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Triangular microwell for single cell trapping and short-term culturing: Cellular study and analysis of Leukemia cell line

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ABSTRACT: Microfluidic system is a single cell analysis (SCA) platform which is useful for cellular biological study. However, the system still needs further development and improvement for a complete high-throughput single cell analysis. The purpose of this study was to design and develop a microfluidic device for trapping and culturing single cells of human leukemia cell line. Trapping achievement and short-term culture capability were also evaluated. Polydimethylsiloxane (PDMS) microfluidic device consisted of two layers, one of which was a main channel with a dimension of 5 mm wide, 15 mm long, 70 μm high; and an array of 40 μm equilateral triangular microwells with 30 μm deep arranged in downward oblique direction. Trapping efficacy results were about 70.8% for single cells. The effects of different cell densities on cell trapping rates showed that the increment of cell density reduced the single cell trapping capability, and the trapping method was not harmful to the cells. Potential short-term culture of single cell trapping examined by Trypan blue dye assay and Calcein AM/EthD-1 fluorescent staining revealed 80% and 75% of cellular viability after 24 and 48 h of incubation, respectively. In summary, our developed PDMS microfluidic device succeeded in single cell trapping pattern and short-term culturing of leukemia cell line single cells.

KEYWORDS: cell culture, cell viability, microfluidic device, polydimethylsiloxane, single cell trapping

INTRODUCTION

Biological study of cell is very important for biological science, and the study revolves around the concept that the cell is the fundamental unit of life. Cellular biology in normal and neoplastic cells can vary their ability in different ways in order to maintain their homeostasis of dynamic changes in responses to extracellular and intracellular microenvironments [1]. Different cellular biological processes, both in the same and the different tissues, lead to the instability of cellular biology, i.e. the cellular heterogeneity. Factors that influence this heterogeneity pattern include microenvironments, cell-to-cell communications, cell-to-acellular component interactions, and genetic and epigenetic elements. Study of cellular biology can be disrupted by these cellular heterogeneities. The confusing and unstable data of biological signals from the real-time biological study of mixed cell subpopulation are always interpreted in many cellular studies. The interference of cellular signals among cells is also observed in small subpopulation of interest interrupted by stronger signals from main subpopulation [2, 3]. Various conventional methods have been used for biological study of cells. One of the methods is the cell culture system used for studying biological changes in normal and pathological cells. Nonetheless, this system has some limitations to be considered, which includes the effect of cellular heterogeneity in a cell population, microenvironmental control and various biological responses of cells [4]. Moreover, data obtained from analytical assays using average measurement techniques were difficult to interpret and did not represent the actual results at the single-cell level [5, 6].

Single cell analysis (SCA) is one of the bioengineering methods that can solve the disruption of cellular biology study, especially in the time-lapse monitoring of dynamic cell changes [7, 8]. Several bioengi-

neering platforms that initialize single cell analysis, including optical tweezers, magnetophoresis, selective de-wetting, negative dielectrophoresis, and chemical cell patterning, are low-throughput, complicate, and expensive. Some applications using external physical forces (e.g., electricity, magnets, and high-frequency optical waves) or chemical intervention could cause biological changes, cellular degeneration and/or death of trapped cells [9, 10]. In addition, many single cell isolation methods, such as fluorescence activated cell sorting (FACS), magnetic-activated cell sorting (MAC), electrophoresis, and laser microdissection (LMD), are available for the study of cellular heterogeneity. Again, these methods have limitations: label-dependence, prolonged and intricate sample preparation causing harms to studied cells [11].

Microfluidic system, a new research technique for manipulating inertial hydrodynamic immobilization with specific microwell designs, was developed to overcome these limitations. The merits of this technique include low-cost simplicity fabrication, high-throughput performance, high portability, and uncomplicated system operation. The system provides unique advantages for cell biological study, such as capabilities to manipulate at single cell level, control and mimic in vivo microscale environments, separate cells for pure population extraction and miniaturization. Additionally, the microwell system can also be combined with other bioanalytical methods such as immunocytochemistry and polymerase chain reaction (PCR) for advance cellular analysis [12, 13].

