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## Cellular calcium oscillations in droplets with different chemical concentrations supplied by droplet-array sandwiching technology

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## ABSTRACT

Digital microfluidics using droplets on a chip, such as droplet-array sandwiching technology, provide efficient tools for biochemistry from the perspective of time and sample consumption. Droplet-array sandwiching technology uses the fusion and separation of droplets on upper and lower substrates for high-throughput screening. In our previous work, we developed independent control of individual droplets for this technology using electrowetting-on-dielectric to control the droplet height and allow different chemical concentrations in droplets on the same chip. In this study, we explored the applicability of droplet-array sandwiching technology to cell-based analysis by observing cellular calcium oscillations in HeLa cells in droplets with different histamine concentrations. Histamine concentrations could be controlled by controlling contact time between upper and lower droplets. We found that calcium oscillations intensified with higher histamine concentrations prepared by controlling contact time. These results suggest that droplet-array sandwiching technology can be used for cell-based analysis, where the chemical concentration for cellular stimulation needs to be controlled.

## 1. Introduction

Recently, remarkable progress has been made in assay methods for biochemical analysis, particularly in lab-on-a-chip technology [1]. Microfluidic technology using microchannels, microchambers, microvalves and micropumps enables efficient handling of a small amount of sample on a chip, which reduces the time and cost of biochemical assays. Moreover, the sensors can be integrated into an assay chip for in situ analysis.

Digital microfluidics uses droplets on a chip, in contrast to conventional microfluidics that uses continuous flow [2]. Each droplet can be regarded as a reactor for a biochemical assay and can even be used for cell culture. A small volume droplet allows an efficient assay in terms of time and cost consumption. The present digital microfluidic technology allows for droplet manipulation, including conveyance, fusion, and separation. Droplets may be manipulated through electrical control based on electrowetting-on-dielectric (EWOD) [3–7]. EWOD enables control of the wettability of the surface, leading to surface tension control when generating and manipulating droplets.

Droplet-array sandwiching technology (DAST) has been used for

spatial droplet manipulation [8–11] in cell-based drug screening, in which chemicals are introduced into droplets for biochemical assays. In DAST, droplets are arranged and distributed on two opposing upper and lower substrates, then fused and mixed. Through this method, it is possible to control the concentration of substances in the droplets. Controlling the concentrations allows for various biochemical processes for assays such as cell culture, medium exchange, and chemical stimulation. Our group has previously studied spatial droplet manipulation technology such as DAST was reported by our group [12–14]. We demonstrated and evaluated the mixing of the red dye compound new coccine and deionized (DI) water using DAST [12]. The upper droplet containing the red dye compound and the lower DI water droplet were fused to transport the red dye into the lower droplet. As a result, the concentrations of the red dye compound in the upper and lower droplets decreased and increased, respectively.

We also previously developed a method for controlling droplet height using EWOD technology [13,14], which enabled selective control of the contact between a pair of droplets in a droplet-array. An electrical field was generated between the central circular electrode and outer electrode under a droplet, changing the wettability and decreasing the

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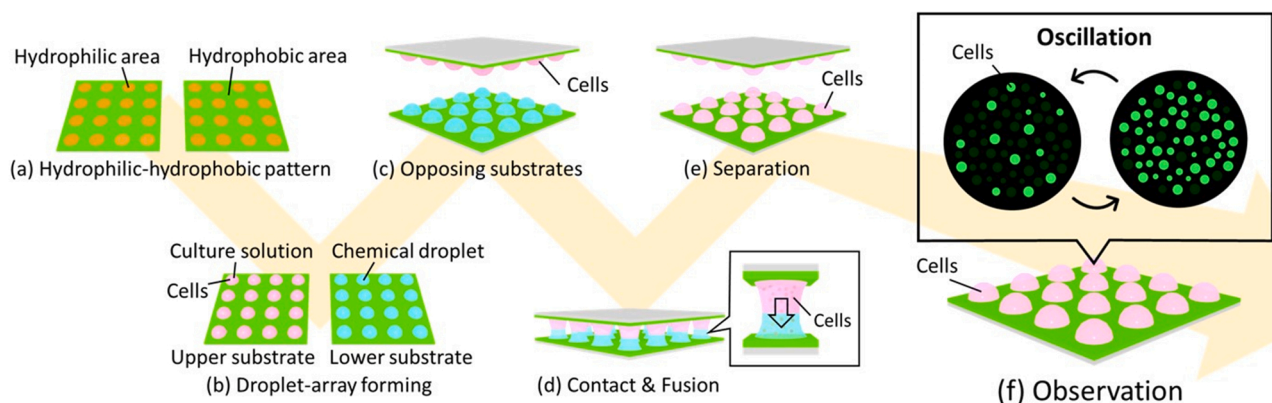
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**Fig. 1.** Conceptual illustration of preparation process of cells and chemicals in droplet arrays for biochemical reaction, such as cellular calcium oscillation. (a) The hydrophilic-hydrophobic patterns form droplets at the designed positions. (b) Droplet arrays can be used as a chamber for cell-culture and biochemical reaction. Droplet-array sandwiching technology (DAST) enables the handling of cells and chemicals. (c) The droplets formed on the two substrates come into contact and fuse when they move closer together in the vertical direction. (d) Cells in the upper droplets fall into the lower droplets, whereas the chemicals mix between the fused droplets. (e) The position of the upper substrate is raised to separate the contact of droplet pairs. (f) Biochemical reaction of cells is evaluated in droplet arrays. Every droplet-array was manipulated in batches. Selective control of the contact and transport between droplet pairs by electrowetting-on-dielectric for DAST was developed.

