Influence of Culture Conditions on Cell Proliferation in a Microfluidic Channel

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Microfluidic devices have emerged as a new cell culture tool, which can mimic the structure and physiology of living human organs. However, no standardized culture method for a microfluidic device has yet been established. Here, we describe the effects of various conditions on cell proliferation in a microchannel with a depth smaller than 100 μm. Primary endothelial cell proliferation was suppressed with a decrease in the culture medium volume per cell culture area. Moreover, cell growth was compared with or without medium flow, and the optimum culture condition was determined to be 1 μL/h flow in a 65-μm-deep microchannel. In addition, glucose consumption was greater under fluidic conditions than under static conditions, and the ability of tumor (HeLa) cells to convert glucose into lactate appeared to be higher in a static culture than that in a fluidic culture. Overall, our results will serve as a useful guide for designing a microfluidic cell culture platform in a channel smaller than 100 μm.

Keywords Microfluidics, cell, cell culture medium, metabolism, perfusion culture

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Introduction

A microfluidic device is a new cell culture tool that can provide complex microscale structures with well-controlled parameters to mimic organ structures and the physical environment in the body. Moreover, the development of a microfluidic organ model, named organ-on-a-chip, has become a widely used tool for bioassays, and has proven to be especially useful for drug development as an alternative approach to animal testing, emerging as a major topic in bioanalytical chemistry.

Since a microchannel has a higher culture area-to-volume ratio compared to a conventional cell culture dish, traditional cell culture techniques cannot be directly transferred to a microfluidic cell culture system. Indeed, the application of macroscale cell culture instructions to a microchannel results in low viability and poor quality. However, no cell culture method has yet been established for microfluidic devices. Therefore, optimization of the culture conditions, including a suitable coating with extracellular matrix, cell seeding density, and the time interval between media changes, is required for maintaining the health and function of cells in a microchannel.

Beebe and colleagues have conducted detailed biochemical analyses of cell culture in a microchannel. Yu et al. used mammary gland epithelial cells (NMuMG) and a polydimethylsiloxane (PDMS) microchannel (0.25 height × 0.5 width × 60 mm length), and examined the effects of the seeding cell density, medium change frequency, and culture platform (microchannel vs. 96-well plate), among other conditions. They showed that the cells exhibited increased growth rates in a microchannel as compared to the 96-well plate, and cell proliferation in a microchannel increased with a decrease in the frequency of media changes (1 h vs. 12 h). Moreover, they demonstrated that the accumulation of factors secreted from cells in the microchannel induced the growth of NMuMG cells, because the secreted factors remained on the cell surface, which does not occur in macroscale open well cultures. These findings predicted the enhanced effects of autocrine and paracrine signaling in low-volume, diffusion-dominated cell cultures. However, not all of the effects of microscale culture are positive. Paguirigan et al. cultured mouse mammary fibroblasts in a PDMS microchannel (0.25 height × 0.75 width × 5.00 mm length), and examined the cell proliferation rates and glucose consumption for 2 days without media changes. They found that cell metabolism in the microchannel culture differed from that in macroscale cultures. Although the proliferation rates were significantly reduced, glucose consumption in the microchannel culture was over 3-fold higher than that in macroscale cultures. Furthermore, Su et al. used three cell lines as a model to characterize cell stress in a microchannel (0.25 mm height × 5.00 mm length); parental human embryonic kidney (HEK) cells, and transfected HEK cells that stably express wild-type and mutant (G601S) human ether-a-go-go related gene (hERG) potassium channel protein. They found that glucose was rapidly depleted, and the metabolic waste accumulation was more rapid in the microfluidic cell culture compared to those in macroscale cultures. Thus, even though cells experienced stress in the microchannel, the stress effects could be partially rescued by properly adjusting the buffering capacity of the culture medium.