There are 2 types of microfluidics, active and passive. Active microfluidics, such as magnetophoresis, acoustophoresis, and dielectrophoresis, require external force fields for protocol stabilization. In passive microfluidics, internal hydraulic properties of fluids are used, and the processes yield high-throughput rate without using external forces that are harmful to the cells [14, 15]. Several microfluidic researches have reported the achievements of single cell entrapment with typical microwell designs. Chen et al [16] revealed 70% success of single cancer cell trapping in their 1,024 microchambers of microfluidic device. Swennenhuis et al [17] reported approximately 67% trapping achievement of single LnCAP, PC3, and SKBR-3 cell lines with a simple array of 6,400 circular microwells and a central pore at the bottom that could be modified to translocate the target cells from the device for further molecular biological study.

Although there were numerous applications of microfluidic device with microwells in biological studies, for example cell behavior, drug delivery, disease detection, and clinical pathology [2, 3], no common protocol for different cell types was established. Hence, studies for each specific application need to be developed and improved. Besides, the complete high-throughput single cell analysis of microfluidic system is still not successful. Therefore, the objective of this study was to design and examine a microfluidic device with triangular microwells for trapping and culturing single cells using human leukemia cell line as an *in vitro* cellular model. The evaluation for trapping achievement and short-term culture capability and method were performed for improvement of the potential application of our in-house microfluidic platform. In addition, novel protocols for cell evaluation inside the microfluidic platform were developed for single cell analysis.

MATERIALS AND METHODS

Design of microfluidic device

The design of microchip has been improved for several years from the study of Park et al [18], Ketpun et al [19] and Tongmanee et al [20]. Entrapping single cells by microwell in a microdevice involved three induction forces. At the beginning, cells were pulled by inertial hydrodynamic force along the main flow inside the microchannel. The floating cells were then pulled across streamlines by gravitational force and moved downward to the bottom where an array of microwells was placed. At the end, hydrodynamic force was generated by fluid flow near the microwells, to which the surrounding cells were attracted. Based on our previous studies [19, 20], among the three types of microwells: triangular, circular and square, the triangular could generate stronger internal recirculation and vorticities comparing to the circular and the square; hence, the triangular were selected. Moreover, one of our research teams reported high trapping rate (up to 80% efficacy) of single polystyrene beads in an array of equilateral triangular microwells [21].

To design microfluidic systems for single cell trapping, COMSOL Multiphysics® version 5.3 software was used to investigate the effects of the size and the arrangement of microwells, as well as the feeding flow rate. From our investigations, the main flow would penetrate deeper to the bottom of the triangular microwells resulting in efficient nutrient delivery and waste removal (Fig. S1). While only a recirculation flow (no inward/outward flow) were observed at the main microchannel bottom of the circular and the square, and that might lead to an accumulation of waste inside them; the main flow could penetrate to the bottom of the triangular with a higher flow velocity at the two-third depth twice higher than the circular and the square.

Moreover, the unique flow structures in the triangular created a vast area from the back to the front contributing to little flow disturbance to the trapped cells in the microwell. From our previous experimental data [20], the triangular sizes of 40, 60, and 80 μm microwells would be suitable for trapping the 10, 15, and 20 μm single cell sizes, respectively.

Fig. S2 shows the flow structures when the cells

were trapped inside the triangular. As mentioned earlier, the cells were dragged by the main flow towards the microwell's tip. The flow velocity decreased when they were occupied by the trapped cells. At the two-third depth, the flow velocity decreased four times comparing to the empty wells. In this study, oxygen concentration was also investigated using laminar fluid flow and dilute species transport capabilities of the software. Cell oxygen consumption was modeled by Michaelis-Menten kinetics. The simulation suggested that the oxygen delivery was relatively high for the triangular as a concentration of oxygen surrounding the trapped cells only one forth reduced from the main flow due to cell's consumption. However, the oxygen concentration in microfluidic system depended on many factors such as flow velocity, growth rate, cell type, and the number of cells [22].

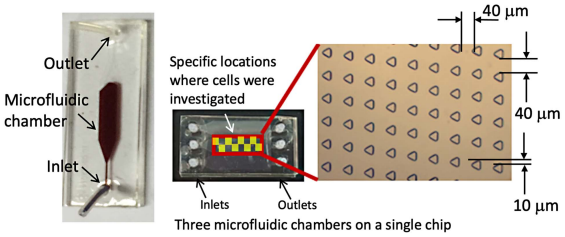

Fig. 1 A PDMS microfluidic device with an array of triangular microwells placed on a bottom surface.