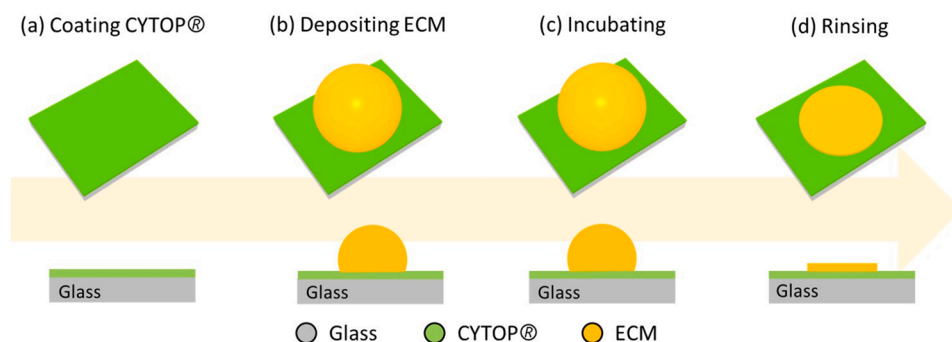
contact angle and height of the droplet. Height control of the droplet allowed control of the contact between the upper and lower droplets, making it possible to individually control contact time between a pair of droplets. This enabled adjusting the concentrations of substances in the droplets through mixing. Selective contact technology using EWOD can manipulate individual droplet pairs independently, whereas conventional DAST can only operate droplet arrays on the same substrate in a batch. Consequently, we were able to prepare a droplet array with different concentrations of substances on a substrate using our individually controlled DAST.

Cell-based drug screening is attracting attention as a promising application for DAST and other droplet microfluidics. For example, a hanging-drop culture plates have been studied for efficient three-dimensional cell culture [15–19]. The hanging drop culture is particularly suitable for three-dimensional cell cultures, such as spheroids, because of its hemispherical shape. A high-throughput super-hydrophobic microwell array based on DAST has also been reported [19]. Droplet arrays can be used as chambers for cell culture in a manner similar to that of hanging droplet cell culture technology because DAST enables the handling of cells and chemicals between vertically connected and separated droplet pairs. The chemicals in the upper droplets diffuse into the lower droplets when the droplets fuse. In our previous work [12,14], a living cellular aggregate was handled using DAST. During culture, the medium was exchanged by spatial contact fusion of the droplets each day and cell viability was confirmed after several days of culture.

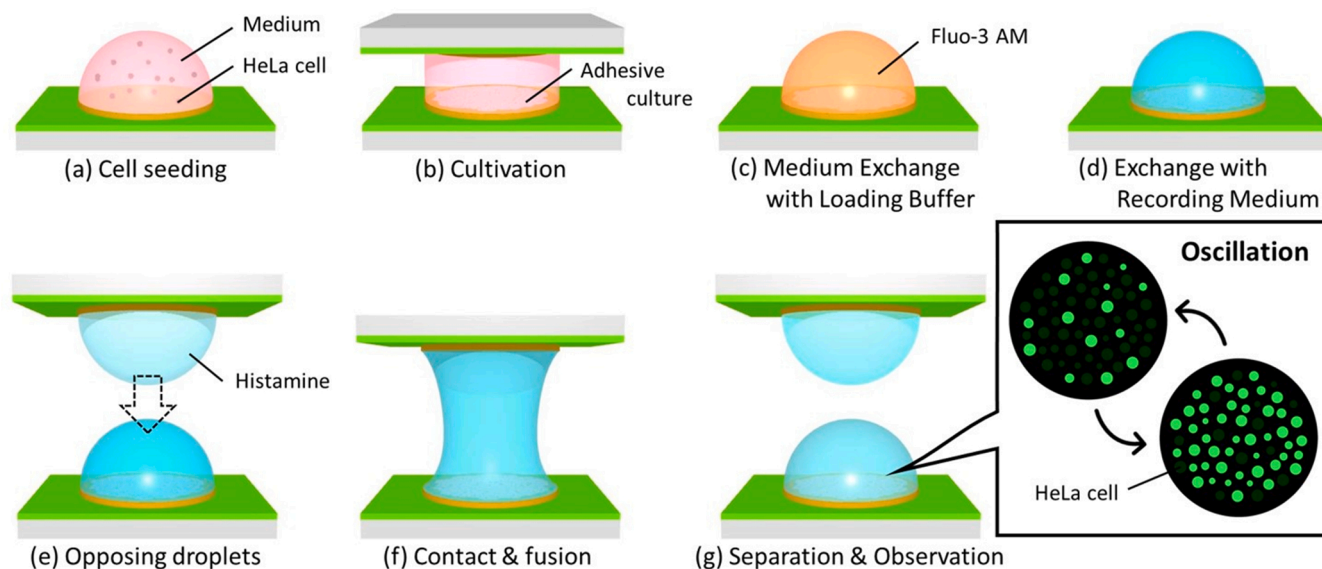
The present study focuses on droplet arrays as chambers for

