Supporting Information

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SI Text

Image Analysis on the Single Cell Level. For a fluorescent video recorded of a cell colony responding to ATP, Fig. S1 demonstrates the procedure to obtain the calcium dynamics on the single cell level. For each cell, its fluorescent intensity is averaged over 40 pixels around the center of mass (Fig. S1*A*) for each frame. The time series obtained this way give the raw intensity profiles as shown in Fig. S1*B*, where the first 100 sec were recorded before ATP reached the cell colony. The baseline intensity I_r can then be obtained by averaging the raw intensity over the first 100 seconds for each cell. Finally the relative changes compared to the baseline result in the response curves $R_i(t)$ as defined in Eq. **1** in the main text and are shown in Fig. S1*C*.

To find the activation time from the response curve R_i, it is first smoothed in Matlab [function: smooth(), method: 'loess'] to remove high frequency fluctuations. A threshold is defined as 10% of the peak response and the activation time is defined as the time when the response curve reaches the threshold level. The activation times obtained this way are shown in Fig. S1*C* with circle symbols.

To evaluate the effect of down-sampling in calculating the fluorescent intensity of a cell, we use randomly selected 100 cells (labeled with $\alpha = 1\ldots100$) in 1,000 frames (labeled with $n = 1\ldots1{,}000$) from 10 independent experiments. First, we locate 45 pixels belong to each cell (when cell density is high, this covers more than 80% of cell area), and calculate the averaged intensity $I_{\alpha n}(45)$ as a benchmark. We then define the down-sampled $I_{\text{alphan}}(m)$ as the intensity averaged over m pixels. These $m < 45$ pixels are randomly chosen from the 45 pixels belong to each cell. Now the relative deviation is calculated as $\Delta_{\alpha n}(m) = \frac{|I_{\alpha n}(m) - I_{\alpha n}(45)|}{I_{\alpha n}(45)}$. The mean and standard deviation of $\Delta_{\alpha n}(m)$ for each m is calculated from the 10^5 data points and are plotted in Fig. S1*D*. The mean deviation of $I_{\alpha n}(40)$ from the benchmark is about 5%.

The cells can also be perturbed by the flow in the 2D culture configuration. To demonstrate the flow effect, we evaluate the mean response curve of 400 cells in a typical experiment where 40 μM ATP was delivered to the cells. As can be seen from Fig. S1*E*, turning on the flow did produce a mild calcium elevation. However, the cells relaxed quickly before ATP arrived. Thus to exclude the influence of flow, we only used the shaded part of the time series for correlation analysis.

Statistical Analysis of Activation Time. To evaluate the probability distribution $P(t)$ of response times t_i within a cell colony, we use a Gaussian estimator to obtain a weighted average:

$$P(t) = \left\langle e^{\frac{(t-t_i)^2}{2\sigma^2}} \right\rangle_i . \qquad \textbf{[S1]}$$

This allows us to quantify the faster response of a high density colony compared to a lower density colony. In addition to the results shown in Fig. 1 in the main text, we report two more experiments in Fig. S2.

Although the cells belong to the high density group systematically responded faster then the low density group in the above experiments, pretreating the cell culture by palmitoleic acid before ATP stimulation, thus blocking gap junction communications, destroyed this temporal order. As can be seen in the Table S1, in 40% of the experiments the cells in the low density group responded earlier.

Here T_l-T_h is the difference of median response times between low density cell groups and high density cell groups. A positive number means the low density group responds later than the high density group in a corresponding experiment.

Locating the Peak Negative Correlations. The cross-correlation functions described in Fig. 2 of main text are noisy, even though average has been taken over all nearest-neighbor pairs. However, the position of peak negative correlation qualitatively represents the time scales for nearest-neighbor cells to "exchange" intensity. To locate the negative, original cross-correlation functions are first smoothed with a long-pass filter for each cell pair [Matlab function: smooth(), method: 'loess'] and then the minimum positions are found (Fig. S3*A*). We plot the smoothed pair-correlation functions of three experiments in Fig. S3 *B–D*, the negative correlation peaks were used to obtain the statistics shown in Fig. 2*B*, *Inset*.

Calcium Dynamics at Low Concentration of ATP. When the cells were stimulated by less than 10 μM ATP, typically only a subpopulation showed detectable calcium responses. If subsequently stimulated again by a high concentration of ATP (approximately 100 μM), this subpopulation also turn out to be the pacemaker cells for the second perturbation. We report two such experiments in Fig. S4.

Correlation Analysis at Intermediate Cell Densities. When excited by a moderate concentration of ATP (<100 μM), cell colonies of intermediate densities (500 cells/mm^2) demonstrate negative correlations (Fig. S5*A*). This is qualitatively similar to high density colonies as well (Fig. 2*B*), which is a signature of intercellular communications between nearby cells.

To better evaluate the influence of intercellular communications during collective chemosensing, we excite the cell colonies of varying densities with 100 μM ATP, where the responses were dominated by a single transient rise and subsequent relaxation. We can then use the nearest-neighbor pair-correlation functions to quantify the strength of intercellular communications.

For each pair of cells, the correlation function $C_{ij}(t)$ at zero time delay $t = 0$ is related to the intercellular signaling. As a result, when cell density increases, $C_{ij}(0)$ also increases (Fig. S5*B*, *Upper*).

When the responses of two cells $\{ij\}$ are not synchronized, the position of their peak correlation T_{ij} is away from 0. Fluctuation in T_{ij} measures the level of synchronization and so is also related to the intercellular signaling. We plot the standard deviation of T_{ij} for approximately 1,000 cell pairs at varying cell densities in Fig. S5*B*, *Lower*. As can be seen, when cell density increases, the nearest-neighbor cells response in a more synchronized fashion.

Statistics of Calcium Oscillations. The calcium dynamics in our experiments can be divided into three phases: spontaneous oscillations when cells were exposed to pure buffer flow, ATP-induced flickering when cells were stimulated with ATP flow, and the subsequent relaxation from the ATP perturbation. To characterize the average frequencies at each phase, we calculate the cumulative count of flickering events and perform linear fits within each phases. As can be seen from Fig. S6, calcium oscillations of cells embedded in hydrogel persisted longer than in the 2D culture conditions.

Fourier Spectrum of Isolated Cells in Hydrogel. The low density cell colony encapsulated in hydrogel demonstrate less clear structures

in the Fourier spectrum as compared with the spectrum of high density colonies (Fig. S7). To calculate the Fourier spectrum, first we perform fast-Fourier-transformation (FFT) to the response curves $R_i(t)$ of all the cells in the field of view and obtain $G_i(f) = |FFT(R_i)|$, then the mean spectrum is calculated by averaging $G_i(f)$, $i \in$ cells in the field of view.

