreases with increasing ATP concentration, and is independent of the substrate's Young's modulus.

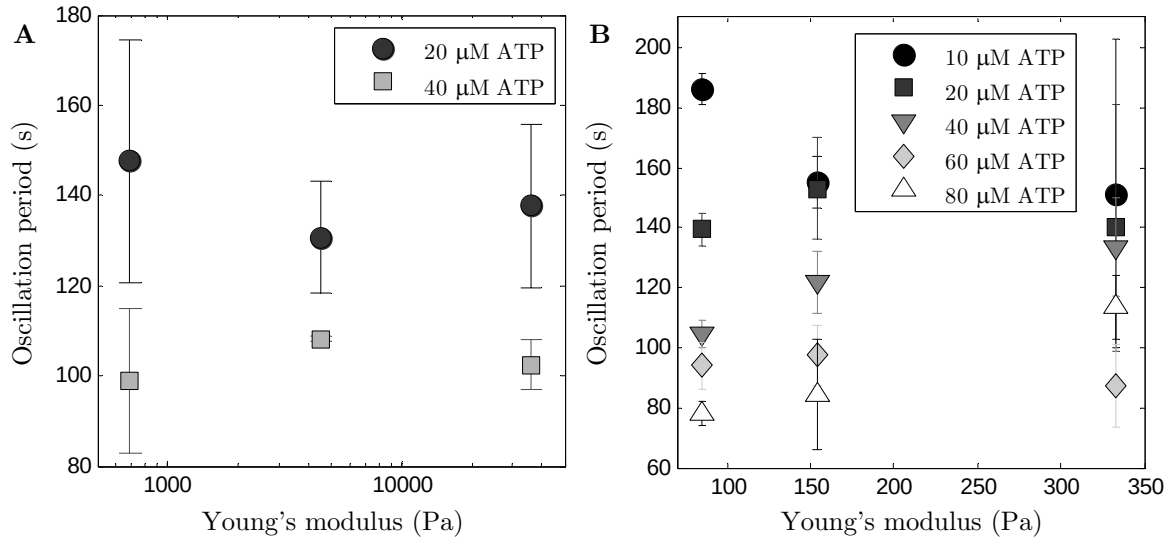

Figure S4. Dynamics of calcium oscillations in fibroblast cells in a 2D culture on PA gels (A) and in a 3D culture inside a hydrogel matrix (B). Values plotted are the average of 3 experiments and error bars are standard errors of the mean of the medians.

Gap junctions immunostaining in 2D fibroblast cultures

We stained gap junctions (connexin-43) in our 2D fibroblast cultures with and without treatment of a gap junction inhibitor, 50 μM palmitoleic acid. Our results show that in the control experiments, Cx43 is preferentially concentrated near cell margins (white arrowheads), suggesting formation of gap junction at cell-cell contacts (Figure S5A), while when treated with 50 μM palmitoleic acid, Cx43 is distributed throughout the cytoplasm, suggesting lack of gap junctions at cell-cell contacts (Figure S5B).

