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Microfluidic Serial Dilution Cell-Based Assay for Analyzing Drug Dose Response over a Wide Concentration Range

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In this paper we report a perfusion culture microchamber array chip with a serial dilution microfluidic network for analyzing drug dose response over a concentration range spanning 6 orders of magnitude, which is required for practical drug discovery applications. The microchamber array chip was equipped with a pressure-driven interface, in which medium and drug solution were added with a micropipet and delivered into the microfluidic network by pneumatic pressure. We demonstrated that the microchamber array chip could be used to estimate the 50% growth inhibitory concentration using the model anticancer drug paclitaxel and the model cancer cell line HeLa. The results obtained by using the microchamber array chip were consistent with those obtained by a conventional assay using microplates. The microchamber array chip, with its simple interface and well-designed microfluidic network, has potential as an efficient platform for high-throughput dose response assays in drug discovery applications.

The cell-based assay has become an important analytical method to reduce screening costs in drug discovery.[1] Currently, high-throughput screening (HTS) using a microplate is the gold standard for the cell-based assay. However, such assay systems are burdened by several issues such as high cost, poor reliability of data, difficulty in achieving rapid and accurate dispensing of very small liquid volumes, and uncontrolled evaporation of dispensed liquids.[2] In contrast, the use of microfluidics is expected to circumvent these issues, and microfluidic technologies are promising candidates to miniaturize assays and to increase experimental throughput and reliability in drug discovery applications.[3−5]

In the drug discovery process, a cell-based drug dose response assay is often used to analyze the 50% growth inhibitory concentration (IC_{50}) or 50% effective concentration (EC_{50}). For these analyses, researchers often use stepwise drug concentrations in a linear dilution series[6−10] or a logarithmic dilution series, for which concentration ranges span 3−6 orders of magnitude.[11−16] Needless to say, preparation of a large number of solutions with different concentrations is tedious and time-consuming work. In addition, the dilution process sometimes causes experimental errors, since serial dilution using a micropipet can induce accumulation of dilution errors.

Recently, several research groups have reported the use of perfusion culture chips equipped with a microfluidic concentration profile generator.[17−19] However, most of the generated concentration profiles are linear, and there are no reports about the use of such a generator to generate a wide concentration range, e.g., 6 orders of magnitude, which is indispensable for the pharmacological drug dose response assay. More recently, we have investigated a serial dilution microfluidic network composed of microchannels with a high fluidic resistance ratio for generating arbitrary monotonic concentration profiles.[20] We have shown that this serial dilution microfluidic network is capable of generating concentration profiles spanning 6 orders of magnitude.

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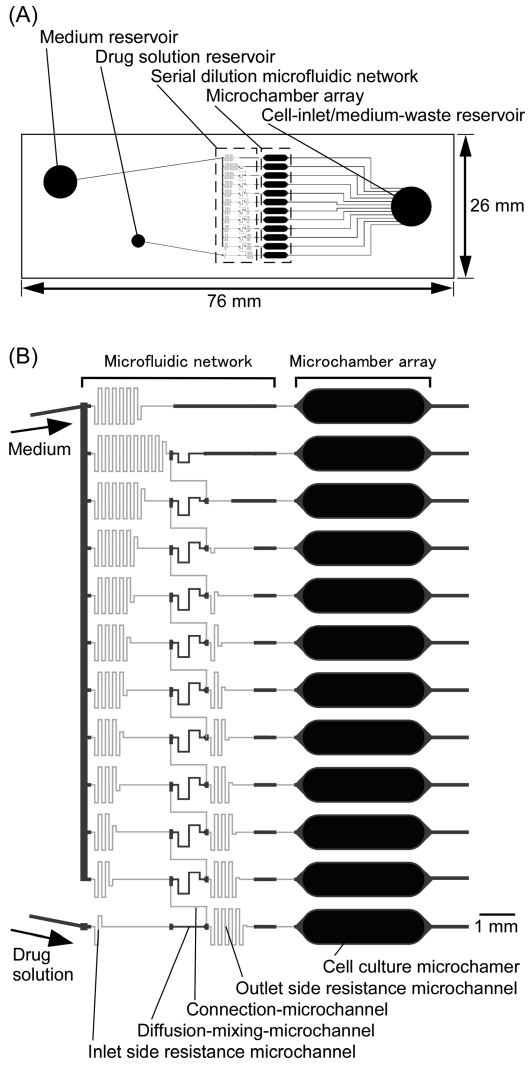