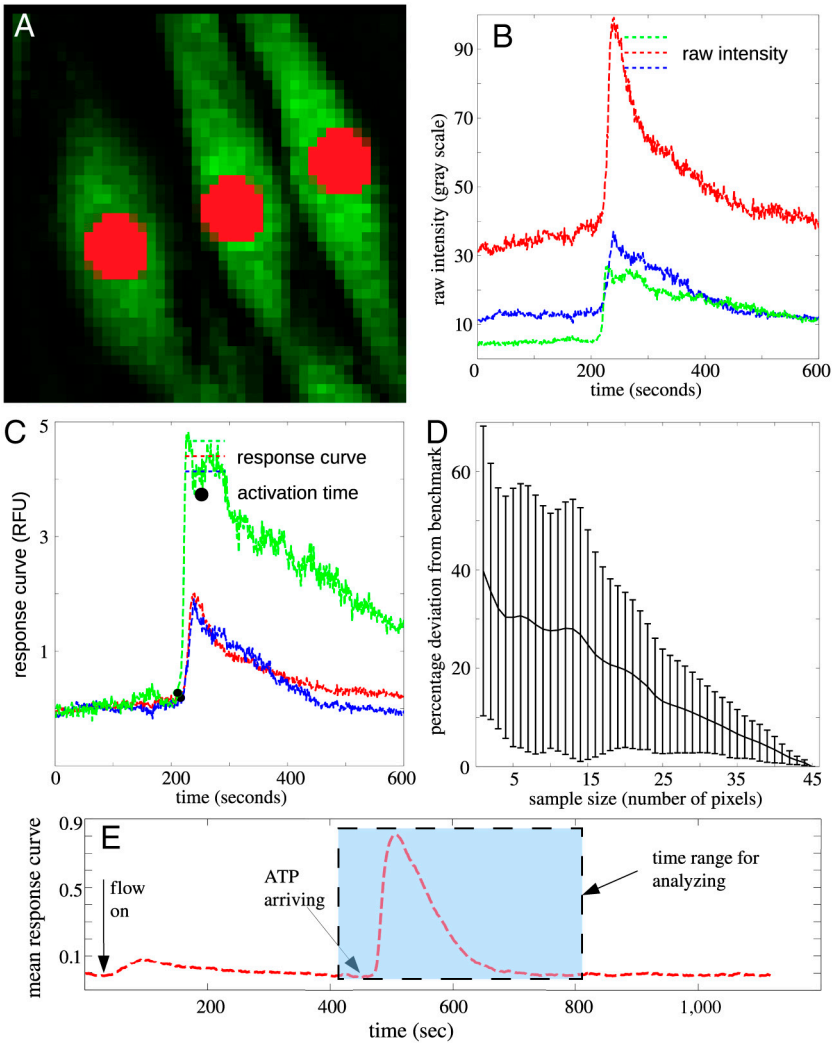


Fig. S1. Image analysis of three representative cells responding to ATP stimulation. (*A*) Green: Fluorescence signal from calcium indicator. Red: The area to be averaged in order to obtain the response of each cell. (*B*) Raw intensities recorded for the three cells as a function of time, averaging over the area indicated in *A*. (*C*) Response curves obtained from the time series in *B*. The filled circle symbols label the activation time for each cell. (*D*) The effect of down-sampling in calculating the fluorescent intensity. (*E*) The mean response curve of 400 cells from a typical experiment. The first peak represents the flow perturbation, and the second peak represents the response to ATP.

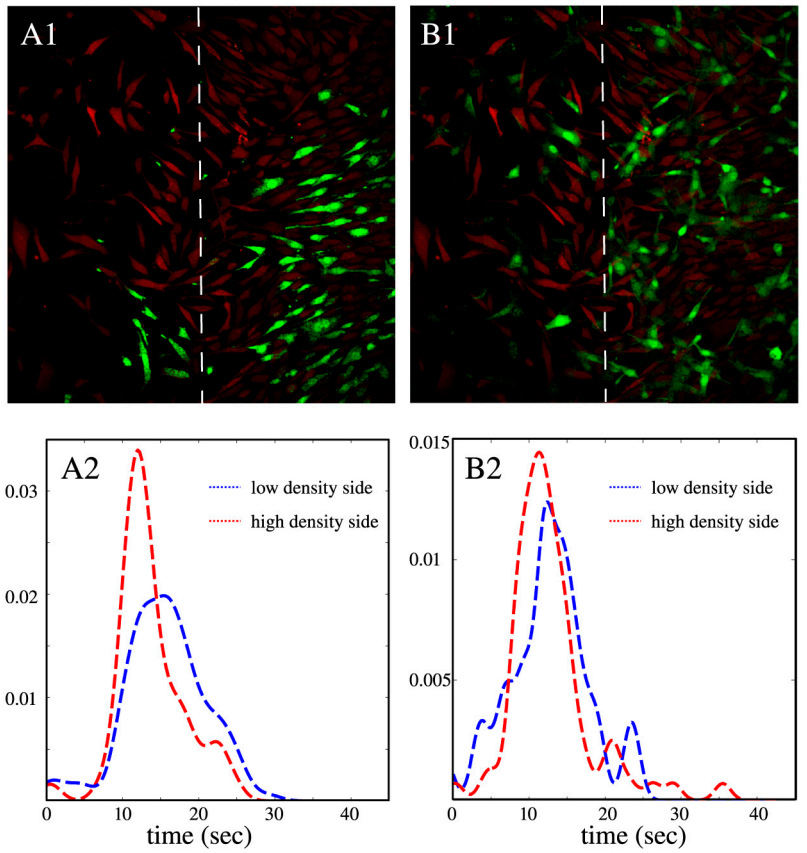


Fig. S2. (*A1* and *A2*) A snapshot and the probability distribution of response times for a cell colony with stepwise density. Seventy-four cells in the low density side and 150 cells in the high density side were sampled. (*B1* and *B2*) Forty-two cells in the low density side and 122 cells in the high density side were sampled.