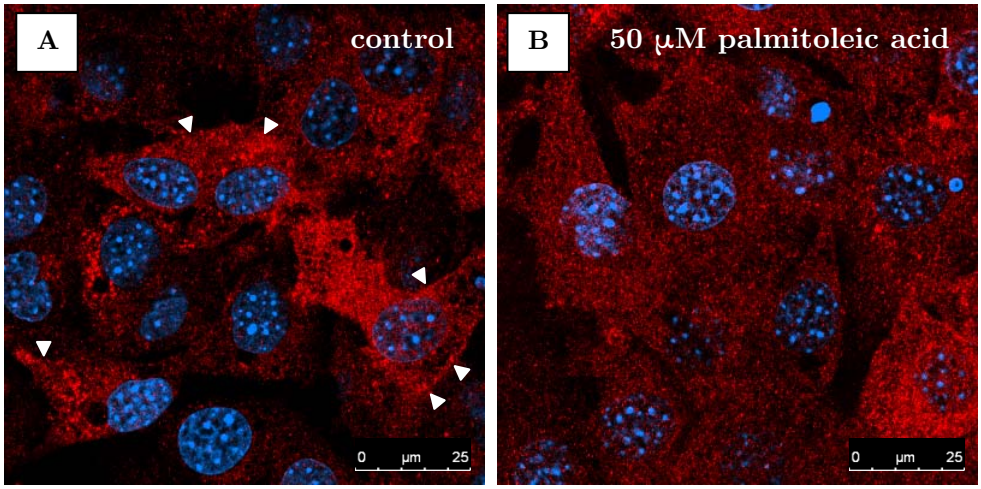

Figure S5. Immunofluorescent microscopy of connexin-43 (gap junctions) in fibroblast cell cultures. Blue: DAPI, Red: Cx43. (A) Cell cultures in the control experiment shows Cx43 concentrated near cell margins (white arrowheads). (B) Cell cultures treated with 50 μM palmitoleic acid shows uniformly distributed Cx43 throughout the cytoplasm.

Actin staining in 2D fibroblast cultures

We stained F-actin in our 2D fibroblast cultures with various jasplakinolide treatment durations. Our results show that 15 minutes of 1 μM jasplakinolide treatment does not result in actin aggregation as reported by Bubb et al. [55], however 2 hours of jasplakinolide treatment does result in some actin aggregation (white arrowheads) near the cell margin (Figure S6).

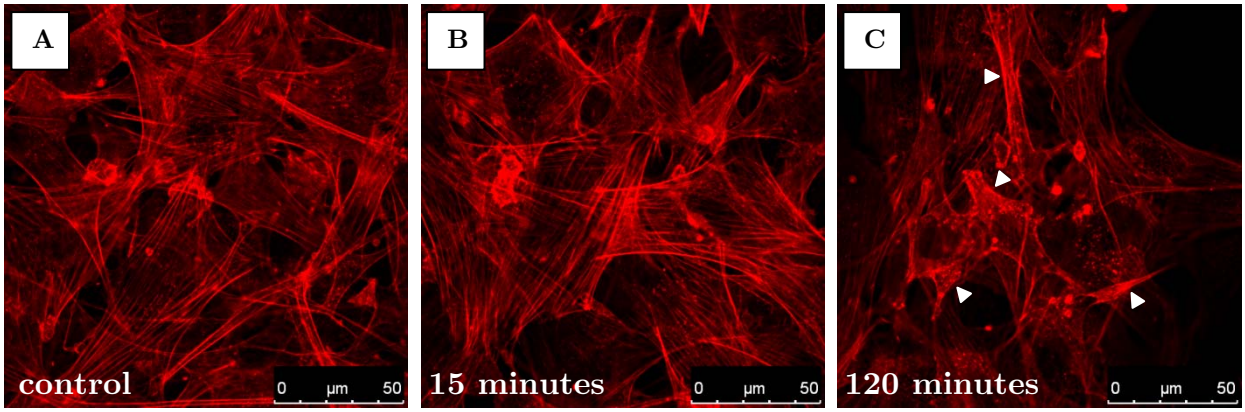

Figure S6. Staining of F-actin in fibroblast cell cultures treated with 1 μM jasplakinolide for various treatment durations. (A) Fibroblast cells show a distribution of actin throughout the cytoplasm. (B) Fibroblast cells after 15 minute treatment of 1 μM jasplakinolide still shows well-distributed actin in the cytoplasm. (C) Fibroblast cells after 2 hour treatment of 1 μM jasplakinolide display actin aggregation near the cell margin (white arrowheads).

Effect of actin inhibitor on cells' responsiveness

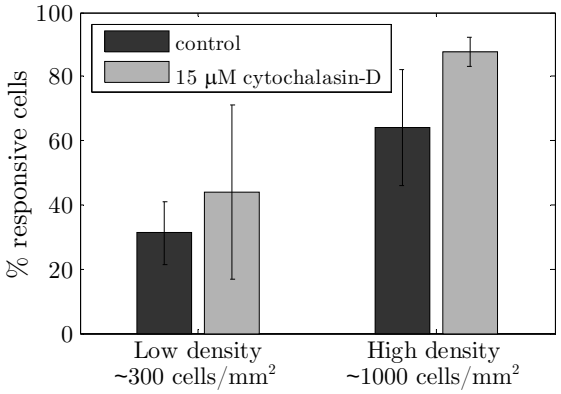

Figure S7. Effect of actin fibers on calcium response in a 2D culture, $E = 36000$ Pa, [ATP] = 20 μM. Cells treated with 15 μM cytochalasin-D for 15 minutes show increased responses; in contrast, individual NIH 3T3 cells show reduced response to ATP upon treatment with 10 μM cytochalasin-D [56]. Values are the average of 3 experiments and error bars are standard errors of the mean.