We have also reported the effects of various culture conditions of vascular endothelial cells, vascular smooth muscle cells, and lymphatic endothelial cells in microchannels.
We confirmed that the number of immortalized human microvascular endothelial cells (HMEC-1) was increased day by day in a microchannel with a depth smaller than 100 μm, whereas there was no such increase in the numbers of human dermal microvascular endothelial cells-neonatal (HMVEC-dBinNeo) and human dermal lymphatic microvascular endothelial cells-neonatal (HMVEC-dLylNeo). Moreover, although these primary cells proliferated in a conventional polystyrene Petri dish culture, no proliferation was observed in our 65-μm-deep microchannel. These findings indicated the importance of seeding primary cells at a sufficient density, i.e., at near confluence, in a shallow microchannel.12

Alternatively, a microchannel cell culture is commonly performed in a sub-millimeter-deep microchannel. For example, the depth of commercially available microchannels provided by ibidi (Germany) ranges from 200 to 800 μm. Beebe et al. also used 250-μm-deep microchannel.13,15 However, considering the size and structure of human tissues, there is a need to develop cell culture methods in a microchannel smaller than 100 μm with or without medium flow. For instance, the diameter of capillaries is smaller than 100 μm, and the blood flow entering the capillary is controlled by small muscles, known as precapillary sphincters, to increase or decrease the flow rate.23 Thus, the aim of the present study was to develop models for resolving the cell culture problem in a shallow microchannel.12

Accordingly, in the present study, primary endothelial cells were seeded in microchannels with different depths at the same density as used in a dish-based protocol, and their growth rates were compared (Fig. 1). Next, the cell growth was compared in a microchannel smaller than 100 μm in depth with or without medium flow. In addition, tumor cell line behavior was investigated in the small microchannel. Finally, the glucose concentration and lactate accumulation were examined, and the difference of metabolic activity of the cells was evaluated under the optimized conditions.

Experimental

Fabrication of the microdevice

Microchannels were fabricated by a molding process as described previously.24 In brief, the degassed PDMS mixture was poured onto a master to a thickness of 4 mm, which has channel structure of 0.065 × 0.3 × 20, 0.25 × 1 × 10, or 0.5 × 1 × 10 mm (H × W × L), and then baked at 65°C for 1 h. The PDMS replica was peeled off from the master and adhered to a glass slide (26 × 76 mm), and then baked further at 100°C for 1 h. Through-holes were made at both ends of the microchannel with a 1.0-mm biopsy punch. Both bonding surfaces of the PDMS sheet and a cover slip were exposed to oxygen plasma at 100 W for 35 s. The PDMS sheet was bound to the cover slip and baked at 65°C for 1 h. Each hole was connected to a polytetrafluoroethylene (PTFE) tube (0.46 mm inner diameter, 0.92 mm outer diameter, 10 mm long; Nichias, Tokyo, Japan). The PTFE tube was glued to the PDMS sheet with the PDMS mixture and then baked at 100°C for 1 h. For a static culture, each free end of the PTFE tube was connected to a TYGON tube (0.79 mm i.d., 2.38 mm o.d., and 20 mm long; Saint-Gobain K.K., Tokyo, Japan), which was closed with plastic clips during culture. For fluidic culture, one of the PTFE tubes was connected to a 1-mL syringe (Terumo, Tokyo, Japan) via a bubble trap,24,25 a PFA capillary (0.1 × 0.3 × 750 mm), and a 23-G needle (Nonaka Rikaki, Tokyo, Japan).

Microfluidic cell culture

Normal human dermal microvascular endothelial cells (HMVEC-d) and normal human lymphatic microvascular endothelial cells (HMVEC-dly) (both from Lonza, Basel, Switzerland) were used as the BECs and LECs, respectively. Cells between passages 3 and 8 were used for all experiments. Cells were grown in EBM-2 supplemented with EGM-2 BulletKit (Lonza) and 1× antibiotic-antimycotic (Thermo Fisher Scientific, Waltham, MA). High-glucose-concentration medium was prepared by dissolving 0.14 g of glucose (Wako Chemical, Osaka, Japan) in 40 mL of EGM-2, and then filtered with a 0.2-μm filter (Millex, Merck Millipore, MA).

Human epitheloid cervix carcinoma (HeLa) cells were supplied by RIKEN BRC (Tsukuba, Japan) and grown in high-glucose Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Thermo Fisher Scientific) and 1× antibiotic-antimycotic (Thermo Fisher Scientific).