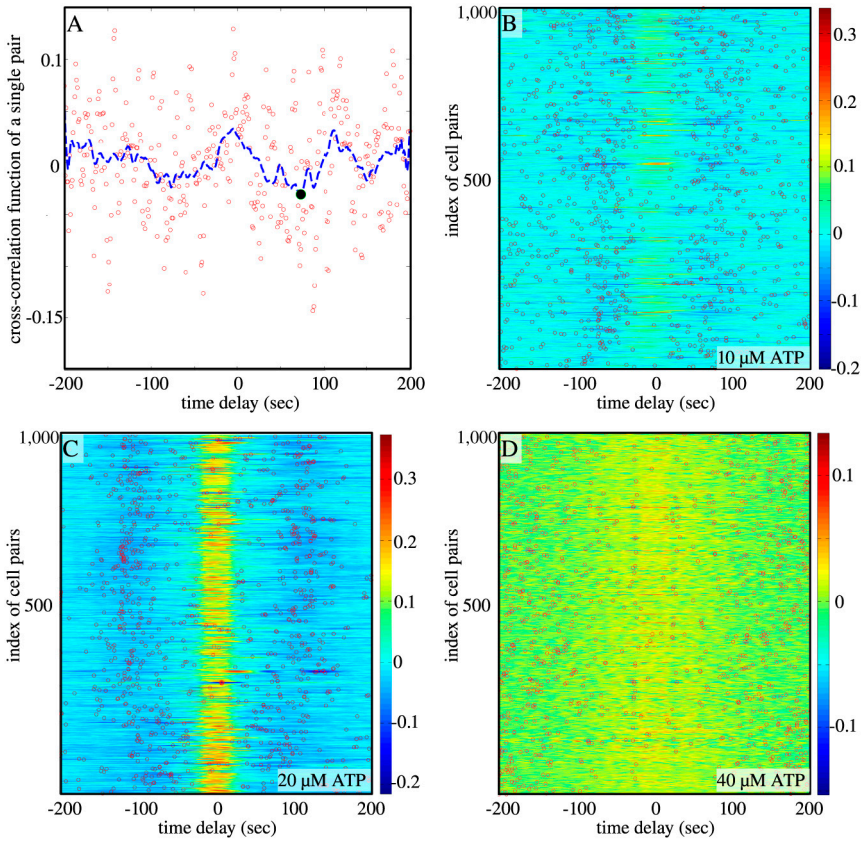


Fig. S3. (*A*) The normalized cross-correlation function for a typical nearest-neighbor pair. Open circle: raw data, dashed line: smoothed curve, filled circle: negative peak located. (*B* and *C*) Smoothed pair-correlation functions of three experiments. The corresponding ATP concentrations are 10 μM, 20 μM, and 40 μM, respectively.

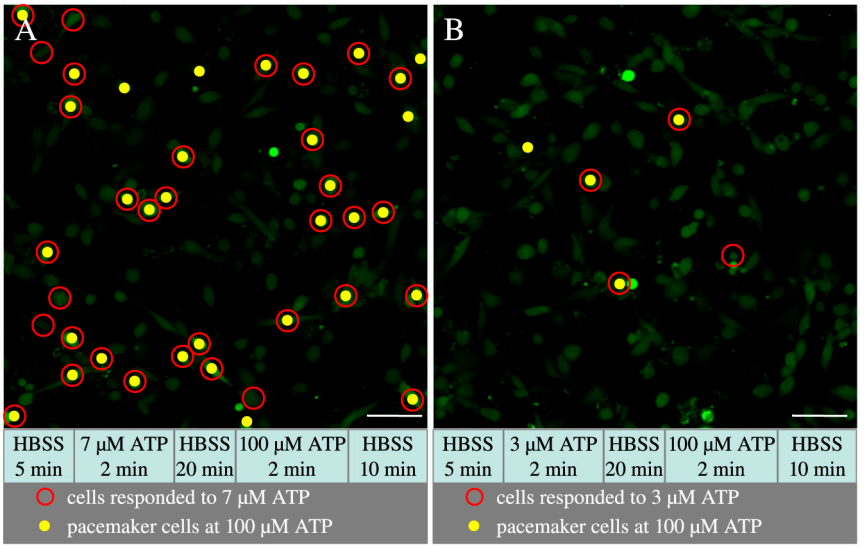


Fig. S4. Cell responses to two pulses of ATP. (*A*) A 2-min pulse of 7 μM ATP followed by a subsequent 2-min pulse of 100 μM ATP were delivered to a cell colony by continuous flow. The red circles label the 34 cells in the field of view showing detectable response to the 7-μM pulse and the yellow dots label the first 34 cells responded to the 100-μM pulse. (*B*) A 2-min pulse of 3 μM ATP followed by a subsequent 2-min pulse of 100 μM ATP were delivered to a cell colony by continuous flow. The red circles label the 4 cells in the field of view showing detectable response to the 3-μM pulse and the yellow dots label the first 4 cells responded to the 100-μM pulse. Scale bar: 100 μm.

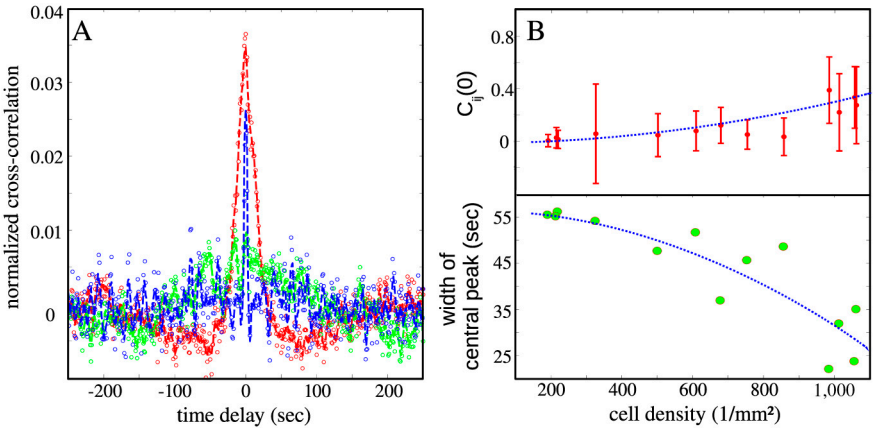


Fig. S5. Cross-correlation analysis for intermediate cell densities. (*A*) Cross-correlation function obtained by averaging apprximately 1,000 nearest-neighbor cell pairs at moderate cell densities (500 cells/mm^2). (*B*) Nearest-neighbor correlation analysis for cell colonies with varying densities excited by 100 μM ATP. *Upper*: Mean and standard deviation of $C_{ij}(0)$, the pair-correlation function at 0 time delay. *Lower*: Standard deviation of T_{ij}. T_{ij} is the time delay corresponding to the positive peak correlation between cell *i* and cell *j*, and $\langle T_{ij} \rangle = 0$. Note this quantity also represents the width of the central peak as shown in main text Fig. 2*A*. Dashed curve: quadratic fits as a guide to the eye.