Diffusion of ATP in 3D hydrogel matrix

We use the dye sulforhodamine-B (MW = 558.66; Invitrogen) to study the diffusion of ATP (MW = 551.14; Sigma-Aldrich) inside the 3D Extracel hydrogel matrix. For all three values of Young's modulus that we employ, we see that ATP diffusion profile is unaffected by the number of cross-links as judged by the gel elasticity. We calculate the diffusion time, t_d, as the time it takes to go from 10% to 90% of the saturation ATP concentration. No dependence of diffusion time on gel elasticity was observed; each t_d has a standard deviation of <7%. The difference in calcium responses of cells seen in Figure 5 is therefore not caused by diffusion of chemicals inside the matrix.

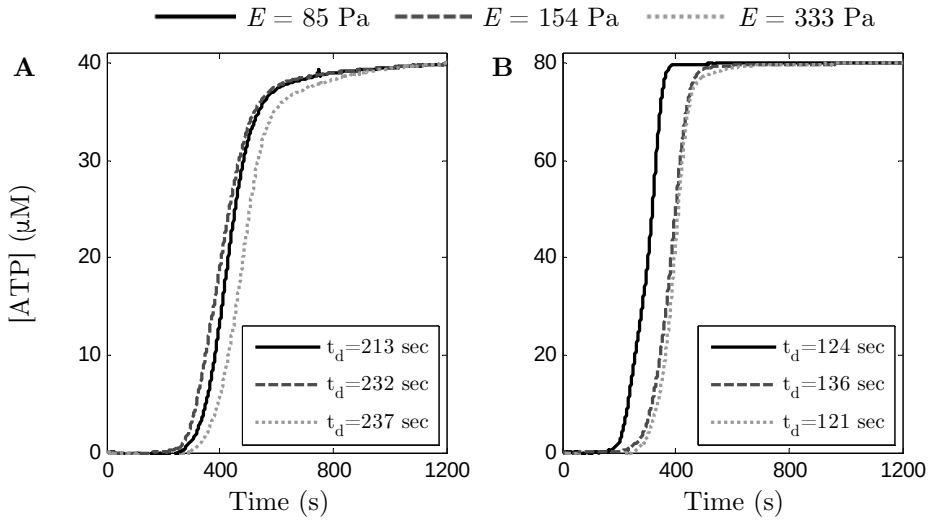

Figure S8. Temporal profile of ATP diffusion inside hydrogel as mimicked by sulforhodamine-B for various values of substrate elasticity for: (A) 20 μM ATP and (B) 80 μM ATP. Intensity is measured in the focal plane of the cells (~30 μm from the bottom of the 3D substrate).

Effect of myosin-based contractility on Ca^{2+} oscillations behavior in 3D substrates

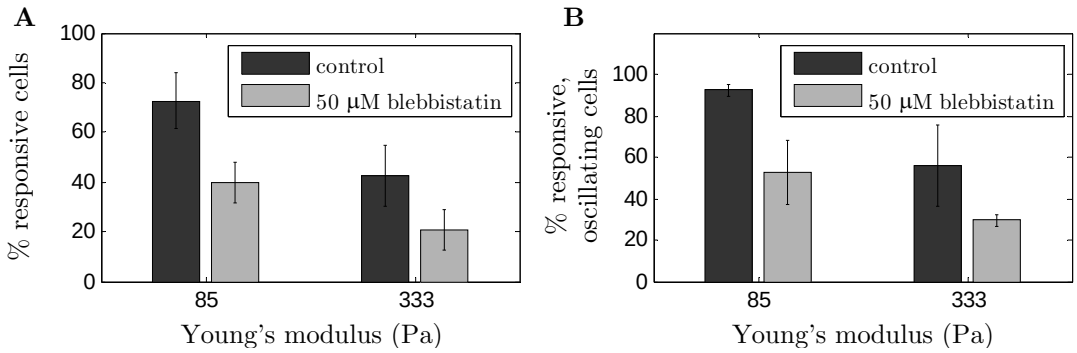

Figure S9. Effect of cell contractility on calcium response in a 3D culture, [ATP] = 60 μM. (A–B) Cells treated with 50 μM blebbistatin for 30 minutes show fewer calcium responses and oscillations. Values are the average of 3 experiments and error bars are standard errors of the mean.