biochemical reactions in cell-based analyses. Cells were cultured in droplets, and the chemical concentrations in the droplets for cellular stimulation were controlled by DAST. Fig. 1 shows conceptual illustration of preparation process of cells and chemicals in droplet arrays for biochemical reaction, such as cellular calcium oscillation. Droplet arrays on hydrophilic patterns surrounded by hydrophobic areas can be used as chambers for cell cultures and biochemical reactions. Biochemical reaction of cells is evaluated in droplet arrays on a lower substrate as shown in Fig. 1(f) after the preparation process. After the preparation process is completed, the position of the upper substrate is raised and separate the droplet pairs in Fig. 1(e). We focused on cellular calcium oscillations in droplets with different concentrations of stimulating chemicals supplied by DAST as shown in Fig. 1(f). We chose to study cellular calcium oscillation because it is a well-known research subject because that controls several vital cellular processes in neurons, bones, skeletal muscles, the liver, etc. [20–23]. The frequency and amplitude of the oscillation signal is decoded to understand various cellular processes [24,25]. Stimuli in the culture environment, such as the intensity and amount of chemical stimulation, are known to affect the oscillations. One such example is the frequency of oscillations in calcium signaling in mammalian liver cells being dependent on the amount of stimulus [26]. Thus, evaluating calcium signaling is an important subject for biological research. Moreover, small-scale evaluations of calcium oscillations are necessary for drug screening and microfluidics technology can reduce the number of tests. To the best of our knowledge, cellular calcium oscillation analysis in droplets has not yet been attempted.

Therefore, in this study we investigated the analysis of calcium



**Fig. 2.** Fabrication process of wettability patterns for droplet formation. (a) A 12 nm thick CYTOP® layer was coated as a hydrophobic layer. The CYTOP® layer was baked at 80 °C for 60 min and 250 °C for 180 min (b) ECM, which was prepared to form 5 µg/cm<sup>2</sup> on the surface, was introduced on the CYTOP® layer as a hydrophilic and insulating layer on the substrate. (c) The substrate was maintained for 3 h in an incubator at 37 °C, 93 % humidity, and 5 % CO<sub>2</sub>. (d) Removal of ECM and rinsing with sterile distilled water.



**Fig. 3.** Procedure for the analysis of cell  $\text{Ca}^{2+}$  oscillations using DAST. Droplets were formed on the wettability patterns fabricated in circular patterns by the material combination of ECM and CYTOP®.

(a) Droplets of cell suspension were formed in 4  $\mu\text{L}$  on the wettability patterns of 2.48 mm in diameter. HeLa cells were seeded in droplets at a concentration between 0.6 and  $1.0 \times 10^4$  cells/droplet. (b) Droplets on the upper substrates came in contact with the droplets on the lower substrate where the HeLa cells existed. After droplet fusion, the HeLa cells were cultured in the fused droplets in an incubator at 37 °C, a humidity of 93%, and an environment of 5 %  $\text{CO}_2$ . (c) After separating and removing the upper substrates, the culture medium of droplets on the lower substrate was exchanged with 4  $\mu\text{L}$  of phosphate-buffered saline (PBS). The HeLa cells were in droplets on the lower substrate. After removing the PBS, loading buffer prepared with 10  $\mu\text{mol/L}$  Fluo-3 AM in DMSO in recording medium was introduced, and the cells in droplets settled in an incubator for 60 min (d) After washing with PBS in the same way as that described in (c), 4  $\mu\text{L}$  of recording medium was introduced. (e) Four-microliter droplets of histamine solution as a stimulating chemical formed on the upper substrate. Droplets of histamine solution moved downwards and contacted the droplets on the lower substrate where the HeLa cells existed. Biochemical stimulation by histamine solution was performed by DAST as an alternative to pipetting. (f) Calcium oscillations of HeLa cells in fused droplets were observed using a fluorescence microscope.

oscillations of HeLa cells in droplet arrays. Different concentrations of histamine in the droplets were adjusted by using the combination of DAST and EWOD. We anticipate that our technology using individually controlled droplet arrays will be a useful and efficient tool for cell-based screening owing to its high-throughput performance.

## 2. Materials and methods

### 2.1. Fabrication of hydrophilic-hydrophobic patterns for droplet formation

Fig. 2 shows the fabrication process of the wettability patterns for droplet formation. The hydrophobic layer was a 12 nm thick CYTOP® layer coated on a glass substrate. The CYTOP® layer was baked at 80 °C for 60 min and 250 °C for 180 min (Fig. 2a). The hydrophilic layer was extracellular matrix (ECM), which was prepared to form 5  $\mu\text{g}/\text{cm}^2$  on a surface then introduced on to the CYTOP® layer (Fig. 2b). The glass substrate was maintained for 3 h in an incubator at 37 °C, 93 % humidity, and 5 %  $\text{CO}_2$  (Fig. 2c). The excess ECM was removed and rinsed with sterile distilled water (Fig. 2d).