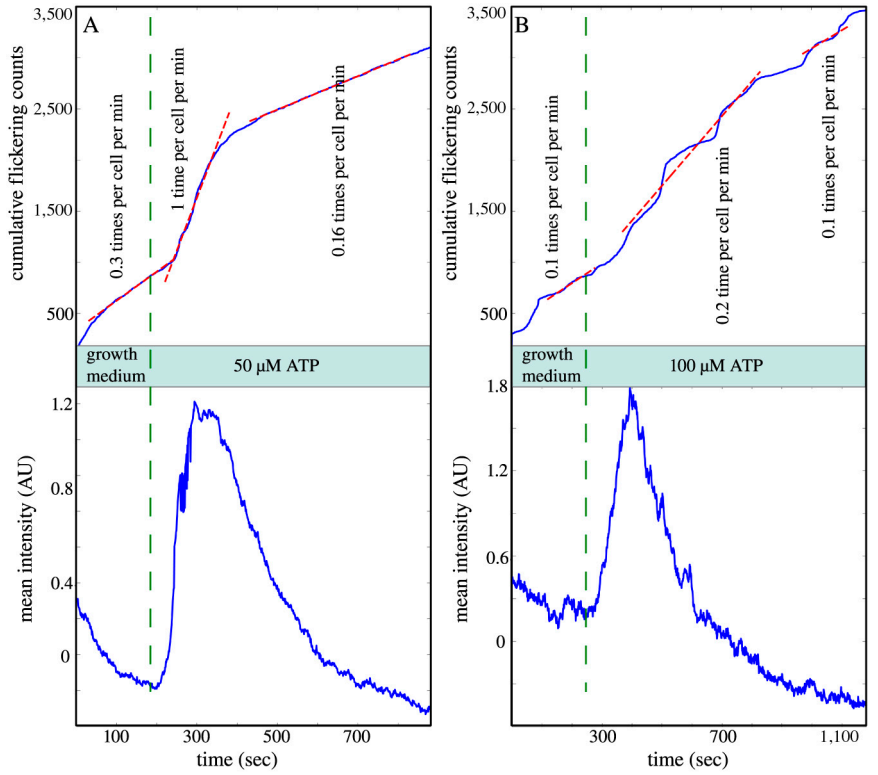


Fig. S6. Cumulative count of calcium flickering events within a high density cell colony is correlated with the mean response curve. The mean response curve can be used to separate the calcium dynamics into three phases: spontaneous oscillations when cells were exposed to pure buffer flow, ATP-induced flickering when cells were stimulated with ATP flow, and subsequent relaxation from the ATP perturbation. The linear fit of the cumulative count at the three phases yields the average flickering frequencies correspondingly. (*A*) Calcium dynamics of a typical experiment where a high density cell colony cultured on glass substrate was stimulated by 50 μM ATP. (*B*) Calcium dynamics of a typical experiment where a high density cell colony embedded in hydrogel was stimulated by 100 μM ATP.

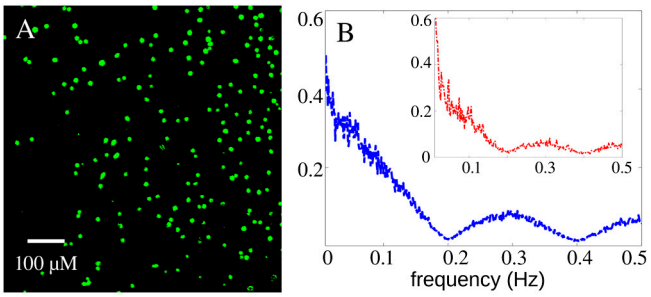
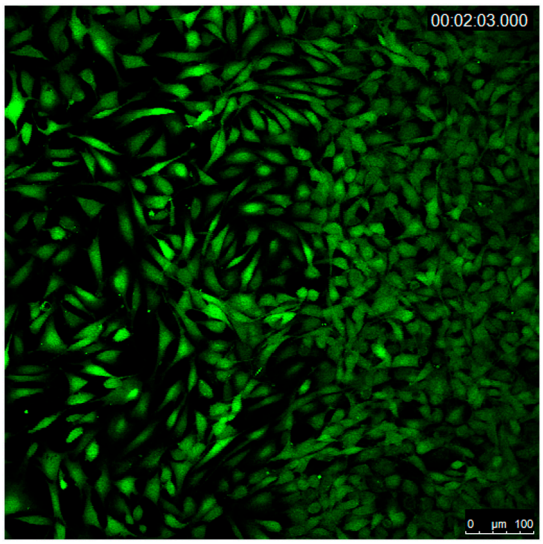
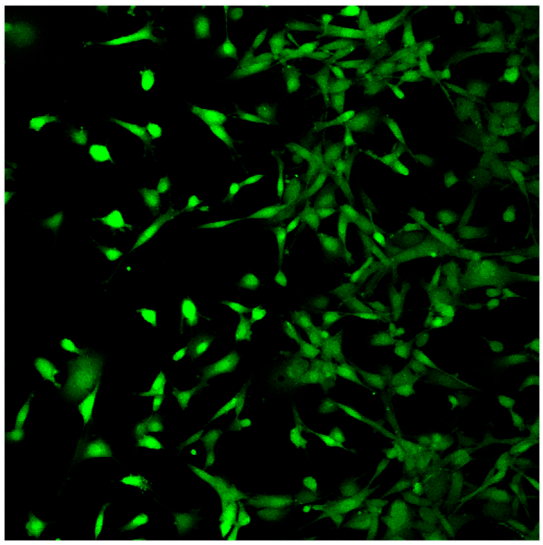


Fig. S7. The mean Fourier spectrum of the response of isolated cells to 100 μM ATP. (*A*) A snapshot of the fluorescent image. (*B*) The Fourier spectrum averaged over all cells shown in *A*. As a comparison the mean spectrum of a closely packed colony is shown as an inset, which is the same as Fig. 5*C* of the main text.