Cell culturing in a microchannel was conducted as described previously with slight modifications.1 The microchannel was coated with 0.1 mg/mL fibronectin (Life Technologies, Carlsbad, CA) for BECs and LECs, or with 2 mg/mL Matrigel (BD Bioscience, San Jose, CA) for HeLa cells, and incubated at 37°C for 16 h and then at 37°C for 1 h. After washing with a fresh medium, 4, 5, or 7 μL of the cell suspension (from 1.0 × 10⁶ to 3.0 × 10⁶ cells/mL, depending on the situation) was introduced into a 65-, 200-, or 500-μm-deep microchannel using a 50-μL microsyringe (Hamilton, Reno, NV) equipped with a 25-G or 27-G needle (Terumo, Tokyo, Japan).

The device was wrapped with a wet lint-free wiper (BEMCOT M-1, Asahi Kasei, Tokyo, Japan) to prevent desiccation, and then incubated under static conditions for 6 h (BECs and LECs) or 3 h (HeLa) in a 5% CO₂ incubator at 37°C. After incubation, the cells were cultured for 72 h under a 1-μL/h or 6-μL/h pulsate flow generated using a miniaturized infusion pump (SMP101-L, Primethech, Tokyo, Japan), 1-μL/h or 6-μL/h continuous flow (shear stress, τ = 0.01 or 0.07 dyn/cm²) generated using a syringe pump (Model 210 or 230, KD Scientific, Holliston, MA), or under static conditions with a medium change every 24 h.

Cell viability assay

The cell viability assay was performed using two fluorescent
dyes (LIVE/DEAD Viability/Cytotoxicity assay kit, Thermo Fisher Scientific). Calcein AM (2 μM) and ethidium homodimer (4 μM) were reacted with the cells in the culture media for 30 min at 37°C under 5% CO₂, and then rinsed with PBS(+).

Cell cycle assay
The cell cycle assay was performed using a Cell-Clock Cell Cycle Assay Kit (Biocolor, Carrickfergus, UK). Cell-Clock Dye Reagent (20 μL) was added to the microchannel, and then the device was returned to the incubator for 60 min. After incubation, the dye reagent was removed, and the cells were washed twice with a fresh warm medium at 23°C. The cell images in the whole microchannel were immediately taken with a microscope, and the number of dyed cells was counted.

Microscopic observation
Cell images were taken under an IX 71 microscope (Olympus, Tokyo, Japan) with a 100-W halogen lamp and a 100-W high-pressure mercury lamp. Images were acquired with a CCD camera WAF-250D2 (Watec, Yamagata, Japan) or ORCA-R2 (Hamamatsu Photonics, Hamamatsu, Japan). The dichroic mirror block U-MWIG3 (excitation 530 - 550 nm, emission >575 nm) or U-MNIBA3 (excitation 470 - 495 nm, emission 510 – 550 nm) was used for observation of ethidium homodimer or Calcein AM staining, respectively.

For cell-growth monitoring, cells cultured in a microchannel or a cell culture flask were observed once every 24 h at three fixed points.

pH measurement
A pH measurement was performed using the combination of a pH electrode (MI-710, Microelectrodes Inc., NH, USA) and a pH meter (KRSE, As One, Osaka, Japan).

Analysis of glucose and lactate concentrations
Glucose and lactate concentrations in the collected culture medium were determined using the Glucose Colorimetric/Fluorometric Assay Kit and the Lactate Colorimetric/Fluorometric Assay Kit (BioVision, CA, USA), respectively. In brief, the medium from the microchannel culture was diluted to a range of the calibration curve with the assay buffer, and then 5 μL of the diluted sample was mixed with 5 μL of glucose or lactate assay reagent (assay buffer:probe:enzyme = 4.6:0.2:0.2 μL) and incubated for 30 min at 37°C for glucose or at 23°C for lactate. After the reaction, samples were diluted 10-fold with the buffer, transferred to a cuvette, and analyzed with an ultraviolet-visible spectrophotometer (V-630 BIO, Jasco, Tokyo, Japan) at 570 nm to compare with the standard curve.

Glucose consumption and lactate production per unit area were calculated by the following equation:

\[
\text{Glucose consumption per unit area} = \frac{\text{Concentration after culture} \times \text{volume of medium}}{\text{Total culture area in the microchannel}}
\]

\[
\text{Lactate production per unit area} = \frac{\text{Concentration after culture} \times \text{volume of medium}}{\text{Total culture area in the microchannel}}
\]

Results and Discussion

Microfluidic culture of BECs and LECs under static conditions
In general, cells in a shallow microchannel are seeded at a higher density compared to that used in a cell culture flask to achieve a sufficient density for confluence.⁴⁻⁶ To investigate the effect of a lower seeding density, the cells were seeded in the microchannel at the normal density used in the conventional culture method (~50 cells/mm²).

