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ORIGINAL RESEARCH PAPER

Mechanical stimuli modulate intracellular calcium oscillations: a pathological model without chemical cues

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Abstract
Objective To control the oscillatory behavior of the intracellular calcium ($[Ca^{2+}]_i$) concentration in endothelial cells via mechanical factors (i.e., various hydrostatic pressures) because $[Ca^{2+}]_i$ in these cells is affected by blood pressure.
Results Quantitative analyses based on real-time imaging showed that $[Ca^{2+}]_i$ oscillation frequency and relative concentration increased significantly when 200 mm Hg pressure, mimicking hypertension, was applied for >10 -min. Peak height and peak width decreased significantly at 200 mm Hg. These trends were more marked as the duration of the 200 mm Hg pressure was increased. However, no change was observed under normal blood pressure conditions 100 mm Hg.
Conclusion We generated a simple in vitro model to study $[Ca^{2+}]_i$ behavior in relation to various pathologies and diseases by eliminating possible complicating effects induced by chemical cues.

Keywords Blood pressure · Calcium signaling · Endothelial cells · Hydrostatic pressure · Intracellular Ca^{2+} concentration · Live imaging · Pathological model

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Introduction

Intracellular Ca^{2+} is a universal second messenger involved in many signal transduction pathways (Bootman et al. 2012). It has attracted much attention in the study of physiological and/or pathological processes in cells. In most cases, elevated levels of $[Ca^{2+}]_i$ are closely related to various pathologies, such as hypertension, inflammation, cardiac and neural diseases (Missiaen et al. 2000). Dynamic oscillatory changes in $[Ca^{2+}]_i$ trigger biochemical signals that affect enzyme activities, gene expression, and other cellular functions (Song et al. 2012). This oscillatory function helps the cell avoid high levels of $[Ca^{2+}]_i$ that are toxic over the long term. These findings suggest that $[Ca^{2+}]_i$ needs to be studied in conjunction with its oscillatory changes.

Therefore, the concentration and/or dynamics of $[Ca^{2+}]_i$ should be determined in advance for in vitro studies. Then, various pathological models can be conducted in relation to $[Ca^{2+}]_i$ and we may further

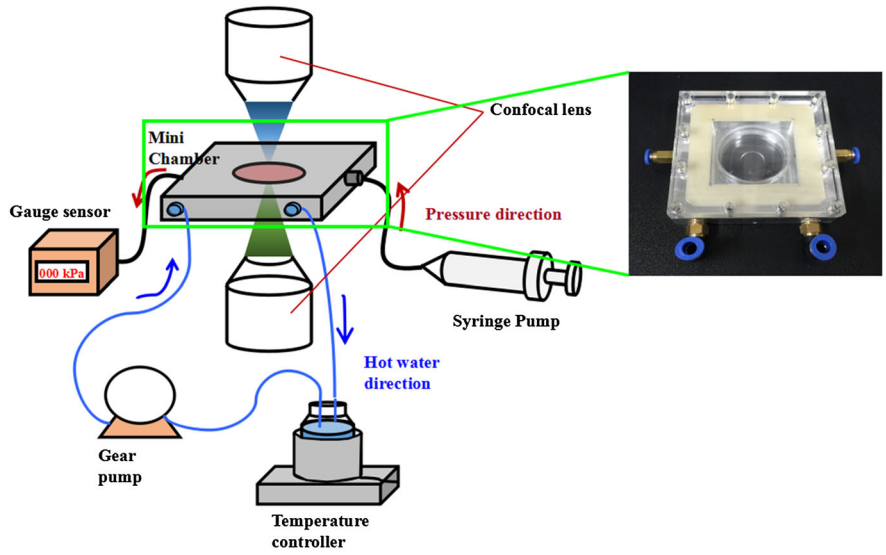

Fig. 1 Schematic diagram of the mini-chamber pressure system developed to expose live cells to hydrostatic pressure (HP). A mini chamber connected to a pressurizing system was mounted on a confocal microscope. A water-circulation system maintained the chamber at 37 °C

consider any treatments to have those pathological patterns to be normal or healthy status. Most studies have employed chemical cues to control $[Ca^{2+}]_i$ behaviors and mimic pathological status for further drug screening (Hongo et al. 2015). However, chemical cues might evoke several signaling pathways or share the receptors with tested drugs which result in unclear effects. On the other hand, various studies of normal and pathological cells have adopted mechanical stimuli to allow for mechanical biomimetic conditions; cellular responses observed under biomimetic environmental conditions are expected to be more similar to those observed in vivo status, as in the human body (Estrada et al. 2011). Also, $[Ca^{2+}]_i$ behavior of vascular endothelial and smooth muscle cells is closely related to blood pressure condition (Adamova et al. 2009). This implies that mechanical pressure may be a potential option for arbitrarily control of $[Ca^{2+}]_i$ and mimic pathological status.