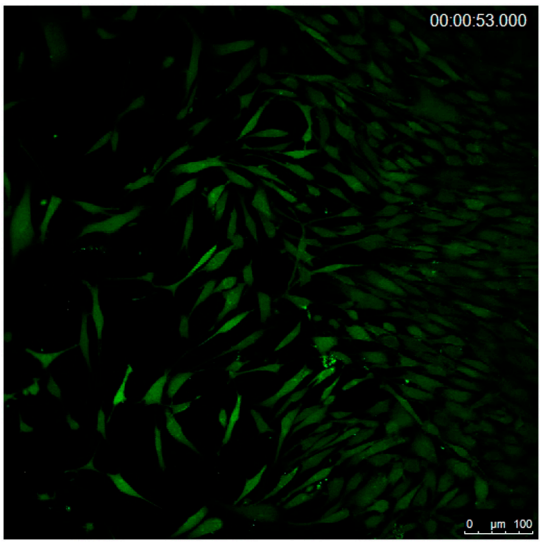
Movie S1. Fibroblast cell colonies with stepwise density grown on a glass substrate was stimulated by 40 μM ATP.

Movie S1 (WMV)



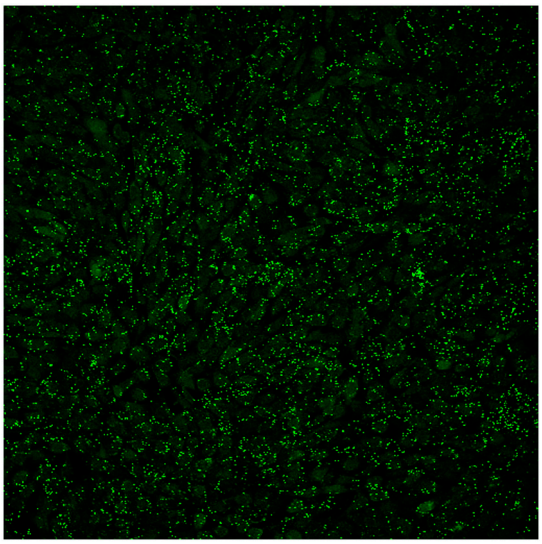
Movie S2. Fibroblast cell colonies with stepwise density grown on a glass substrate was stimulated by 40 μM ATP.

Movie S2 (WMV)



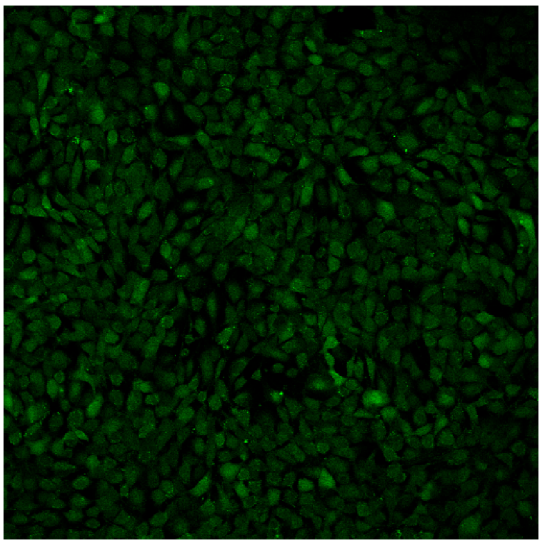
Movie S3. A fibroblast cell colony with stepwise density grown on a glass substrate stimulated by 100 μM ATP.

Movie S3 (WMV)



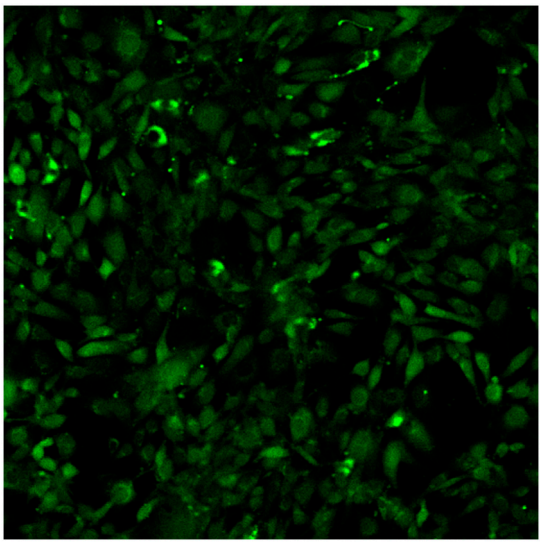
Movie S4. High density colonies of fibroblast cells were stimulated by 10 μM ATP.

Movie S4 (WMV)



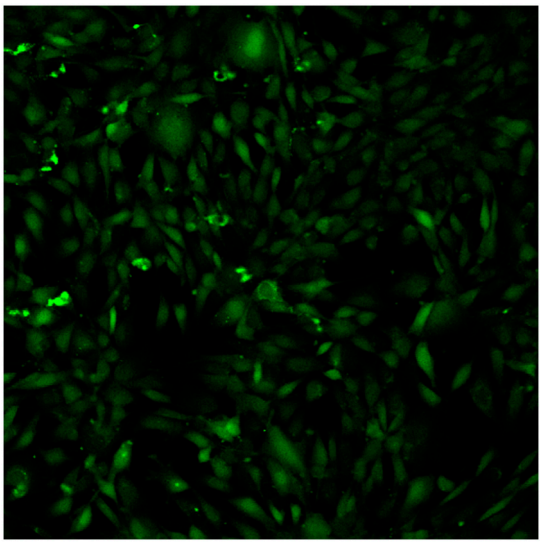
Movie S5. High density colonies of fibroblast cells were stimulated by 10 μM ATP.

Movie S5 (WMV)



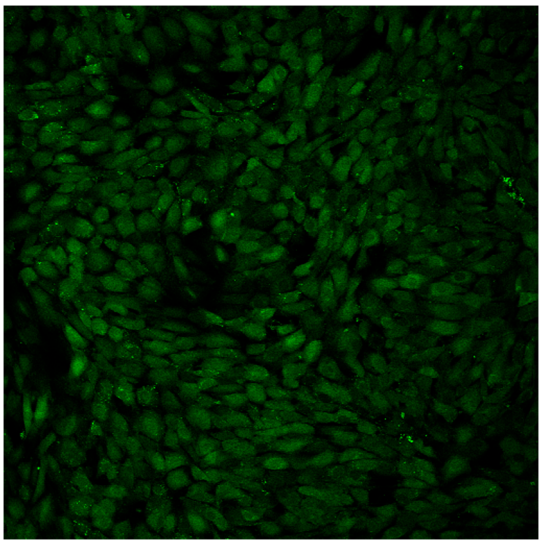
Movie S6. High density colonies of fibroblast cells were stimulated by 10 μM ATP.