Viability / cytotoxicity test for fibroblast cells in 3D hydrogel matrix treated with blebbistatin

We tested the fibroblast cells viability in the 3D hydrogel matrix when treated with blebbistatin using LIVE/DEAD® Viability/Cytotoxicity Kit (Life Technologies). Tests with various blebbistatin concentrations and treatment durations were performed. Our results show that >95% of encapsulated fibroblast cells treated with blebbistatin with concentration of 100 μM or lower for 90 minutes or less are viable (Figure S10–S11, Table S4–S5).

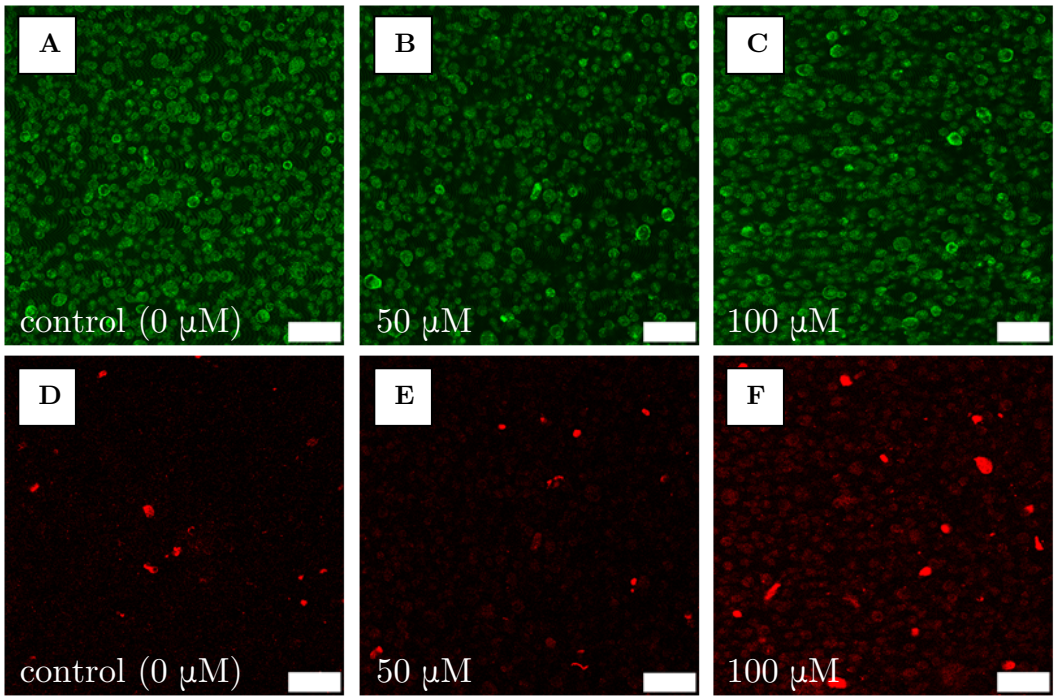

Figure S10. Viability / cytotoxicity test for cells treated with blebbistatin of various concentrations for 30 minutes. Green cells (A–C) are alive and red cells (D–F) are dead. Scale bar = 100 μm.

Table S4. Percentage of live cells in 3D-encapsulated fibroblasts treated with blebbistatin for 30 minutes.