In this study, we employed hydrostatic pressure at different magnitudes and durations to investigate the potential of controlling $[Ca^{2+}]_i$ concentration and/or oscillations in vitro to mimic pathological conditions without any chemical cues. The results are expected to contribute to additional studies on screening of potential drugs and explaining the basic mechanisms of normal or pathological signaling pathways by eliminating possible unclear effects of chemical cues currently adopted to control $[Ca^{2+}]_i$ behavior.

Materials and methods

Acquisition, culture, and seeding of endothelial cells

Human umbilical vein endothelial cells were purchased from Lonza. A commercially-available endothelial growth medium (Lonza) was used for culture. The cells were kept at 37 °C under 5% CO_2.

HP in a mini-chamber system

The custom-designed mini-chamber system used in the present study is shown in Fig. 1. It consists of a small chamber with a confocal dish, syringe pump, and pressure gauge (SMC Pneumatics). It was maintained at 37 °C by a water reservoir and gear pump.

After seeding the cells at passage #5 onto a confocal dish, we allowed 24 h for stabilization. Then, 100 or 200 mm Hg hydrostatic pressure (HP) was applied to simulate normal and hypertensive status, respectively. The duration of pressure engagement varied: 10 min for 100 mm Hg and 1, 5, 10, or 15 min for 200 mm Hg. Each application started 10 min after mounting the chamber onto a confocal microscope. For all groups, real time images were recorded during pressurization and another 10 min after de-pressurization. A group that was not exposed to pressure was included as a control. Therefore, the groups included were as