Images of BECs and LECs cultured in the microchannel are shown in Fig. 2a. The cells were seeded at a density ranging from 50 to 80 cells/mm² and the culture medium was changed every 24 h. The results indicated clear suppression of cell proliferation in the microfluidic system. Viability of both types of cells could not be maintained in the 65-μm-deep microchannel. With an increase of the channel depth, the cell proliferation rate improved, and the initial cell density was maintained in the 200-μm-deep microchannel. In addition, cell proliferation was observed only in part of the 500-μm-deep microchannel. The time course of the relative cell density (i.e., cell density divided by the initial cell density) is shown in Figs. 2b and 2c. Both types of cells in the 500-μm-deep microchannel grew more slowly than those in a culture flask. These results indicated that cell proliferation is suppressed with a decrease in the culture medium volume per cell culture area.

Microfluidic culture of BECs and LECs under static conditions with high-glucose medium
Since the cells did not grow in the 65-μm-deep microchannel under static conditions, we hypothesized that the nutrition was not sufficient for cells in the microchannel to sustain growth. Therefore, the cells were next cultured in a medium containing a higher concentration of glucose (4.5 g/L). The high-glucose medium did not improve BEC growth, and the number of BECs seeded at high and low densities reduced day by day (Fig. 3a). In contrast, the high-glucose medium positively affected the LEC culture. LECs seeded at 400 cells/mm² (confluence) maintained their initial density and those seeded at 150 cells/mm² proliferated by 1.5 times (p = 0.017) the initial density for 3 days in the 65-μm-deep microchannel.

Microfluidic culture of BECs and LECs under fluidic conditions
BECs and LECs were also cultured under fluidic conditions after 6 h of static culturing for cell attachment in the 65-μm-deep microchannel, in which fresh medium was continuously supplied. The cells were seeded at a density of approximately 80% confluence, which helped to maintain their viability. In contrast to the static condition in which the cells maintained their initial density (Figs. 3c and 3d, blue closed circles), those cultured under fluidic conditions showed different growth patterns. BECs proliferated under all fluidic conditions except for 6-μL/h continuous-flow culture (Fig. 3c). Although the specific effect of continuous-flow and pulsating-flow on the cell’s environment is currently unknown, we speculate that this may involve differences in autocrine secretion. The cell density for 3 days under these flow conditions was higher than that observed under the static condition (p = 0.0015 for 1-μL/h continuous-flow, p = 0.0018 for 6-μL/h pulsating-flow, and p = 0.0711 for 6-μL/h pulsating-flow). Almost all of the cells were alive under these three conditions, whereas dead BECs were observed under the 6-μL/h continuous-flow condition (Fig. S1, Supporting Information). The LEC density was also increased under both the 1-μL/h pulsating-flow and 1-μL/h continuous-flow conditions, and the cell density for 3 days under these flow conditions was higher than that observed under the static condition (p = 9.00 × 10⁻⁷ for 1-μL/h continuous-flow and p = 0.0043 for 1-μL/h pulsating-flow). Unlike BECs, the density of LECs decreased under both the 6-μL/h pulsating-flow and continuous-flow conditions. This suggests that BECs might be more resistant to flow stress than LECs. Taken together, these results indicate that cell growth under fluidic conditions differs depending on the type of cells seeded. In the 65-μm microchannel, an optimal culture condition was determined to be 1-μL/h flow culture for both BECs and LECs. In case of static culture, nutrients in the medium may be insufficient to
support the cell growth during the initial 6-h and the following 24-h static cultures in the 65-μm microchannel.

Microfluidic culture of HeLa cells

HeLa cells are tumor cells that proliferate much faster than BECs and LECs, and thus easily grow in a microchannel. HeLa cells were seeded at a density of approximately 80 cells/mm² and cultured for 3 days in the 65-μm-deep microchannels under static conditions or 6-μL/h continuous-flow conditions, or in a cell culture flask for comparison. The culture medium was changed every 24 h for the static culture. Unlike BECs and LECs, HeLa cells were able to grow in the 65-μm-deep microchannels (Fig. S2, Supporting Information), although the growth rate was reduced compared to that in the culture flask. In addition, the growth rate under the static condition was reduced compared to that observed under the continuous-flow condition.