Movie S6 (WMV)



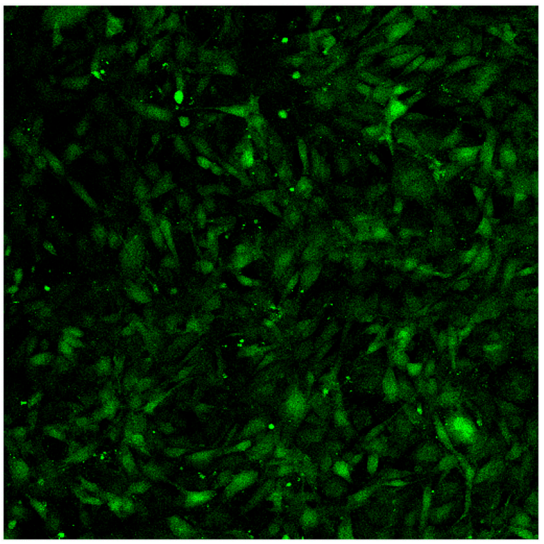
Movie S7. High density colonies of fibroblast cells were stimulated by 10 μM ATP.

Movie S7 (WMV)



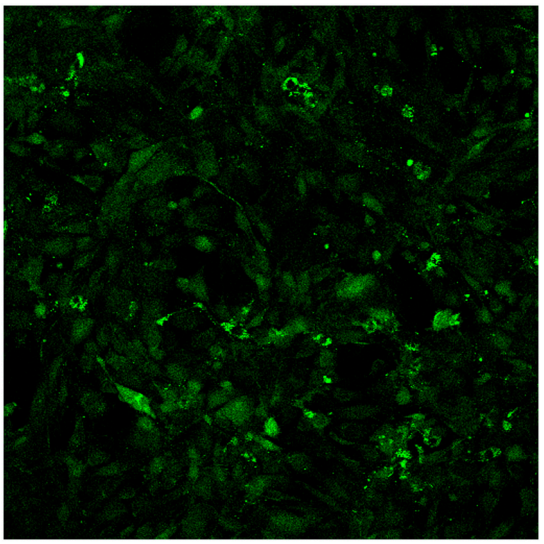
Movie S8. High density colonies of fibroblast cells were stimulated by 20 μM ATP.

Movie S8 (WMV)



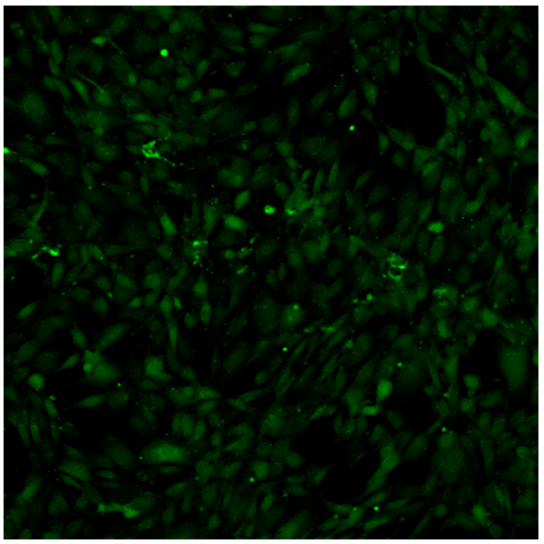
Movie S9. High density colonies of fibroblast cells were stimulated by 20 μM ATP.

Movie S9 (WMV)



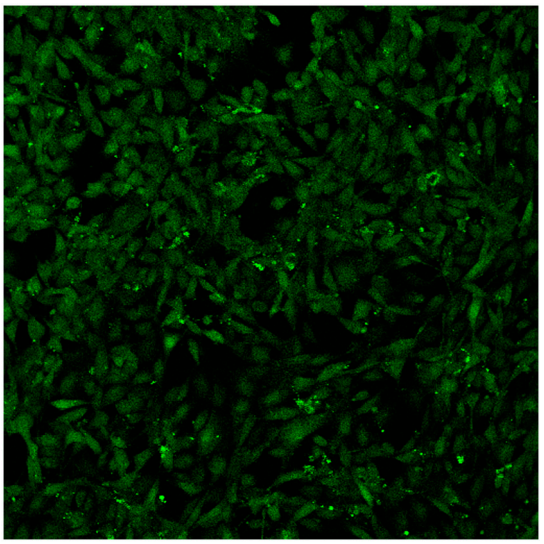
Movie S10. High density colonies of fibroblast cells were stimulated by 20 μM ATP.

Movie S10 (WMV)



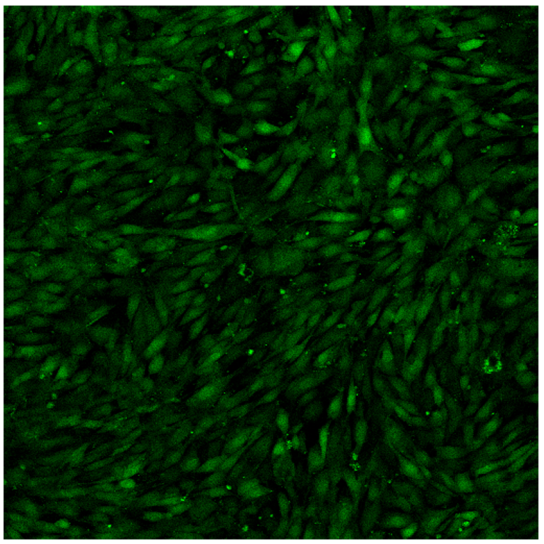
Movie S11. High density colonies of fibroblast cells were stimulated by 20 μM ATP.

Movie S11 (WMV)



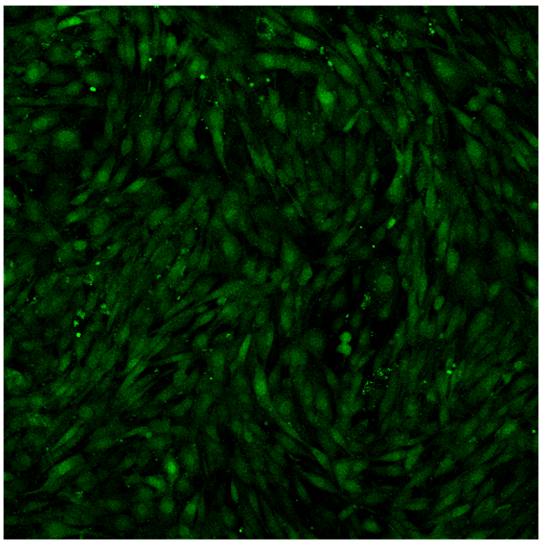
Movie S12. High density colonies of fibroblast cells were stimulated by 40 μM ATP.