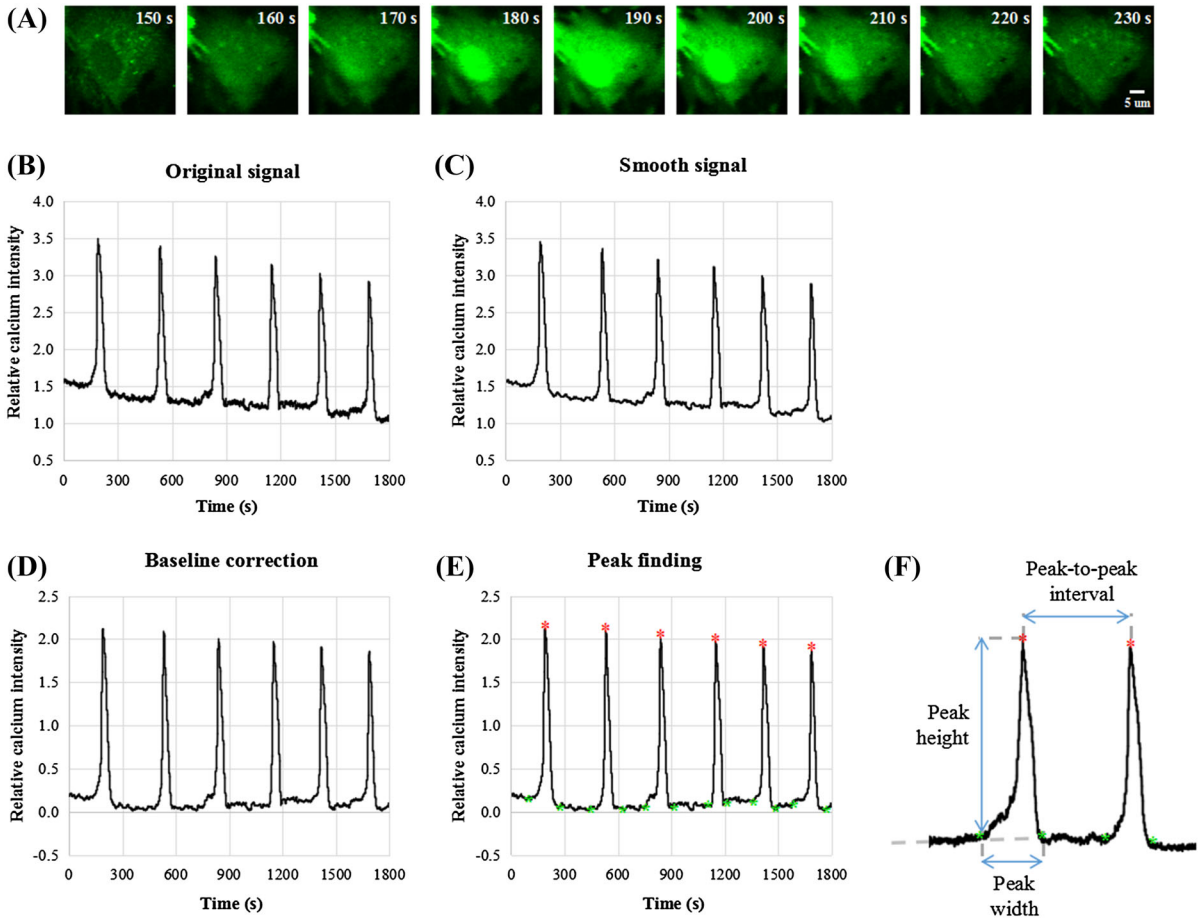

Fig. 2 Typical images observed along the time and procedure for oscillatory calcium intensity analysis. **a** Representative calcium images show relative changes in calcium intensity. **b** Original signal. Calcium intensity is expressed as a ratio of the baseline value. **c** Noise was removed by a low-frequency pass filter. **d** Baseline shift was determined using least squares methods; this shift was beneficial for peak finding. **e** Then, the peak and its width were determined. **f** Illustration of selected parameters

follows: P0 (control), P100/10, P200/1, P200/5, P200/10, and P200/15, where the first and second numbers represent the pressure engaged and its duration, respectively.

Real-time recording of intracellular calcium behavior

Cells were loaded with 5 μM Fluo-4 AM calcium indicator (Molecular Probes) for 30 min. The solution was replaced with endothelial growth medium before pressurizing the cells. Real-time images were obtained during the experiments using a confocal laser microscope and recorded in a 12-bit format at 1 frame/s. $[Ca^{2+}]_i$ intensity was detected using an argon laser at 488 nm. Five independent experiments were performed for each group.

Data analysis

Real-time changes in $[Ca^{2+}]_i$ intensity in a single cell (Fig. 2a) were obtained using the LSM image browser (Carl Zeiss) based on a region of interest. Several oscillatory parameters which can explain signal characteristics were extracted and quantitatively analyzed. They were: frequency, area under the curve within a specific duration, peak height, peak-to-peak interval and width of each typical peak. These parameters have been widely chosen for $[Ca^{2+}]_i$ behavior analyses (Song et al. 2012).