Figure 1. Structure of the perfusion culture microchamber array with a serial dilution microfluidic network. (A) Schematic of the microfluidic network and microchamber array on a glass slide chip. (B) Enlargement of the microfluidic network and microchamber array. The color density designates the three different depths of microchannels and microchambers on the perfusion culture microchamber array chip: the black microchambers are 260 μm deep, dark gray microchannels and terraces are 58 μm deep, and light gray microchannels are 6 μm deep.

In this paper we describe an on-chip drug dose response assay using a perfusion culture microchamber array chip equipped with a serial dilution microfluidic network, in which a logarithmic concentration profile spanning 6 orders of magnitude is automatically generated. We demonstrated the efficacy of this approach by performing an on-chip IC_{50} assay for the model anticancer drug paclitaxel and the model cancer cell line HeLa. We compared the experimental data obtained by this microchamber array chip with data obtained by a conventional experiment using a microplate.

EXPERIMENTAL SECTION

Design of the Perfusion Culture Microchamber Array with a Serial Dilution Microfluidic Network. Figure 1 shows the structural design of the perfusion culture microchamber array with a serial dilution microfluidic network. The perfusion culture microchamber array chip is composed of microchannels and microchambers with different depths. Culture medium and drug solution were supplied through macroscopic liquid reservoirs (left side in Figure 1A). The microchannels connect from liquid reservoirs to 12 microchambers through a serial dilution microfluidic network and from the microchambers to a macroscopic cell-inlet/medium-waste reservoir.

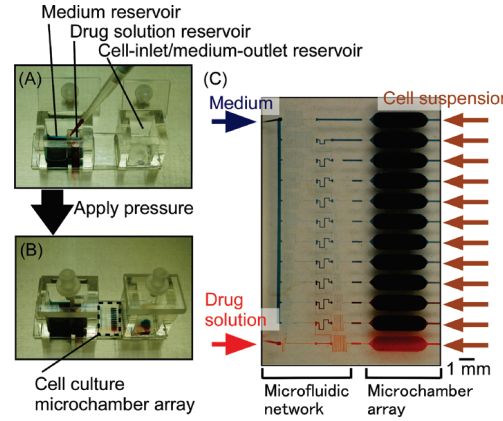

Figure 2. Photographs of the perfusion culture microchamber array chip equipped with a serial dilution microfluidic network. (A) Medium and drug solution were added to the macroscopic reservoirs by means of a micropipet. (B) Solutions were delivered by applying a pressure from the external pressure source through a sterile air-vent filter. (C) Microscope photograph of the generated concentration profile in the microchamber.

The serial dilution microfluidic network used in this study has a structure similar to that designed in our previous study, in which the drug solution was diluted at a ratio of $10^{0.5}$ in each dilution step to create a concentration profile spanning 6 orders of magnitude in 10 dilution steps.[20] In the serial dilution microfluidic network, fluidic resistances of the thin resistance microchannels of 40 μm width and 6 μm depth are dominant, and the concentration profile is determined by the dimensions of the thin resistance microchannels.[20] The thick diffusion-mixing microchannels, which have a width of 50 μm and a depth of 58 μm, induce a long residence time and allow sufficient mixing of microfluids to generate accurate concentration profiles. The details of the structure and dimensions of the serial dilution microfluidic network are shown in Figure S1 and Table S1 in the Supporting Information.