### 2.2. Setup for height positioning and voltage supply for EWOD

The initial distance between upper and lower droplets on substrates was adjusted by positioning stages. EWOD was used to switch the contact and separation of droplets for controlling contact time of droplets. The upper substrate for the droplet-array sandwiching technology was lowered and raised using a precise positioning setup. The positioning setup was consisted of motorized X-axis and Y-axis stages (KXL06050-N1-CA, SURUGA SEIKI Co., Ltd.) and a motorized horizontal Z-axis stage (KHE06008-C, SURUGA SEIKI Co., Ltd.). The horizontal alignment was accomplished in combination with observation by a fluorescence microscope (BZ-X710, KEYENCE) and the X-Y stage. The vertical position

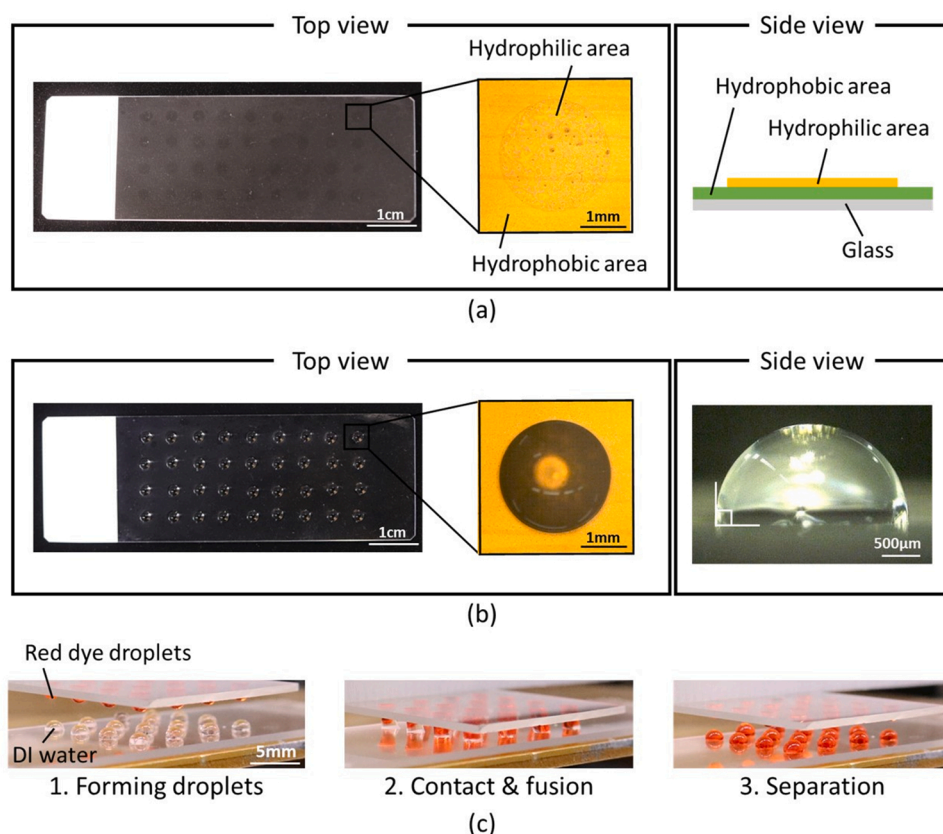
was controlled by the Z stage and fixed by a spacer. A DC power supply (DC160-7.2, NF Corporation) was used to supply voltage to the EWOD. The EWOD voltage was 160 V as described in our previous report [14].

### 2.3. Cell preparation for calcium oscillation in droplets

HeLa cells, which were provided by RIKEN BRC through the National Bio-Resource Project of MEXT, Japan, were prepared for cellular calcium oscillation in droplets. First, 10 % fetal bovine serum, 1 % MEM non-essential amino acids solution (100 $\times$ ) and 1 % mixed antibiotic stock solution (Nacalai Tesque, Japan) were added to DMEM (4.5 g glucose/L) to culture the cells. HeLa cells were cultured in the above DMEM solution and substituted on a glass substrate with hydrophilic (ECM)-hydrophobic (CYTOP®) patterns when the culture status of the HeLa cells became more than 90% confluent. HeLa cells were seeded in droplets concentrations between 0.6 and  $1.0 \times 10^3$  cells/droplet. After droplet fusion, HeLa cells were cultured in an incubator at 37 °C, 93 % humidity, and  $\text{CO}_2$  of 5 %.