The high-glucose medium of the static culture was recovered every 24 h to analyze the glucose concentration, which was 3.26 ± 0.13 g/L for 3 - 24 h, 3.52 ± 0.43 g/L for 24 - 48 h, and 3.45 ± 0.37 g/L for 48 - 72 h, representing an increase of more than 70% of the initial concentration (4.5 g/L). Thus, glucose seems to be sufficient for static culture in a microchannel when the medium is changed daily.

HeLa cell cycle in a microchannel

Three hours after HeLa cells were seeded in the microchannel (Figs. 4b and 4c, day 0), the proportion of cells in the G0-G1 phase (yellow), S-G2-phase (green), and M-phase (dark blue) was 28, 52, and 19%, respectively. Under the static condition, cell density increased 2-fold after 3 days of culture in the microchannel (Fig. 3b), although the growth rate was reduced compared to that in the culture flask. In addition, the growth rate under the static condition was reduced compared to that observed under the continuous-flow condition.

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density increased 4-fold after 3 days (Fig. 3c). The proportion of cells in G0-G1 phase increased day by day, and 96% of cells were in G0-G1 by day 2. A potential reason for this G0/G1 arrest may be that cells reached confluence by the second day of culturing, resulting in reduced cell growth. In addition, the typical duration of G2 or M phases is 4 or 1 h, respectively, which is shorter than that of the G1 phase (11 h), so that these phases are hard to detect. In contrast, more than 50% of the cells were in S-G2 or M phase on day 3 in the static culture. Glucose deficiency induces G2/M transition arrest in a specific type of tumor cells. A potential reason why S-G2 and M-phases accounted for a high rate under the static conditions on day 3, is that the glucose concentration in the culture medium may be insufficient for the cell cycle progression. It may cause a reduction of the growth rate.

Taken together, these findings demonstrated that cell division was still not stopped completely in the static culture, although the cell doubling time was longer than that achieved with the continuous-flow culture.

**Lactate levels in the HeLa cell culture medium**

A potential reason for reduction of the cell growth rate in the microchannel under the static condition is accumulation of waste metabolites in the culture medium. Therefore, lactate, one of the main metabolic waste products in the culture media, was measured.

We detected a clear increase in the lactate concentration in the conventional flask culture each day of culturing (Fig. 5a), which corresponds to the results of cell growth. By contrast, a high lactate concentration was observed as of day 2 of static culture in the microchannel (Fig. 5b), whereas a low lactate concentration was maintained throughout the culture under the fluidic condition (Fig. 5c), indicating that lactate was diluted by the large volume of culture medium used in the flow culture. Moreover, the lactate concentration depended on the surface area-to-volume ratio, which was 0.5 mm²/μL for the flask, 17.5 mm²/μL for the static culture in the microchannel, and 0.04 mm²/μL for 24-h continuous-flow culture in the microchannel. Furthermore, even though the cell density increased daily in the static culture in the microchannels, the lactate concentration in the medium was not affected. This suggests that the cell metabolism was regulated by the high concentration of lactate in the medium.

Although the cells did not reach confluence by 24 h after...
seeding in the static culture, the lactate concentration was still high. Therefore, the lactate concentration was measured at 3 h after seeding, which reached 20.12 ± 2.62 nmol/μL, representing half of the 24-h accumulation value (40.71 ± 6.50 nmol/μL). In general, lactate has an adverse effect on the cell growth, productivity, and viability.29 Although concentrations below 20 mM lactate do not affect the cell growth or productivity, a lactate concentration of 20 – 40 mM impairs the productivity and more than 40 mM inhibits cell growth.30 Therefore, a medium exchange once every 3 h is recommended for a static culture in a microchannel.

In the HeLa cell culture medium

Since lactate can reduce the pH, we measured the change in the pH value of the culture media. However, the volume of the culture medium in the 65-μm channel and the inlet tube was only 5 μL, which was not sufficient to measure the pH with a standard electrode system. Therefore, we measured the pH of the culture media from the fluidic culture and flask culture, with a sufficient volume of 140 μL per day in 6 μL/h continuous-flow culture.