The microfluidic device composed of two functional layers: the upper and the lower. The upper layer was the main flow microchannel, with a dimension of 5 mm × 15 mm × 70 µm. The lower layer contained an array of 12,480 equilateral triangular microwells with the side length of 40 µm and the depth of 30 µm. They were arranged in an oblique downward (Fig. 1 and Fig. S3). The distance between rows and adjacent microwells was 40 µm, and the indenting distance was 10 µm to create a uniformity of oxygen concentration between rows. As shown in Fig. S3, cells in an inline arrangement in the back row tended to expose to lower oxygen concentration as the oxygen transported by flow convection from the main flow was not fast enough. The longer downstream distance for two microwells located inline would create a suitable space for the oxygen from the main flow to be fed for the cell consumption, but longer space is needed resulting to a decrement of trapping capacity.

Microfluidic chip was fabricated using a standard soft lithography with elastomer polydimethylsiloxane (Sylgard 184, Dow Corning, USA) casting on a silicon mold. The two layers of PDMS films were then bonded together with oxygen plasma of 30 W and 40 standard cubic centimeters per minute (sccm) of oxygen for 90 s and later placed on a glass slide. Fig. 1 shows a single fabricated microfluidic chip with three chambers and the specific locations where cells were investigated (marked in yellow).

Cell preparation

Leukemia cell line (Jurkat cell line), ATCC number CRL-2063, was kindly obtained as a gift from Dr. Navapon Techakriengkrai, Department of Veterinary Micro-biology, Faculty of Veterinary Science and Prof. Apiwat Mutirangula, Department of Anatomy, Faculty of Medicine, Chulalongkorn University, Thailand. The cells were cultured in T-25 flasks and maintained in a complete medium consisting of RPMI-1640, 10% (v/v) fetal bovine serum (FBS), and 1% (v/v) penicillin/streptomycin (Invitrogen, USA). All cultures were maintained in a humidified incubator at 37 °C and 5% CO_2.

Evaluation of trapping efficacy of the designed device

For the evaluation of trapping efficacy, cell concentration of 1.5×10^6 cells/ml with ⩾ 90% viability measured by Trypan Blue dye exclusion test was used. Cell trapping was started by injecting phosphate-buffered saline (PBS) solution with a flow rate of 0.5 ml/h to rinse and remove air bubbles from the system, then, a continuous flow of 0.2 ml/h for cell trapping was begun and continued for 30 min. Cells outside the microwells were removed by flushing with complete medium at 0.2 ml/h. Specific locations for cell counting were established within a marking grid in the trapping chip chamber.

Fig. 1 shows the eight squares for cell counting at the specified locations. The counting was observed under a stereomicroscope. Total of 6,240 microwells, or approximately 50% of total microwells in the main chamber, were investigated. Cells in each square were counted and identified as single or multiple (⩾ 2 cells inside a microwell). An average number of trapped cells was calculated from three independent trapping experiments.

Comparison of cell density for trapping

To find the suitable cell density for trapping, three cell densities of 1×10^6, 1.5×10^6, and 2×10^6 cells/ml were prepared. The flow rate was reduced to 0.2 ml/h when loading cells into the system. The cell density of those three concentrations with ⩾ 90% viable cells tested by Trypan Blue as-say were introduced through the device for 30 min. Counting protocols as mentioned above were used.

Potential short term culture capability

For cell culturing in microfluidic device, Jurkat human leukemic cells were cultured with a complete medium of RPMI-1640 supplemented with 10% FBS (Invitrogen, USA). The device was incubated under a controlled condition of 37 °C and 5% CO_2. The

culture medium was replenished every 24 h with flow rate at 0.15 ml/h. After culturing in the device for 24, 48 and 72 h, Trypan blue dye exclusion assay and the Calcein AM/Ethidium homodimer I (EthD-1) fluorescent dyes were performed for cell viability evaluation. The staining protocols in the microfluidic device were developed and briefly described as follows. For Trypan blue dye exclusion test, a working solution composed of 500 µl of 0.4% Trypan blue (Hyclone, GE Healthcare Life Sciences, Marlborough, MA, USA) and 200 µl of 1x PBS was used. For straining processes of trapped cells, the medium in the microchannel was drained; and the channel was slowly flushed with the working solution and incubated in the solution for 10 min in a dark cabinet. Evaluation of viability cells was performed under a light microscopy. Dead cells were positively stained with Trypan blue dye and seen as bright blue color (Fig. 2).