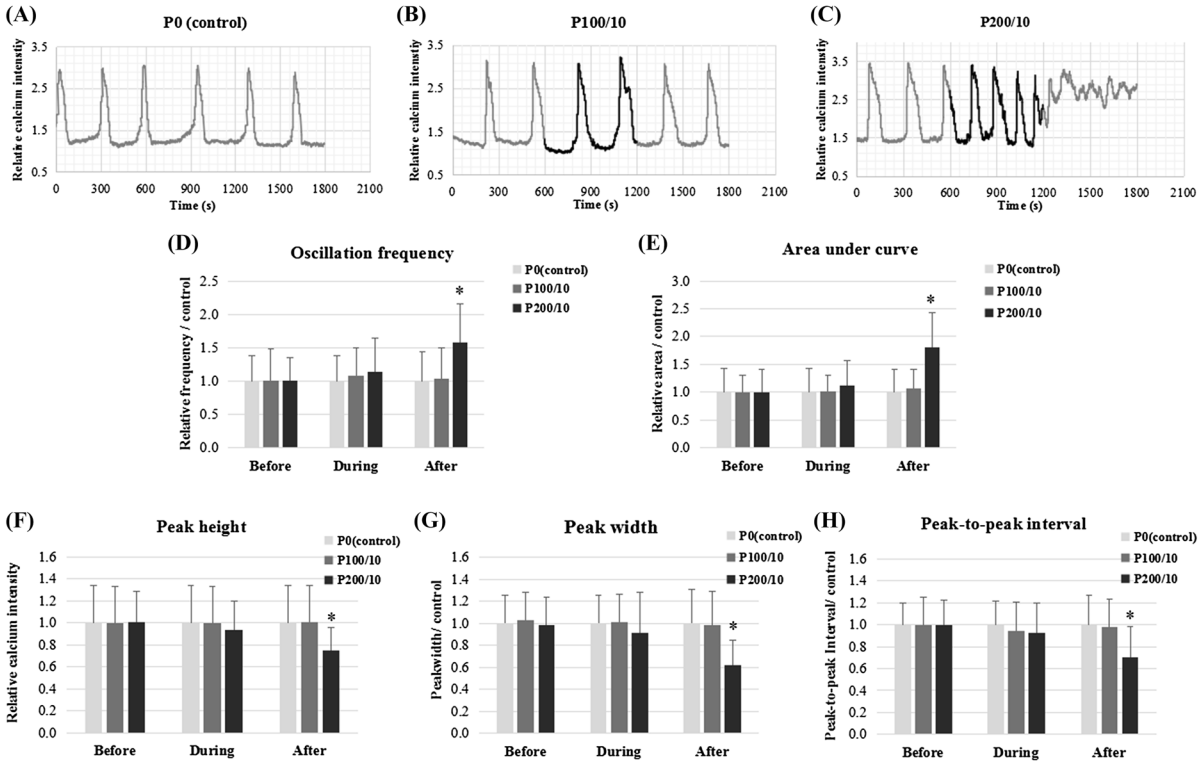

Fig. 3 Typical signals showing relative intracellular calcium concentration, $[Ca^{2+}]_i$ oscillatory behavior and their quantitative analyses under different magnitudes of hydrostatic pressure for 10 min. **a**–**c** Representative calcium oscillation pattern in each group. **a** P0 (control) group with no pressure. **b** Normal pressure for 10 min (P100/10). **c** High pressure for 10 min (P200/10). **d**–**h** Quantitative analyses of oscillatory parameters: **d** Oscillation frequency, **e** Area under the curve, **f** Peak height, **g** Peak width and **h** peak-to-peak interval. (*$P < 0.05$)

MATLAB (R2015a, Mathworks, Inc.) was used in all processes.

Raw oscillatory curves produced heterogeneous data with high noise (Fig. 2b), as reported previously (Christo et al. 2015). Therefore, noise was reduced using the moving-average low-pass filtering technique (Fig. 2c). The baseline for each curve was corrected using the asymmetric least squares method (He et al. 2014) (Fig. 2d). Peaks were detected with the peak-finding function in MATLAB and the selected parameters were extracted and/or calculated (Fig. 2e, f). The number of peaks within specific duration (before, during and after pressurization) was divided by the corresponding duration. Then frequency was obtained. However, the area under the curve during a specific time was calculated without baseline correction.

To avoid the long-term effect of fluorescence exposure on calcium intensity, all parameters were normalized to those in the control group. A one-way analysis of variance (ANOVA) was performed to identify any differences using SPSS ver. 19.0 K software (SPSS Inc.). $P < 0.05$ was considered statistically significant.

A total of 300–500 cells (>20% of all cells on a dish) were observed to examine oscillatory behavior in each group over five independent experiments. Assuming they were normal distribution we eliminated some data which were beyond 95% confidence interval.

Results

The typical signal of control group was shown in Fig. 3a. For 30 min consistent pattern was observed. This implies that $[Ca^{2+}]_i$ oscillatory behavior was not affected by experiment duration, 30 min. No differences in any of the parameters were observed among the groups before the pressure was engaged, as