The initial pH value of the medium, incubated in a CO₂ incubator for 30 min, was 7.71 (Figs. 5d and 5e). In the fluidic culture, the medium maintained a constant pH of 7.20 for 3 days, whereas that of the medium recovered from the flask decreased day by day, which is in line with the results for lactate concentrations (Fig. 5a). After 2 days of culture, the pH of the flask medium was 7.01; because the lactate concentration in the medium from the flask was lower than that of the static culture in the microchannel (30 vs. 40 nmol/μL), the pH value of the medium in the microchannel would inevitably be lower than 7. Borsi et al.31 reported that fibroblasts exposed to an acidic culture medium completely lost their ability to proliferate; Kruse et al.32 showed that the proliferation of fibroblasts and keratinocytes decreased at pH 6.5 compared to pH 7.4. These reports suggest that the cause of the growth rate reduction in our microchannel under the static condition might be the low medium pH.

Glucose consumption and lactate production

Glucose consumption and lactate production were compared to assess the glycolytic activity of the cells cultured in the microchannel. As shown in Fig. 6, in all cases, the amount of glucose consumption under the fluidic conditions was greater than that consumed under the static conditions.

Although lactate production was almost the same in BECs and LECs, HeLa cells showed much higher lactate production: the conversion rate from glucose to lactate in BECs and LECs was 15 - 30%, whereas that of HeLa cells was greater than 80%. This difference is likely to be derived from an intrinsic difference in the metabolism of the cell types. Our results confirmed the well-known Warburg effect, in which tumor cells show enhanced conversion of glucose to lactate, even in the presence of normal levels of oxygen.33 Thus, despite lower lactate accumulation in the BEC or LEC culture in the microchannel than that of HeLa

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*Fig. 4* Cell cycle assay of HeLa cells in the microchannel. (a) Photograph of HeLa cells cultured in the microchannel. Scale bar: 100 μm. G0-G1 phase (yellow), S-G2-phase (green), M-phase cell (dark blue). Time course of cell density with cell cycle phases in (b) static culture and (c) fluidic culture.
cells (Fig. 6), BECs and LECs still could not grow (Fig. 2), suggesting that the resistance to lactate stress might differ between normal cells and HeLa cells. In addition, HeLa cells consumed more glucose in the fluidic culture than in the static culture, despite producing the same levels of lactate in the two conditions (Fig. 6). Therefore, the ability to convert glucose into lactate might be enhanced in the static culture, implying a lower oxygen concentration in the medium than that under fluidic cultures.

Conclusions

Our results provide insight into the differences between microfluidic and conventional dish-based cultures with respect to cell proliferation and glucose metabolism to serve as guidance for establishing standardized and optimized microchannel culture conditions. Overall, the findings are consistent with a previous report showing that the accumulation of metabolic waste is more rapid in microfluidic cell culture than in macroscale cultures. Our results further indicate that the slow growth in a 65-μm-deep channel can be improved by frequent or continuous medium exchanges with low shear stress. These results will be useful for designing a microfluidic cell culture platform with a channel smaller than 100 μm, which mimics small blood vessels. Because the blood vessel device can link other tissue chip devices, these results will help development of a new organ/body-on-a-chip technology. However, further research is needed to uncover the underlying factors contributing to the lack of growth of BECs and LECs in the 65-μm-deep microchannel when the cells were seeded at a density of 50 cells/mm², despite low lactate accumulation. To further optimize the culture conditions, the effects of cell-secreted growth factors and other metabolites such as amino acids and ammonia should be investigated.

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Supporting Information

BECs and LECs viability assay (Fig. S1) and photographs of HeLa cells cultured in a channel and a flask (Fig. S2). This material is available free of charge on the Web at http://www.jsac.or.jp/analsci/