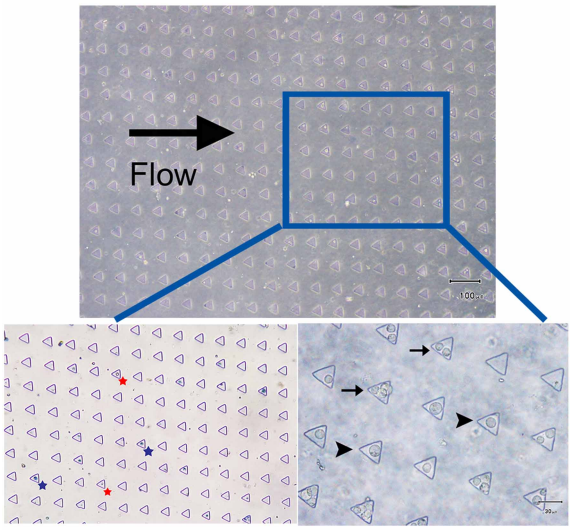

Fig. 2 Left, Trypan blue staining of trapped cells at 48 h: positive dead cells (Blue stars) and negative living cells (Red stars), 200×; Right, cell trapped in triangular microwells in conventional culture media: single cell (arrowheads) and multiple cells (arrow).

For the fluorescent staining of Calcein AM and EthD-1, a dye solution composed of 4 µm Calcein AM and 2 µm EthD-1 mixed with PBS solution (18912-014, Gibco, UK). After the drainage of culture medium, the microchannel was slowly flowed with dye solution at a flow rate of 0.2 ml/h and incubated with the solution for 5–10 min in a dark cabinet. Viability cells were evaluated under fluorescence microscopy (BX51TRF, Olympus) using cell Sens Standard computer software. The living cells converted the nonfluorescent Calcein AM to a green fluorescent Calcein after acetoxymethyl ester hydrolysis by intracellular esterase, while dead cells were detected via checking damaged cell membrane integrity strained with EthD.

Statistical analysis

Percentage of viable cells was calculated by the following equation: $C_v = 100 \times (1 - C_i/N)$, where C_v is the percentage of viable Jurkat cells, C_i is the number of dead cells, and N is total number of single trapped cells. The numbers of single cell and multiple cell trapping microwells were represented as mean with standard error. The correlation of trapping efficacy of the device at different cell concentrations was analyzed by Student's t-test using PROC FREQ of SAS Software. The p-value < 0.05 was determined as significant different of all statistical methods.

RESULTS

Trapping efficacy evaluation

The number and percentage of cell trapping was shown in Table 1. The cell trapped microwells accounted for 42.63% (2,660 from the total of 6,240). The numbers of single-cell and multiple-cell trapped microwells were 1,911 (71.84% of all the occupied ones, 30.63% of the total) and 749 (28.16% of all the occupied ones, 12% of the total), respectively.

Table 1 Percentage of cell trapping in microfluidic chip.

	Average no. of occupied microwell	% of all occupied microwell (2,660)	% of total microwell (6,240)
Total trapping	2,660 ± 26.45	–	42.63
Single cell	1,911 ± 14.84	71.84	30.63
Multiple cell	749 ± 10.26	28.16	12

Three independent trapping experiments were performed.

Effect of cell density on trapping rate

It was observed that the trapping rate increased with the increment of cell density. The percentages of occupied wells at 1×10^6, 1.5×10^6, and 2×10^6 cells/ml were accounted for 20%, 42%, and 64%, respectively (Table 2). In addition, the ratios of single-cell and multiple-cell trapping percentages were approximately equal, except at the highest cell concentration. At 2×10^6 cells/ml concentration, the percentage of single cell trapping was significantly decreased, but it was

Table 2 Trapping efficacy of the device at different cell concentrations.

Concentration	No. of occupied microwell	% of total microwell (6,240)
1.0×10^6 cells/ml	1,248 ± 23.69	20
1.5×10^6 cells/ml	2,660 ± 43.14*	42.63
2.0×10^6 cells/ml	4,005 ± 18.92*	64.18

* $p < 0.05$ compared between the different cell concentrations.

Table 3 Effects of cell concentration on single-cell and multiple-cell trapping efficacy.

Concentration	No. of occupied microwell		% of all occupied microwell	
	Single cell	Multiple cell	Single cell	Multiple cell
1.0×10^6 cells/ml	583 ± 6.02	665 ± 7.09	46.71	53.29
1.5×10^6 cells/ml	1,286 ± 7.21*	1,374 ± 10.01*	48.35	51.65
2.0×10^6 cells/ml	919 ± 10.81*	3,086 ± 8.5*	22.95	77.05

* $p < 0.05$ compared between the different cell concentrations.

significantly increased for the multiple cell trapping, as shown in Table 3.