### 2.4. Evaluation of the cellular calcium oscillation in the droplets

The fluorescence intensity of HeLa cells was measured as a function of time and evaluated using time-lapse imaging and an image processing software (ImageJ). A fluorescent probe (Fluo-3 AM) was used for fluorescence observation. We prepared loading buffer with 10  $\mu\text{mol/L}$  Fluo-3 AM in DMSO in a recording medium of 20 mmol/L HEPES, 115 mmol/L NaCl, 5.4 mmol/L KCl, 0.8 mmol/L  $\text{MgCl}_2$ , 1.8 mmol/L  $\text{CaCl}_2$ , and 13.8 mmol/L glucose, pH 7.4. Histamine was added and replaced with recording medium. A fluorescence microscope (BZ-X710, KEYENCE) equipped with a 20 $\times$  objective lens (NA 0.45) was used for the evaluation at an excitation wavelength of  $470 \pm 40$  nm and an emission wavelength of  $525 \pm 50$  nm. Fluorescence images were recorded every 1 s and observed at room temperature for 5 min.



**Fig. 4.** Fabrication results of the substrates with wettability patterns for droplet formation and demonstration of droplet arrays preparation. (a) A 2.48 mm diameter circular pattern of the hydrophilic-hydrophobic surface was formed using ECM and CYTOP®. (b) A hemispherical droplet-array on the circular pattern of the wettability pattern was formed. Each droplet could hold a 4  $\mu\text{L}$  of volume with an assumed contact angle of  $90^\circ$ . (c) Demonstration result of supplying the red dye from the upper to lower droplets through the procedure including droplets formation, contact for fusion of droplets, and separation of fused droplets. Every droplet-array was manipulated in batches.

The intensity change over time for a single cell was evaluated and averaged using an image processing software (ImageJ). The multiplicity of the intensity for image processing was 5.0. Five cells were used for the evaluation. The starting point of the fluorescence rise was adjusted by averaging the data from the five cells because of variations in the timing of the fluorescence rise.

### 2.5. Sequential procedure of cell-based evaluation using the combination of DAST and EWOD

Fig. 3 shows the sequential procedure of cell-based evaluation using selective control for DAST. A single pair of droplets are extracted and depicted for the explanation in Fig. 3. We applied DAST to cell  $\text{Ca}^{2+}$  oscillations in droplets. Biochemical processes by DAST can be used for assays such as cell culture, medium exchange, and chemical stimulation.

A 100  $\mu\text{mol/L}$  aqueous solution of histamine was prepared for the upper droplets, and the recording medium was prepared for the lower droplets. The Check Color Histamine assay kit (Kikkoman Biochemifa Company, Japan) was used.

Hydrophilic-hydrophobic wettability patterns were created and 4  $\mu\text{L}$  droplets for cell suspension were formed on them. HeLa cells were seeded in the droplets (Fig. 3a). Droplets on the upper substrate came in contact with the droplets on the lower substrate where HeLa cells existed (Fig. 3b). The HeLa cells were cultured in the fused droplets. After separating and removing the upper substrates, the culture medium from the droplets on the lower substrate was exchanged with 4  $\mu\text{L}$  of phosphate-buffered saline (PBS) (Fig. 3c). HeLa cells in the droplets adhered to the lower substrate. After removing the PBS, the loading buffer was prepared with 10  $\mu\text{mol/L}$  Fluo-3 AM in DMSO in the recording medium and introduced, and the lower substrate with cells in the droplets was placed in an incubator for 60 min. After washing with PBS in the same manner as before (Fig. 3c), 4  $\mu\text{L}$  of recording medium was introduced (Fig. 3d).

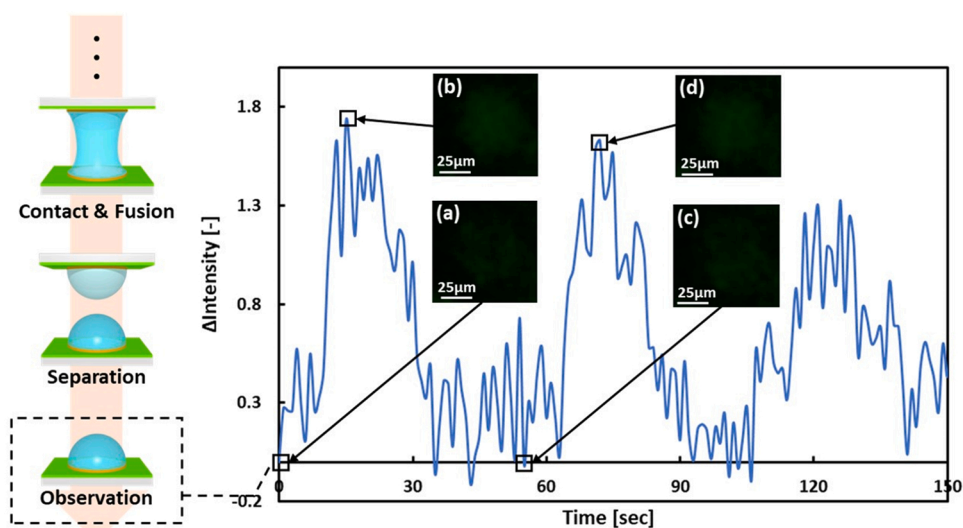
Biochemical assays using DAST were performed to stimulate the HeLa cells. Droplets of histamine solution (4  $\mu\text{L}$ ) were formed on the upper substrate (Fig. 3e). Droplets of histamine solution moved downwards and contacted the droplets on the lower substrate where the HeLa cells were present. Histamine was provided from the upper droplets to the lower droplets by DAST (Fig. 3f). The concentrations of histamine could be controlled by the contact time of a pair of droplets. Fused droplets were separated to stop the transportation of histamine and to fix the concentration. Fluorescence signal oscillations were then observed in the HeLa cells (Fig. 3g).