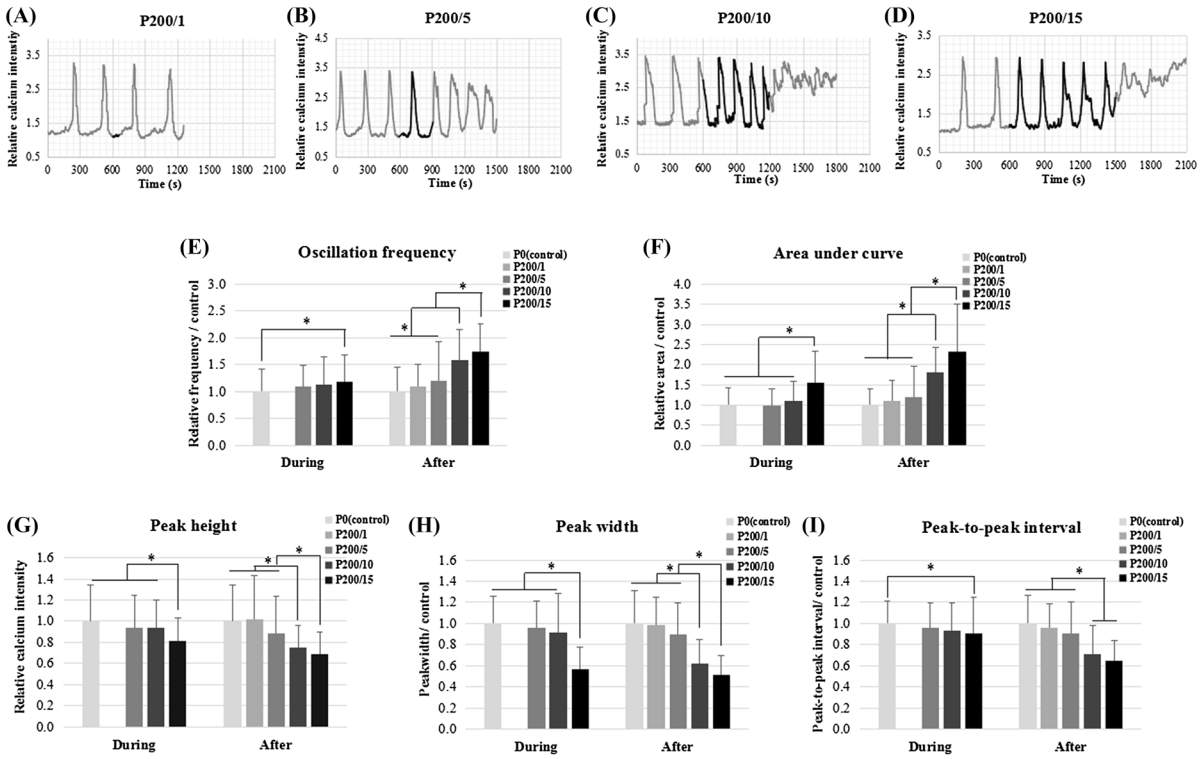

Fig. 4 Typical signals showing the effects of high pressure (200 mm Hg) duration. **a–d** Representative oscillation pattern in each group. **a** 200 mm Hg for 1 min (P200/1), **b** 200 mm Hg for 5 min (P200/5), **c** 200 mm Hg for 10 min (P200/10), (D) 200 mm Hg for 15 min (P200/15). **e–i** Quantitative analyses of oscillatory parameters: **e** oscillation frequency, **f** area under the curve, **g** peak height, **h** peak width, and **i** peak-to-peak interval. (*$P < 0.05$)

expected (see the group "Before" in Fig. 3d–h). This finding suggests that the conditions, including temperature, were well controlled in all groups. However, $[Ca^{2+}]_i$ oscillation pattern changed and became more complex during the 10 min pressurization (Fig. 3b, c). When lower pressure was engaged for 10 min (P100/10), the signal tended to return to the form before pressure was applied (Fig. 3b). However, the signal did not return to the form under higher pressure (P200/10; Fig. 3c). The accumulated 200 mm Hg HP made the pattern more complex, especially after pressurization. This trend was quantitatively analyzed and was shown in Fig. 3d–h. Frequency in P200/10 was significantly increased after pressurization (Fig. 3d). Moreover, the area under curve was also significantly increased (Fig. 3e). Meanwhile, peak height, peak width and peak-to-peak interval were significantly decreased in P200/10 after pressurization (Fig. 3f–h). All these findings suggest that higher pressure significantly affected and changed original $[Ca^{2+}]_i$ oscillatory characteristics.