[Blebbistatin] (μM)	Total number of cells	Number of dead cells	% Live cells
0	807	17	98
25	771	4	99
50	665	17	97
75	624	27	96
100	691	21	97

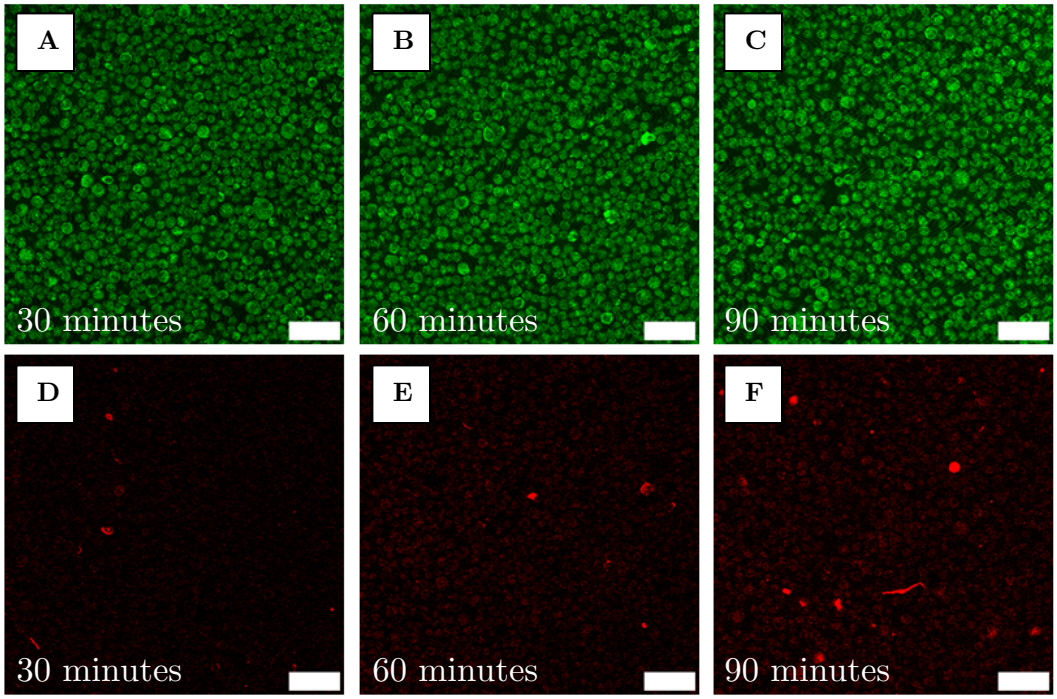

Figure S11. Viability / cytotoxicity test for cells treated with 50 μM blebbistatin for various treatment durations. Green cells (A–C) are alive and red cells (D–F) are dead. Scale bar = 100 μm.

Table S5. Percentage of live cells in 3D-encapsulated fibroblasts treated with 50 μM blebbistatin.

Blebbistatin treatment duration (min)	Total number of cells	Number of dead cells	% Live cells
30	1073	7	99
60	1031	7	99
90	923	14	98

Response of fibroblast cell cultures to various nucleotides

We performed experiments where we excite 2D cell cultures on PA gel with ATP and ADP. We observe that the fractions of cell cultures that show Ca^{2+} response and oscillations are similar at high density (~1000 cells/mm^2). However, at intermediate density (~700 cells/mm^2), the response strongly depends on the nucleotide.

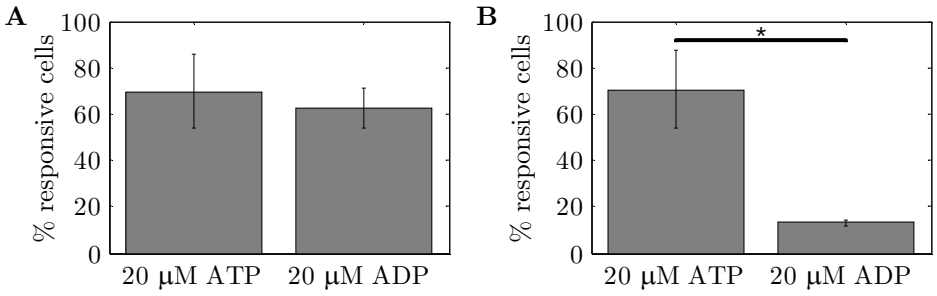

Figure S12. Response of 2D cell cultures ($E = 4500$ Pa) to 20 μM ATP and ADP at high density (A) and intermediate density (B).