The cell culture microchambers are rectangular (1×2.5 mm) with a depth of 260 μm. Shallow terrace structures with 58 μm depth were fabricated around each cell culture microchamber to assist with the removal of air (i.e., avoidance of bubble formation) during cell loading.[21] The perfusion culture microchamber array chip features a pressure-driven liquid delivery system,[21] which is capable of delivering multiple liquids simultaneously and is suitable for highly integrated drug dose response assays. Figure 2 shows a photograph of the fabricated perfusion culture chip equipped with the serial dilution microfluidic network. The drug solution and culture medium were handled by means of a micropipet (Figure 2A) and were delivered by pressure applied through the air-vent filter (Figure 2B). The drug solution and dilution media were continuously introduced into the microfluidic

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network by applying 5−20 kPa of pressure. In the microfluidic network, the drug solution was repeatedly diluted with the diluting medium, and a logarithmic concentration profile spanning 6 orders of magnitude was successfully generated in the microchamber array (Figure 2C).

Fabrication Method. Perfusion culture microchamber arrays were made of poly(dimethylsiloxane) (PDMS) and were fabricated by photolithography and replica molding using photoresist patterns as master templates.[22,23] The master templates with three photoresist layers were fabricated by multilayer photolithography[24] with a modification.[21] First, negative photoresist SU-8 2002 (MicroChem, Newton, MA) was patterned by photolithography on a silicon wafer to create 6 μm depth resistance microchannels and connection microchannels. An SU-8 50 layer then was spin-coated over the SU-8 2002 pattern and patterned by photolithography to create 58 μm depth microchannels and terraces. Finally, a thicker photoresist, SU-8 2075, was patterned by photolithography to create 260 μm depth microchambers. After this three-cycle photolithography process, the photoresist pattern was developed in ethyl lactate (Wako Pure Chemical, Osaka, Japan). After washing, the master template was treated with tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane (Gelest, Morrisville, PA).[21,22]

PDMS prepolymer and curing agent (Sylgard 184, Dow Corning, Midland, MI) were mixed thoroughly and poured onto the negative masters. After being cured in an oven at 120 °C for 1 h, the PDMS plate was peeled from the master template. The macroscopic medium and drug solution reservoirs and cell-inlet/medium-outlet reservoir were fabricated in the same manner using acrylic resin plates and rods as templates. After surface oxidization by O_2 plasma using a plasma reactor (PR500, Yamato Scientific Co. Ltd., Tokyo, Japan), the PDMS plates and reservoirs were bonded. The obtained PDMS microchamber arrays were annealed at 90 °C for 1 h to make the microchannel surface hydrophobic and were sterilized by ultraviolet light irradiation.

Cell Culture. Human cervical carcinoma cell line HeLa (Riken Bioresource Center, Tsukuba, Japan) was maintained in Eagle's minimum essential medium (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (Gibco), 100 units/mL penicillin, 100 μg of streptomycin, and glutamine at 37 °C in a humidified atmosphere containing 5% CO_2.

Dose Response Assay Procedure. Figure 3 shows the experimental procedure for a dose response assay using the perfusion culture microchamber array with the serial dilution microfluidic network. Paclitaxel was used as a model anticancer drug, and HeLa cells were used as model cancer cells to demonstrate the dose response assay.

The HeLa cells were harvested by trypsin and suspended in the culture medium. The cell suspension (3.5×10^5 cells/mL) was added to the cell-inlet/medium-outlet reservoir, and cells were loaded into each of the microchambers by applying 20 kPa of pressure to the reservoir. During the cell introduction procedure, the flow of the cell suspension became very slow after the microchamber was filled up, since the thin microchannel between the microchambers and the microfluidic network provided a high fluidic resistance: the fluidic resistance of the microchannels (cross-section area of 40×6 μm) is more than 10^6 times higher than the fluidic resistance of the microchamber (cross-section area of 1000×260 μm) in unit length. As a result, the same amount of cell suspension was introduced into each microchamber during the cell introduction procedure, and thu,s the number of introduced cells was homogeneous in each microchamber.