Potential short-term culture capability

After 24, 48 and 72 h, the viability of cells was examined by two techniques: Trypan blue dye assay and Calcein AM/EthD-1 fluorescent staining as shown in Fig. 3. Table 4 shows the results of the number and percentage of viable single cells at various time observations evaluated by Trypan Blue assay. Viable single cell rate at 24, 48 and 72 h were 82.1% (1,568 from 1,911 cells: $C_v = 82.1$; 100 times magnification), 74.5% (1,423 from 1,911 cells) and 49.56% (947 from 1,911 cells) of all trapped single cell, respectively.

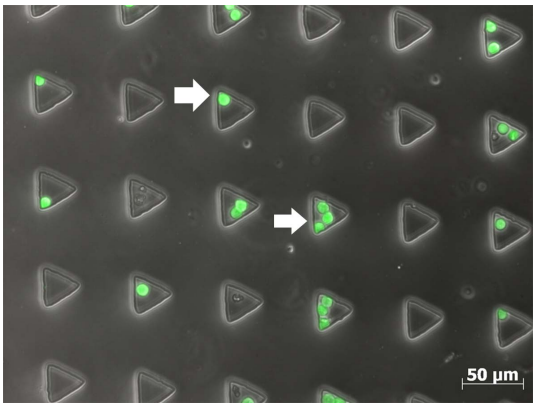

Fig. 3 Left, cell viability tests at 72 h: Trypan blue assay showing positive non-viable cell (arrowhead) and viable cell (arrow); and right, Calcein AM fluorescent staining with positive living cells in bright green color.

Table 4 Culture capability at various time observations by Trypan Blue assay.

Time observation	No. of viable single cells	% of viable single cells
24 h	1,568 ± 28.91	82.1
48 h	1,423 ± 25.51	74.5
72 h	947 ± 18.73*	49

* $p < 0.05$ compared between the different time observations.

Table 5 shows the results of the number and percentage of viable single cells at various time observation that were evaluated by Calcein AM fluorescent staining. Viable single cell rate at 24, 48 and 72 h were 79.69% (1,523 from 1,911 cells: $_{Cv} = 79.7$; 100 times magnification), 75% (1,433 from 1,911 cells) and 51.56% (985.31 from 1,911 cells) of all trapped single cell, respectively.

Table 5 Culture capability at various time observations by Calcein AM fluorescent staining.

Time observation	No. of viable single cells	% of viable single cells
24 h	1,523 ± 12.50	79.69
48 h	1,433 ± 15.04	75
72 h	985 ± 18.02*	51.56

* $p < 0.05$ compared between the different time observations.

DISCUSSION

Our study demonstrated potential application of microfluidic chip with triangular microwell array for trapping and short-term culturing of cancer cell line. Study of viability of culture cells was done inside the microfluidic chip to exhibit the efficacy of our model for single cell analysis.

In the design of Park and colleagues [18], the system composed of an array of triangular microwells with a dimension of 50 µm long and 20 µm deep extending downward into the bottom layer making up a total of 10,000 microwells. The long narrow main channel, a dimension of 5 mm wide, 15 mm long, and 200 mm high, was equipped with the 5 mm in diameter of inlet reservoir for mixing the cell suspension. A 1 ml syringe was used for the loading system by pulling the fluid of various velocities from 0.05 to 0.18 ml/h (average velocity in the channel = 14–50 µm/s) with 20 min perfusion time. Park and colleagues showed 62% of single cell trapping efficacy when randomly counted of 3,000 microwells from the total of 10,000.

In this study, the microfluidic chip consisted of two layers of a main microchannel with dimension of 5 mm wide × 15 mm long × 70 µm high, and an array of 40 µm equilateral triangular microwells (a total of 12,480) with 30 µm deep arranged in downward oblique direction. Syringe pump was used to inject fluid with cell suspension at a flow rate of 0.2 ml/h and 30 min loading time. The current device had shorter flow chamber and smaller but deeper microwells comparing to the design of Park and colleagues.

Our study showed that the single cell trapping could reach 71.84% of all occupied microwells or 30.63% of the total number. Differences in the depth and the dimension of microwells as well as the loading time might play important roles for single cell trapping rate achievement. From our investigation, longer loading time with lower flow rate led to the increment of multiple cell trapping.