The 200 mm Hg treatment (i.e., high pressure group) changed the oscillatory patterns in the P0, P100/10, and P200/10 groups, and the changes remained after de-pressurization. Therefore, we further investigated the effects of 1-, 5-, 10-, and 15-min pressure duration of high pressure on oscillatory behavior. The typical patterns observed are presented in Fig. 4a–d, depending on the duration of higher pressure (200 mm Hg). Figure 4e–i present the results of quantitative analyses for various parameters selected. After 1 min of 200 mm Hg HP, the pattern tended to recover after the pressure was released (Fig. 4a). This can be confirmed in the graphs (Fig. 4e–i) that no significant difference of selected parameters between group P200/1 and control group was observed even after pressure was released. The typical signal of group P200/5 shows pattern irregular and heterogeneous (Fig. 4b). Frequency and area under the curve tended to increase when compared with those of control group. Meanwhile, peak height, peak width and peak-to-peak interval tended to

decrease after pressurization. However, significant difference was not still found. When duration was 10 min pattern irregularity and heterogeneity were more observable, especially after pressurization. It can be supported by Fig. 4e–i that significant difference was begun to be observed in all parameters. When duration was extended to 15 min, remarkable irregularities were observed, even during engagement of HP (Fig. 4d). Again, significant differences in all parameters were confirmed (Fig. 4e–i).

Discussion

Our results show that both pressure magnitude and duration affected EC calcium oscillatory behaviors. More interestingly, $[Ca^{2+}]_i$ homeostasis was disrupted when higher pressure ($P = 200$ mm Hg) was applied for a certain periods (>10 min in this study) even after pressure was released. The group of P200/10 showed smaller peaks (lower peak height, narrow peak width) with higher frequency and higher calcium concentration relatively (increased area) than the group of P100/10. These trends were more observable when duration was increased up to 15 min under 200 mm Hg. Other previous studies (Gurkan et al. 2014; Hasel et al. 2005; Ohashi et al. 2007) observed the changes in endothelial cell morphology, related protein expressions and their function under various magnitudes and duration of pressurization. The changes in calcium oscillatory behaviors in this study were acquired within 1.0 h at most. Therefore, further study of morphological changes, related protein expression and cellular functions in relation to $[Ca^{2+}]_i$ oscillatory behaviors is highly recommended.

We hypothesized that either higher pressure or longer time would cause cellular stress and abnormal calcium homeostasis and overload in the absence of chemical cues. In addition, the phenomenon became more observable when higher stress was applied. These findings can be interpreted similar to other reports demonstrating that $[Ca^{2+}]_i$ overload under pathological conditions can cause cellular dysfunction or even death (Frosali et al. 2009; Vassalle and Lin 2004). Calcium overload has been used as an indicator in certain in vitro disease models for drug selection (Hongo et al. 2015; Katnik et al. 2006). The most notable advantage of this kind of simple experimental technique without any chemical cues is that a large amount of information can be obtained over a short time. Thus, the suggested method is suitable for use as a preliminary screening of drugs without using chemical cues which have been used to control $[Ca^{2+}]_i$ concentration in vitro because those chemical cues may cause complex, unclear, or unexplainable outcomes. Based on our results, we suggest that our experimental model could be used as an in vitro pathological model for drug selection, which is related to dysfunction of $[Ca^{2+}]_i$ in some types of cells, such as vascular cells under hypertension or neurons during edema.

Our study has some limitations that should be mentioned. We did not investigate the permeability of calcium ions during pressurization. However, it is well established that high pressure can alter the dynamics and structural characteristics of the cell membrane, in which permeability to molecules changes. Previous studies on the effects of high pressure on membrane structural or functional changes have been reported in various cell types. Nirmalanandhan et al. (2015) showed that elevated pressure ($\sim$300 mm Hg) caused the structure of the myeloid leukemia cell membrane to change to a solid gel-like state; consequently, reduced small molecule intake was detected. Bravim et al. (2010) demonstrated that high stress could damage or cause leaky membranes in Saccharomyces cerevisiae, which may also increase calcium intake from the extracellular environment. However, the HP magnitude used was more than 50 MPa, which is much higher than 200 mm Hg. Therefore, we set a hypothesis that the intake of calcium ion from outside during the pressurization is limited.

In addition, we did not investigate changes in gene or protein expression due to bio-mechanically invoked $[Ca^{2+}]_i$ oscillation changes, as no specific pathological conditions or diseases were clarified beforehand. Therefore, further investigation is necessary focusing on a specific pathology or disease utilizing the suggested simple experimental techniques and related analyses.

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