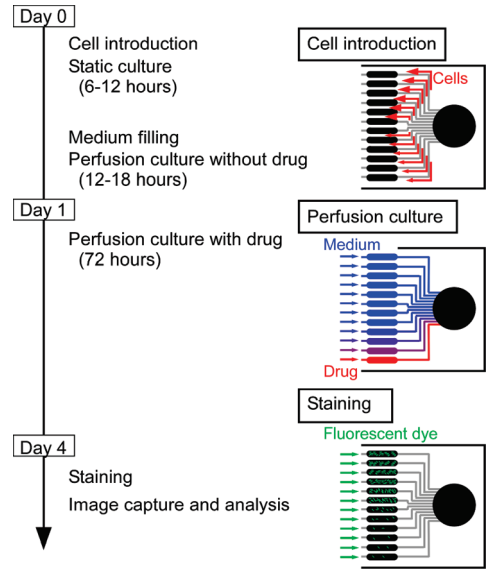

Figure 3. Experimental procedure for the dose response assay using the perfusion culture microchamber array with the serial dilution microfluidic network.

To cultivate the cells in the microchamber array, the array containing the cells was first incubated without perfusion (i.e., under the static culture condition) for 6−12 h to induce cell adhesion on the PDMS surface. After cell adhesion, the microfluidic network was filled with medium by evacuation for 5−8 min from the medium and drug solution reservoirs and through the PDMS layer on the microfluidic network using an evacuation adaptor (Figure S2, Supporting Information). During the liquid filling process, the medium was delivered from the cell-inlet reservoir to the medium and drug solution reservoirs through the microfluidic network, and the air in the connection microchannel was removed through the PDMS layer. As a result, the whole microfluidic network was filled with the medium. The liquid filling by evacuation enhances the removal of the air in the microfluidic network; without evacuation, a small amount of the air will remain in the connection microchannel.

After the liquid filling, a perfusion culture was carried out using the medium without drug by applying 8 kPa of pressure. Twenty-four hours after cell loading, a medium with 10 μg/mL paclitaxel was added to the drug solution reservoir. The paclitaxel was dissolved in dimethyl sulfoxide, and the concentration of the remaining organic solvent in the medium was 1%, which had no effect on cell viability or growth. The perfusion culture was carried out by applying 8 kPa of pressure for 72 h.

After the perfusion culture, the cells were stained with calcein-AM (Dojindo Laboratories, Kumamoto, Japan). A 2 μg/mL solution of calcein-AM in phosphate-buffered saline (PBS) was added to the medium and drug solution reservoirs and was

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delivered into the microchambers at 40 kPa of pressure for 15 min. After 30 min of static incubation at 37 °C, a fluorescence image was obtained with a cooled CCD camera (VB7010, Keyence, Osaka, Japan) equipped with a zoom lens (Navigator Zoom 7000, Nikon, Tokyo, Japan), a fluorescence filter block (U-MNIBA2, Olympus, Tokyo, Japan), and an excitation light source (LC6, Hamamatsu Photonics, Hamamatsu, Japan). The captured fluorescence image was analyzed by Winroof image analysis software (Mitani Corp., Fukui, Japan). After subtraction of the background intensity, the total fluorescence intensity from the microchambers was calculated. The IC_{50} value was calculated from the cell growth curve plotted as a function of the paclitaxel concentration. Three independent microchamber arrays were fabricated and were used for calculation of the IC_{50}.

Dose Response Assay Using a Microplate. The cytotoxicity of paclitaxel was also evaluated using a conventional microplate assay. HeLa cells (1400 cells/well) were seeded on cell culture polystyrene 96-well plates (Becton, Dickinson and Co., Tokyo, Japan) in 100 μL of culture medium without anticancer drugs. After 24 h of cultivation, the medium was exchanged with media containing 10 μg/mL to 0.1 ng/mL paclitaxel, which were prepared by manual serial dilution. After incubation for 72 h at 37 °C, the number of cells was evaluated fluorometrically by staining living cells with calcein-AM. The culture media were exchanged with a 0.2 μg/mL solution of calcein-AM in PBS. After 30 min of incubation at 37 °C, the fluorescence intensity was measured with a GENios plate reader (Tecan, Kawasaki, Japan). To investigate the effect of the surface property of the microplate on the result of the dose response assay, the same experiment was carried out on the PDMS-coated 96-well plate. For this experiment, a small amount of PDMS prepolymer was dropped in the wells of the 96-well plate and incubated at 70 °C for 2 h. The dose response assay then was carried out by the same procedure described above.