## 3. Results and discussion

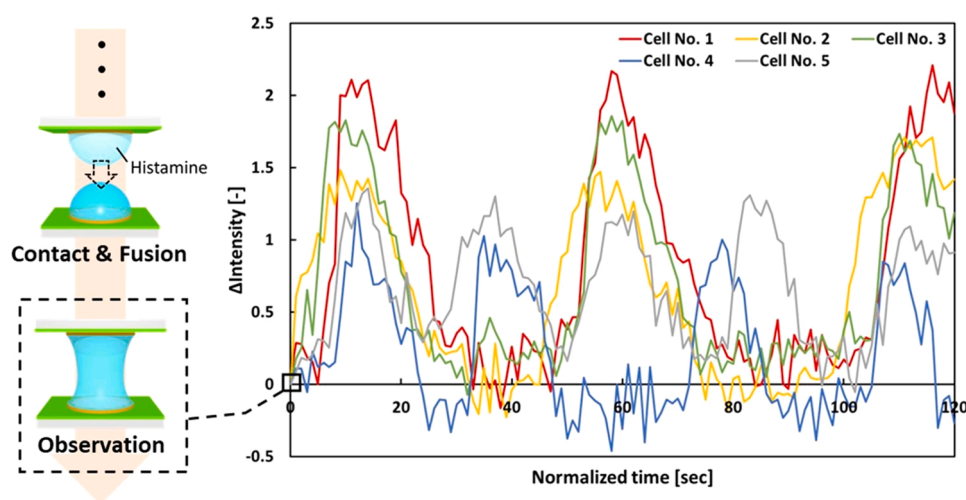
The concentration of histamine in each droplet was successfully controlled using DAST to provide different intensities of stimuli to the cultured HeLa cells. The culturing of the HeLa cells on the lower substrate and the preparation of droplets with histamine on the upper substrate is shown in Fig. 3. Calcium oscillations of HeLa cells in a droplet were observed and evaluated in accordance with the concentration of histamine in the droplet, as shown in Fig. 3f.

### 3.1. Droplet arrays preparation for droplet-array sandwiching technology

Our work has previously presented selective height control of droplets in DAST using EWOD technology [13,14]. The electrodes were covered by parylene-C to generate an electric field for the EWOD. The present study modified a combination of hydrophilic/hydrophobic materials for droplet formation and employed ECM and CYTOP® for hydrophilic and hydrophobic materials, respectively. In our previous studies,  $\text{TiO}_2$  and octadecylphosphonic acid self-assembled monolayer (ODP-SAM) were used as the hydrophilic and hydrophobic materials, respectively. The combination of  $\text{O}_2$ -plasma-treated parylene-C and CYTOP® was also used to form the wettability patterns. In this study, the



**Fig. 5.** Calcium oscillation of HeLa cell in droplets. Droplets with histamine were prepared using droplet sandwiching technology as explained in Fig. 2. An upper droplet with a histamine concentration of 100  $\mu\text{mol/L}$  was contacted to a lower droplet for 120 s. A fluorescent probe (Fluo-3 AM) was used for detecting calcium in the cells. Fluorescence intensity in time lapse images was analyzed by image processing software (ImageJ). Captured images at the characteristic time point were shown in (a)–(d). (a) A cell immediately after separating two droplets, (b) A cell at the first peak of fluorescence intensity, (c) A cell at the first trough of fluorescence intensity, (d) A cell at the second peak of fluorescence intensity.



**Fig. 6.** Calcium oscillations of a single HeLa cell in a droplet. In a droplet, HeLa cells were treated with a final concentration of 100  $\mu\text{mol/L}$  histamine. Time-lapse imaging of cells started immediately after droplet contacting and fusing. The time courses of fluorescence intensity in five cells were normalized by the interval between the first and second peak.

combination of ECM and CYTOP® was selected after considering its biocompatibility.