Movie S12 (WMV)



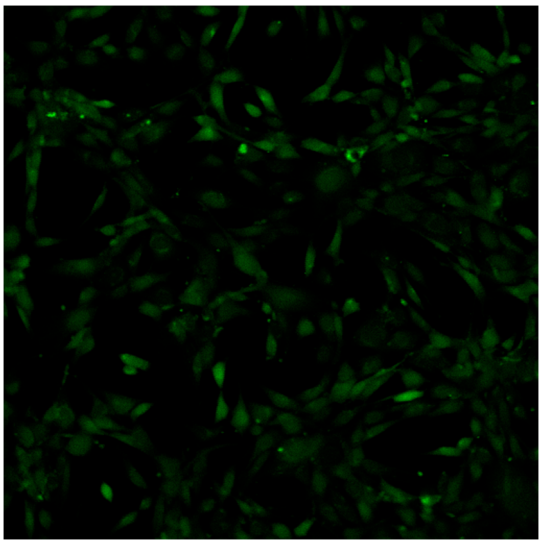
Movie S13. High density colonies of fibroblast cells were stimulated by 40 μM ATP.

Movie S13 (WMV)



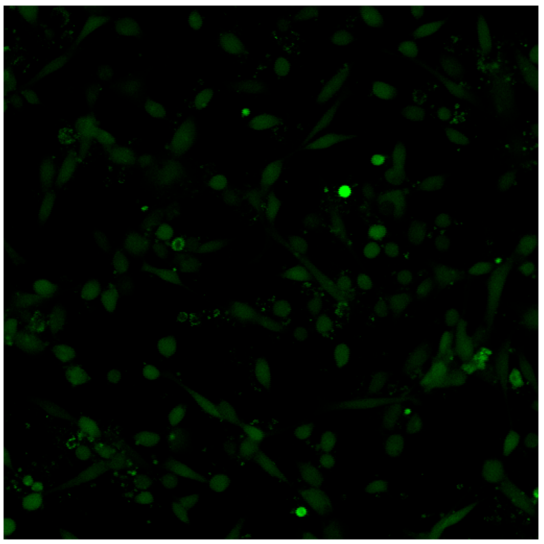
Movie S14. High density colonies of fibroblast cells were stimulated by 40 μM ATP.

Movie S14 (WMV)



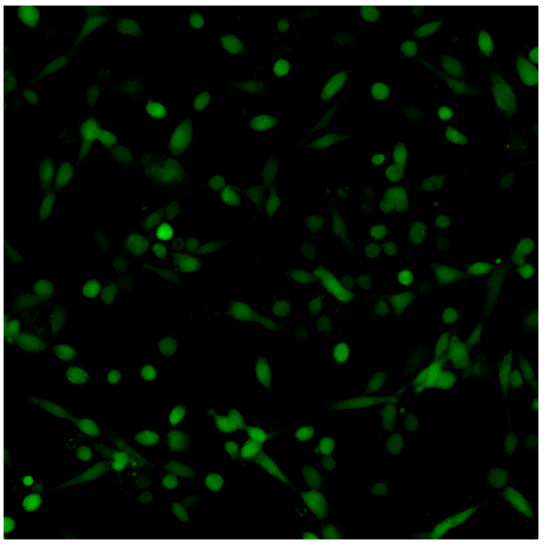
Movie S15. High density colonies of fibroblast cells were stimulated by 40 μM ATP.

Movie S15 (WMV)



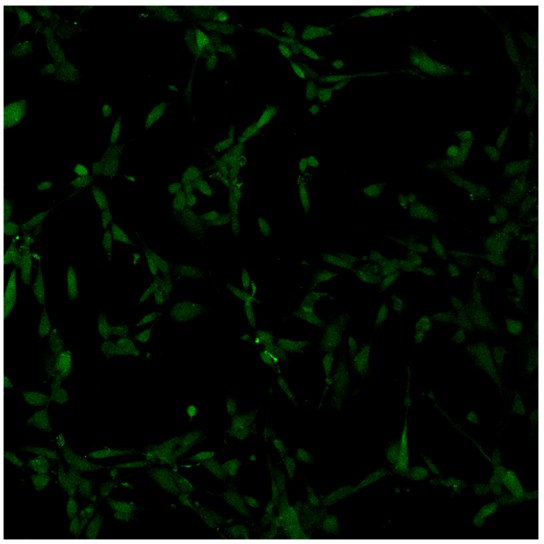
Movie S16. A medium density colony of fibroblast cells was stimulated by 7 μM ATP.

Movie S16 (WMV)



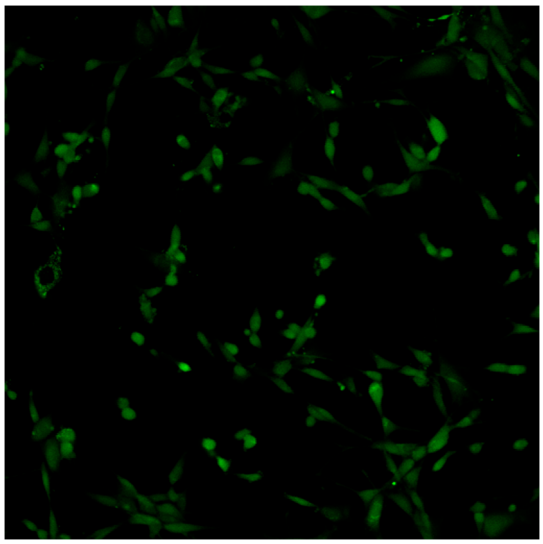
Movie S17. A medium density colony of fibroblast cells was stimulated by 100 μM ATP.