References

1. M. Sato, N. Sasaki, M. Ato, S. Hirakawa, K. Sato, and K. Sato, *PLoS One*, 2015, 10, e0137301.
2. K. Sato, M. Nakajima, S. Tokuda, and A. Ogawa, *Anal. Sci.*, 2016, 32, 1217.
3. K. Sato and K. Sato, *Anal. Sci.*, 2018, 34, 755.
4. S. Lee, J. Ko, D. Park, S. R. Lee, M. Chung, Y. Lee, and N. L. Jeon, *Lab Chip*, 2018, 18, 2686.
5. B. Sebastian and P. S. Dittrich, *Annu. Rev. Fluid Mech.*, 2018, 50, 483.
6. J. Zhang, X. Wei, R. Zeng, F. Xu, and X. Li, *Future Sci. OA*, 2017, 3, FSO187.
7. Y. Liu, E. Gill, and Y. Y. Shery Huang, *Future Sci. OA*, 2017, 3, FSO173.
8. A. B. Shrirao, F. H. Kung, A. Omelchenko, R. S. Schloss, N. N. Boustany, J. D. Zahn, M. L. Yarmush, and B. L. Firestein, *Biotechnol. Bioeng.*, 2018, 115, 815.
9. M. Rothbauer, H. Zirath, and P. Ertl, *Lab Chip*, 2018, 18, 249.
10. H. Kimura, Y. Sakai, and T. Fujii, *Drug Metab. Pharmacokinet.*, 2018, 33, 43.
11. A. U. R. Aziz, C. Geng, M. Fu, X. Yu, K. Qin, and B. Liu, *Bioengineering (Basel)*, 2017, 4, E39.
12. B. Y. Zhang, A. Korolj, B. F. L. Lai, and M. Radisic, *Nat. Rev. Mater.*, 2018, 3, 257.
13. S. Ishida, *Drug Metab. Pharmacokinet.*, 2018, 33, 49.
14. S. J. Hachey and C. C. W. Hughes, *Lab Chip*, 2018, 18, 2893.
15. A. Bein, W. Shin, S. Jailili-Firoozinezhad, M. H. Park, A. Sontheimer-Phelps, A. Tovaglieri, A. Chalkiadaki, H. J. Kim, and D. E. Inger, *Cell Mol. Gastroenterol. Hepatol.*, 2018, 5, 659.
16. R. Prantil-Baun, R. Novak, D. Das, M. R. Somayaji, A. Przekwas, and D. E. Inger, *Annu. Rev. Pharmacol. Toxicol.*, 2018, 58, 37.
17. H. Yu, C. M. Alexander, and D. J. Beebe, *Lab Chip*, 2007, 7, 726.
18. A. L. Paguirigan and D. J. Beebe, *Integr. Biol. (Camb)*, 2009, 1, 182.
19. X. Su, A. B. Theberge, C. T. January, and D. J. Beebe, *Anal. Chem.*, 2013, 85, 1562.
20. Y. Tanaka, Y. Kikukawa, K. Sato, Y. Sugh, and T. Kitamori, *Anal. Sci.*, 2007, 23, 261.
21. K. Jang, K. Sato, K. Mawatari, T. Konno, K. Ishihara, and T. Kitamori, *Biomaterials*, 2009, 30, 1413.
22. N. Sasaki, M. Shinjo, S. Hirakawa, M. Nishinaka, Y. Tanaka, K. Mawatari, T. Kitamori, and K. Sato, *Electrophoresis*, 2012, 33, 1729.
23. T. Sakai and Y. Hosoyamada, *J. Physiol. Sci.*, 2013, 63, 319.
24. Y. Ishigaki and K. Sato, *Micromachines*, 2018, 9, 272.
25. Y. Imura, Y. Asano, K. Sato, and E. Yoshimura, *Anal. Sci.*, 2009, 25, 1403.
26. J. W. Posakony, J. M. England, and G. Attardi, *J. Cell Biol.*, 1977, 74, 468.
27. G. M. Cooper, “The Cell: A Molecular Approach”, 2nd ed., 2000, Chap. 14, Sinauer Associates, Sunderland, MA.
28. T. Isono, T. Chano, A. Kitamura, and T. Yuasa, *PLoS One*, 2014, 9, e96168.
29. B. C. Mulukutla, S. Khan, A. Lange, and W. S. Hu, *Trends Biotechnol.*, 2010, 28, 476.
30. C. Altamirano, J. Berrios, M. Vergara, and S. Becerra, *Electron J. Biotechn.*, 2013, 16, fulltext-2.
31. L. Borsi, G. Allemanni, B. Gaggero, and L. Zardi, *Int. J. Cancer*, 1996, 66, 632.
32. C. R. Kruise, M. Singh, S. Targosinski, I. Sinha, J. A. Sorensen, E. Eriksson, and K. Nuutila, *Wound Repair Regen.*, 2017, 25, 260.
33. M. V. Liberti and J. W. Locasale, *Trends Biochem. Sci.*, 2016, 41, 211.