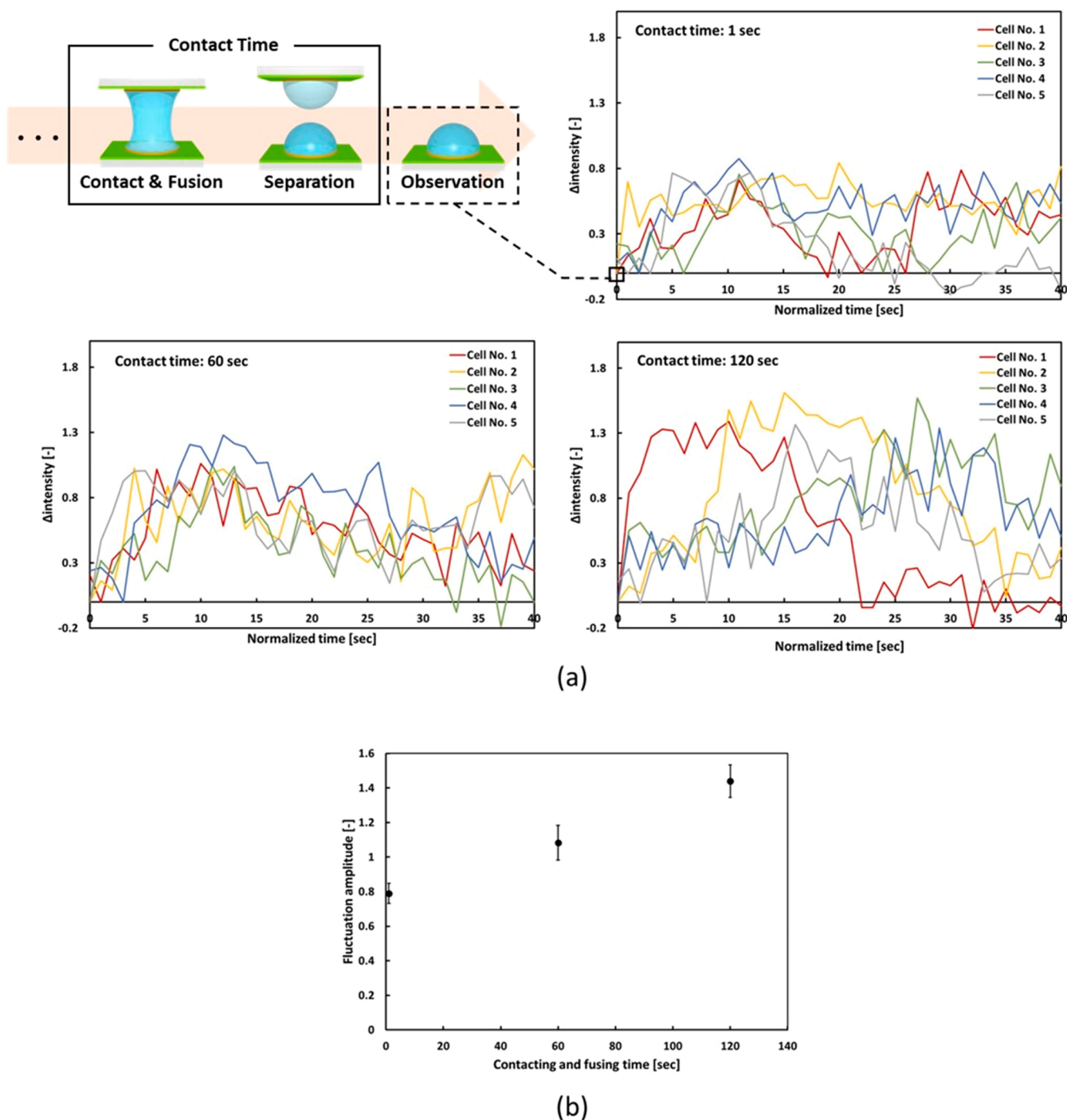
Fig. 4 shows the fabrication results after generating a hydrophilic/hydrophobic pattern for droplet formation. A 2.48 mm diameter circular pattern of ECM and CYTOP® was formed (Fig. 4a). Both the top and side views for a single droplet were magnified and are attached in Fig. 4a. A hemispherical droplet array was formed on the circular wettability pattern, as shown in Fig. 4b. Each droplet had a volume of 4  $\mu\text{L}$  with an assumed contact angle of 90°. The proof-of-concept test of supplying red dye from the upper to lower droplets was successful (Fig. 4c), which corresponds to Fig. 1c. The success with the red dye allowed us to proceed with diffusing histamine into the cell culture droplets.

### 3.2. Cellular calcium oscillation in a droplet induced by chemical stimulation

We confirmed that it was possible to control the concentration of histamine using droplet sandwiching technology. In the first experiment, the histamine concentration of the upper droplets was 100  $\mu\text{mol/L}$ . In the following experiments, positioning stages were used to set the relative position and to control a contact between droplets as shown in

Figs. 5 and 6. The shape control of droplets by EWOD was used in combination with positioning stages in the experiment in Fig. 7 for the comprehensive demonstration. Fig. 5 shows the calcium oscillation of HeLa cells in a droplet with histamine which was prepared by contact with droplets for 120 s. The captured time-lapse images at specific times are shown in Fig. 5(a)–(d). Fig. 5(a) shows a fluorescent image of HeLa cells immediately after two droplets were separated. The HeLa cell and the time at which the first peak appeared is shown in Fig. 5(b). The HeLa cell when the intensity showed the first trough is shown in Fig. 5(c). An image of the second peak of fluorescence intensity is shown in Fig. 5(d). The fluorescence intensity of HeLa cells repeatedly rose and fell owing to histamine stimulation, successfully displaying calcium oscillations in HeLa cells in the droplets.