Movie S17 (WMV)



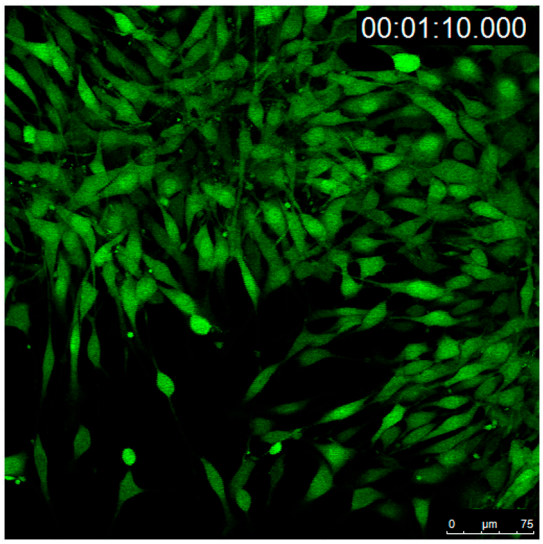
Movie S18. A medium density cell colony of fibroblast cells was stimulated by 20 μM ATP.

Movie S18 (WMV)



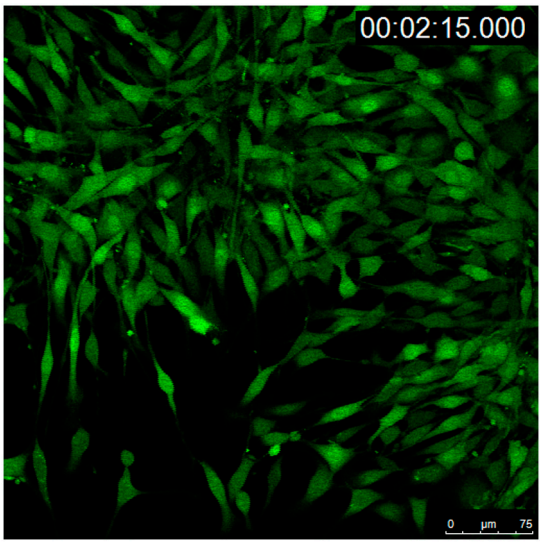
Movie S19. A medium density cell colony of fibroblast cells was stimulated by 40 μM ATP.

Movie S19 (WMV)



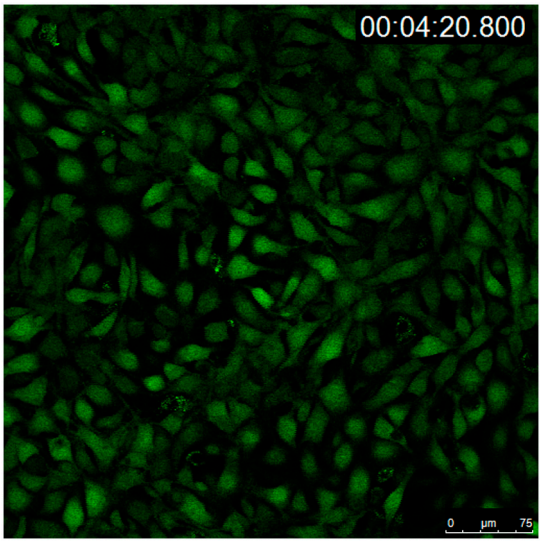
Movie S20. A high density cell colony excited by two pulses of ATP. The first pulse containing 40 μM ATP lasted for 1 min. The second pulse containing 100 μM ATP lasted for 10 min (Mov. S21). In between the two pulses, cells were allowed to relax for 20 min in HBSS.

Movie S20 (WMV)



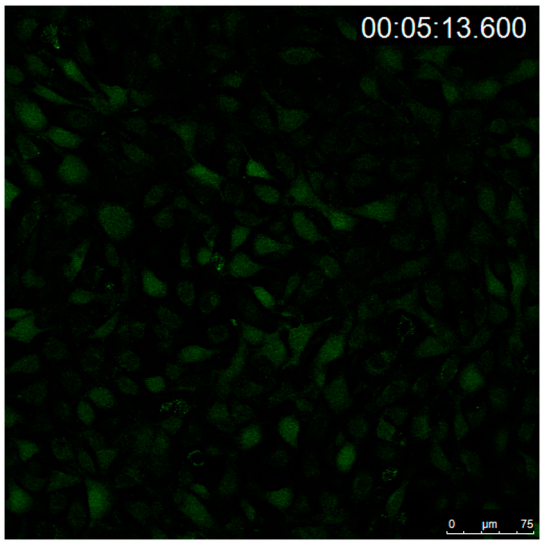
Movie S21. A high density cell colony excited by two pulses of ATP. The first pulse containing 40 µM ATP lasted for 1 min (Mov. S20). The second pulse containing 100 µM ATP lasted for 10 min. In between the two pulses, cells were allowed to relax for 20 min in HBSS.

Movie S21 (WMV)



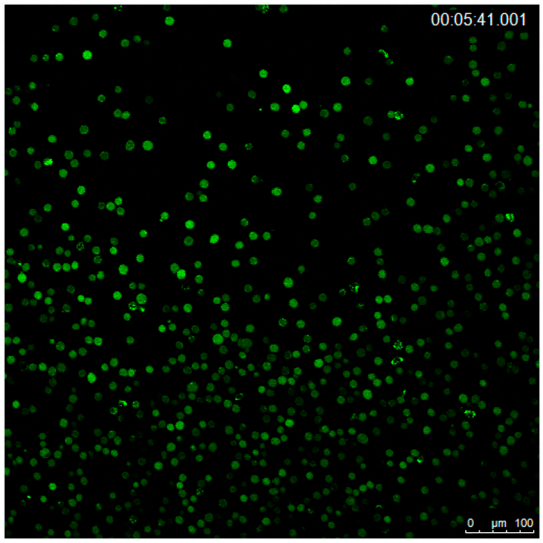
Movie S22. A high density cell colony excited by two pulses of ATP. The first pulse containing 40 µM ATP lasted for 1 min. The second pulse containing 100 µM ATP lasted for 10 min (Mov. S23). In between the two pulses, cells were treated by palmitoleic acid for 20 min.

Movie S22 (WMV)



Movie S23. A high density cell colony excited by two pulses of ATP. The first pulse containing 40 μM ATP lasted for 1 min (Mov. S22). The second pulse containing 100 μM ATP lasted for 10 min. In between the two pulses, cells were treated by palmitoleic acid for 20 min.

Movie S23 (WMV)



Movie S24. Fibroblast cells encapsulated in hydrogel with stepwise cell density were stimulated by 100 μM ATP.

Movie S24 (WMV)

Table S1 Difference in median response times between high density cell group and low density cell group of cell colonies with stepwise densities

Experiment index	1	2	3	4	5	6	7	8	9	10
T_l-T_h (sec)	1	3	−3	−2	1	2	−1	−2	3	0

The cell colonies were pretreated by palmitoleic acid. Ten independent experiments are reported.