RESULTS AND DISCUSSION

The dose response assay of paclitaxel on HeLa cells was demonstrated using the perfusion culture microchamber array with the serial dilution microfluidic network. The cell suspension, culture medium, and drug solution were conveniently handled by the pressure-driven microchamber array (Figure 2). HeLa cells were introduced into the cell culture microchamber array from the cell-inlet/medium-waste reservoir. The coefficient of variation of the introduced number of cells in each microchamber was estimated as 5.3%, assuming that the number of the introduced cells follows the Poisson distribution as expected on the basis of the results obtained in our previous study;[21] the standard deviation (σ) of the Poisson distribution was calculated as $\sigma = \lambda^{0.5}$, where λ is the average number of introduced cells (Table 1). The estimated number of introduced cells was homogeneous enough for a reliable dose response assay. After 6 h of static culture, the HeLa cells were adhered to the bottom of the microchamber. The microfluidic network then was filled with medium, and a perfusion culture was carried out in the absence of the drug paclitaxel. Twenty-four hours after the initial cell loading, the drug-free medium was replaced with a drug solution (10 μg/mL paclitaxel), and a perfusion culture was carried out for 72 h at a logarithmic concentration profile spanning 6 orders of magnitude. The logarithmic concentration profile spanning 6 orders of magnitude was successfully generated as described in our previous study.[20]

Table 1. Dimensions, Culture Conditions, and Hydrodynamic Parameters of the Microchamber Array

culture area of the microchamber	3.3 mm^2
volume of the microchamber	850 nL
cell concentration	3.5×10^5 cells/mL
average number of introduced cells in each microchamber	300
perfusion pressure	8.0 kPa
flow rate of the medium (U)	1.3 μL/h
retention time in the microchamber	40 min
Pe[a]	6.7
average flow velocity in the microchamber	1.3 μm/s
shear stress in the microchamber[b]	3.1×10^{-5} Pa

[a] Pe estimated as LU/D, in which L is the length of the microchamber and D is the diffusion coefficient. The value of D was assumed to be 5×10^{-10} m^2/s. [b] Shear stress was calculated as $6\eta U/H$, in which η is the fluid viscosity and H is the microchamber depth.

In the control microchamber without drug, the loaded HeLa cells adhered to the PDMS surface and grew during the perfusion culture (Figure 4). The cell growth reached the confluent level after a 4 day culture period. These results indicate that the perfusion culture microchamber array chip could be useful for cell growth analysis applications, since the chip provides oxygen through the gas-permeable PDMS wall and provides nutrients to the cells by means of perfusion.

Table 1 shows the culture conditions and hydrodynamic parameters during the perfusion culture. The perfusion culture provided mild culture conditions, the flow velocity was 2 μm/s, and the shear stress was less than 10^{-4} Pa in the microchamber. These flow conditions are orders of magnitude below those at which adverse effects are observed in cell cultures under shear stress.[25−27] As shown in Figure 4, the cell growth was similar in both the upstream and downstream areas in the rectangular microchamber under the culture conditions shown in Table 1. However, cell growth worsened in the downstream area at a medium flow rate as slow as 0.24 μL/h, which is approximately 1/5 as fast as the flow rate used in the present experiments. Under this condition, the Peclet number (Pe), which is the dimensionless number relevant to the ratio of the convection rate and the diffusion rate of the solute, was estimated as 1.3 (Table 1), which indicates that the mixing by diffusion in the microchamber is not enough and that the nutrient in the medium was spent in the upstream area and was exhausted in the downstream area. Therefore, control of the medium flow rate is important for a successful cell-based assay using the perfusion culture microchamber array.

After 72 h of perfusion culture with the drug solution, the live cells were stained with calcein-AM (Figure 5A). The dose response of paclitaxel on HeLa cells was successfully evaluated by fluorescent image analysis. The IC_{50} was successfully calculated from the data obtained from three independent microchamber arrays.