Following the preliminary experiment, the droplets with different histamine concentrations were prepared. Evaluation of the histamine concentration dependence on the fluorescence intensity showed that the highest histamine concentration (100  $\mu\text{mol/L}$ ) caused the highest intensity in fluorescence oscillations. Time-lapse imaging of the cells began immediately after droplet contact and fusion. The time course of the fluorescence intensity in each of the five cells was normalized by the interval between the first and second peaks and was averaged after



**Fig. 7.** The calcium oscillation of HeLa cells in a droplet after different contacting and fusing time. In a droplet, HeLa cells were treated with histamine for a different time; 1, 60, and 120 s. Time lapse imaging of cells started immediately after separating droplets. The time courses of fluorescence intensity in five cells in different concentrations of histamine were normalized by the interval between the first and second peak (a). The fluctuation amplitude of the first peak was summarized (b). Data are presented as the mean  $\pm$  SD.

normalization. Fig. 6 shows recording traces from five cells are shown in each treatment group. Interspike intervals of HeLa cell with histamine treatment, depending on the concentration, ranged from 30 s to > 600 s [27]. In Fig. 6, the interval ranged from 30 s to 60 s, which are similar to those previously studied for HeLa cells with 200  $\mu$ mol/L of histamine [28]. These results suggest that it is possible to evaluate the chemical concentration dependence of biochemical reactions in droplets.

### 3.3. Cellular calcium oscillation in a droplet depending on the intensity of chemical stimulation

We previously reported that the concentration of the fused droplets depends on contact time [14]. An aqueous solution of histamine (100  $\mu$ mol/L) was prepared for the upper droplets, while the recording medium solution was prepared for the lower droplets. The volume of each droplet was 4  $\mu$ L. The substrate for the upper droplets was moved closer to the opposing droplets on the lower substrate. A mixture of the

liquids and diffusion of histamine occurred between the droplets. The droplets were separated after sufficient time for histamine transportation had passed. In our previous study [14], the concentrations of the red dye compound in the upper and lower droplets intersected at the intermediate value and appeared to asymptotically converge to the intermediate value with repeated increases and decreases. We found that it is possible to introduce different concentrations of histamine into the lower droplets using the upper droplets based on DAST.

Based on these results, the next comprehensive demonstration (Fig. 7) used EWOD to control the shape of the droplets in combination with the positioning stages. Droplets whose height is lowered by EWOD cannot contact paired droplets when the substrates with the droplets are approached by the positioning stage (Z-stage), whereas droplets without EWOD control can contact together. The EWOD voltage for changing the height of a droplet was 160 V. Fig. 7(a) shows calcium oscillations of HeLa cells in droplets after different contact times. The concentration of histamine was controlled by the contact time between a pair of droplets. Droplets were placed in contact for 1, 60, and 120 s to prepare different concentrations of histamine in the lower droplets. Time-lapse imaging of the cells was started immediately after separating the droplets. The time course of the fluorescence intensity in each cell was normalized to the interval between the first and second peaks. The hypothesis, based on our previous report [14], that the length of contact time could provide different histamine concentration was supported. As shown in Fig. 7(a), the fluctuation amplitude, which indicates the degree of response, increased in a contact time-dependent manner. The relation between the fluctuation amplitude and the contact time was determined by Pearson's correlation coefficient ( $r = 0.9986$ ) using means (Fig. 7(b)). A significant correlation ( $P = 0.0340$ ) was observed between them. This advances our previous work demonstrating that the final concentration of the substrate in the lower droplet can be regulated by contact time between the upper and lower droplets [12–14].

Consequently, DAST is valuable both in preparing droplets with different concentrations of substrate and in providing the substrate to living cells. DAST is an attractive technique that provides an easy and simple preparation of buffer with different concentrations and a small-scale evaluation of living cell functions.

#### 4. Conclusion

This study reports cellular calcium oscillations in HeLa cells in droplets with different concentrations of histamine supplied by droplet-array sandwiching technology as an example of typical cell-based drug screening. The histamine concentration dependence of the fluorescence intensity was evaluated and found to increase in a time-dependent manner. It is, therefore, possible to control the concentrations of substances in the droplets by controlling the contact time of the upper and lower droplets using DAST. In this study, we positioned the upper and lower substrates using the positioning stage to control contact time for fundamental experiments. In addition, the switching of the contact of droplets in a batch by EWOD was executed and demonstrated in the last experiment. As reported in our previous work, EWOD technology was applied to the control contact time of individual droplets to satisfy the requirements for advanced assays. These results suggest that DAST can be used for cell-based analysis with different chemical concentrations for cellular stimulation.

#### Author statement

Satoshi Konishi conceived the idea and direction of research and experiments with Yuriko Higuchi, Asuka Tamayori, and Satoshi Konishi conducted the experiments. The results were analyzed by all authors. All authors reviewed the manuscript.

#### CRedit authorship contribution statement

**Satoshi Konishi:** Conceptualization, Formal analysis, Funding acquisition, Resources, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Yuriko Higuchi:** Conceptualization, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – review & editing. **Asuka Tamayori:** Formal analysis, Investigation, Visualization, Writing – review & editing.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Data Availability

All data generated or analyzed during this study are included in this published article.

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