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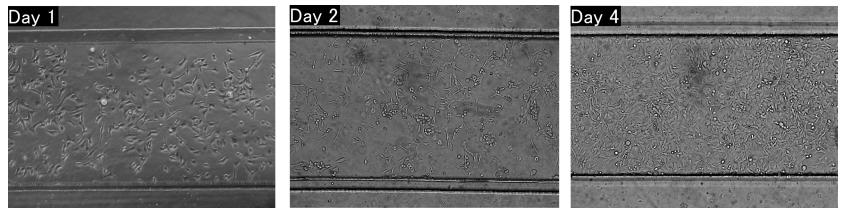
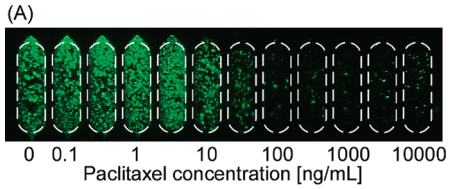
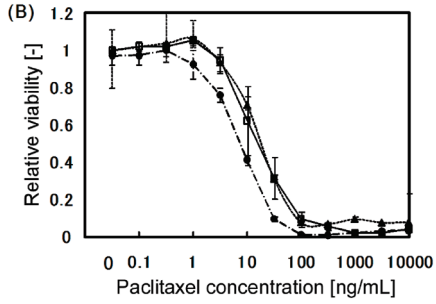


Figure 4. Microscope images of cell growth in the microchamber.





	IC_{50} [ng/ml]
Microchamber array	15.8
PS-Microplate	7.4
PDMS-Microplate	18.3

Figure 5. Dose response assay of paclitaxel on HeLa cells using the microchamber array and microplates. (A) Fluorescence microscope image for the drug dose response assay by the perfusion culture microchamber array. (B) Comparison of the cell growth inhibitory effect of paclitaxel on HeLa cells in the microchamber array (□), in a polystyrene (PS) microplate (●), and in a PDMS-coated microplate (▲). Error bars indicate the standard deviation of the data obtained by three independent microchamber arrays or data obtained from four wells on each microplate.

The experimental data obtained with the perfusion culture microchamber array made out of PDMS were compared with the data obtained with a polystyrene 96-well microplate and with a PDMS-coated 96-well microplate (Figure 5B). The calculated IC_{50} of paclitaxel on HeLa cells was 16, 18, and 7.4 ng/mL for the perfusion culture microchamber array, PDMS-coated microplate, and polystyrene microplate, respectively. From these results, we concluded that the serial dilution microfluidic network was successfully applied to the drug dose response assay, since IC_{50} obtained by the perfusion culture microchamber array was similar to the value obtained by the PDMS-coated microplate.

The average standard deviation of the fluorescence intensity was 8.6% of the relative viability for the perfusion culture microchamber array and 5.3% for the 96-well polystyrene microplate. The large standard deviation for the smaller microchamber array is inevitable because of the smaller number of introduced cells: the average number of introduced cells and corresponding standard deviation were 350 cells and 5.3% for the perfusion culture microchamber array and 1400 cells and 2.8% for the 96-well polystyrene microplate, respectively, assuming the Poisson distribution of the cell introduction. However, in the conventional cell-based assays using the microplate, the transition from 96- and 384-well plates to 1536-well plates is challenging, largely because edge effects and uncontrolled evaporation from very small wells produce poorly defined culture conditions. We believe that the perfusion culture microchamber array chip is capable of obtaining reliable and reproducible data if compared with the 1536-well assay systems, since the perfusion culture microchamber array chip can avoid the unexpected evaporation and bubble contamination.

The IC_{50} depended on the surface materials of the microchamber array or microplate. We attributed the difference in the IC_{50} between the PDMS-coated microplate and polystyrene microplate to the absorption of small and hydrophobic molecules into the PDMS.[28,29] More specific experimental and detailed data focusing on the surface absorption are necessary to investigate this absorption effect on the result of the dose response assay, and currently such a study is in progress.

CONCLUSIONS

A perfusion culture microchamber array equipped with a serial dilution microfluidic network was successfully applied to a dose response assay. The perfusion culture chip, with its simple interface and well-designed microfluidic network, will likely become an advantageous platform for future drug screening using microfluidic devices.

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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