To compare the different cell densities and cell trapping rates, 1×10^6, 1.5×10^6, and 2×10^6 cells/ml with $\geqslant$ 90% viable cells tested by Trypan Blue assay were introduced through the device for 30 min. Trapping rate of overall trapping pattern was more strongly dependent on cell density in the suspension. However, the highest cell density of 2×10^6 cells/ml resulted in a significant decrement of single-cell trapping rate, and loading with over-abundant cells led to the increment of multiple cell trapping due to the significantly reduced speed of suspended cells.

However, the cell death examined at 72 h was about one third of trapped cells. The possible cause of cell death could be the slow flow velocity at the bottom of the well where metabolic waste accumulation occurred. With flow simulation, the flow velocity at the depth between 5 and 20 μm into the well was in an order of 1 to 10 μm/s, which was very slow compared with the order of 100 μm/s of the main flow. This low velocity might limit the removal of waste from the microwell resulting in a poor environment for the cell culture.

To extend the survival of trapped cells, several methods could be used. For example, in a 3D microwell method, a bottom channel under the microwell was arranged to deliver waste to the outlet of microfluidic chip [23]. However, the method required a highly precise fabrication process to align three layers of the material together and especially was not applicable for single cell trapping using a very small size of microwell. Another simple method was to continuously feed culture medium to the culture chamber, but the flow rate needed to be carefully selected to prevent any adverse effect due to a shear force. Hence, the simple structure of a microwell array was more applicable due to the fact that modification to improve its efficacy could be accomplished in many ways.

Although cell culturing using the current protocol could be effective for short term study, it was employed for single cell analysis as reported in our previous work [19]. If the protocol is modified, and the survival of trapped cells can be extended, further investigations to imply our microfluidic device for various purposes of cell analysis will be performed. Optical detector, electrical analysis, and mass spectrometry are the diverse analytical techniques that could be used to develop a protocol to integrate with our platform. For optical detection, various techniques such as fluorescence, infrared and surface plasmon resonance and various types of microscopy could be used for imaging cell morphology and movement, specifically labeled cellular contents aa well as quantification of fractionated biomolecules [24]. Cellular activities such as secretion, permeability, and membrane electrophysiologic activity could be studied by electrical analysis using amperometry or electrochemical impedance spectroscopy incorporated in microdevice [25]. Mass spectrometry might be used for the analysis of cellular contents and metabolites [26]. Combining the microfluidic platform with these various analytical techniques could enhance the advancement of basic biological studies and clinical diagnostic and therapeutic researches.

In the past, several microfluidic studies had used Jurkat cells in different aspects. Segalinya et al [27] created the droplet microfluidics to screen and real-time monitor single TCR T cell activation for recognition of candidate T cell therapeutics. The study of Ramji et al [28] used an array of traps penetrating into the flow chamber to capture single Jurkat T cells for tracing the dynamic of drug-induced response and evaluation of latent HIV activation heterogeneity. The droplet microfluidics might have similar problem with the microwell as the waste removal was impossible. In addition, the nutrient supply was also limited so that the cell culturing in this platform should be conducted in a short time. On the other hand, the trapping microfluidics could not retain trapped cells for long time intervals, for example 12 h. With respect to the short-term culturing, the microwell was promising over the others.

In our previous work, a canine mast cell tumor model was used in the similar microfluidic system, and the results revealed a potential application of triangular microwell array to trap single primary cells harvested from clinical specimens for the first time. The trapping efficacy was 53% within 30 min at a slow flow rate of 0.1 ml/min [19]. The factors affecting the trapping rate when compared with our current study was the difference of their dynamic biophysical properties including pleomorphic shape and anisotropic size of fresh MCT cells that might differ from the rather unique cell line. Moreover, the inertial hydrodynamic factors in the system were detrimental to sorted MCT cells, but not to our Jurkat cell line.

CONCLUSION

By varying various parameters, our microfluidic device succeeded in trapping single cell around 70% of occupied microwells, which indicated a high-throughput trapping system. In the short-term culture, around 75% of single leukemia cells survived for 48 h indicating that our model could be modified and applied for more practical clinical uses. The cell trapping rate was increased with higher cell density; however, the possibility of multiple cell trapping increased as well. In addition to culture efficacy, our results showed that

the cell viability at 72 h was significantly decreased to about one-third of trapped single cells. To improve the success of trapping and culturing, the arrangement of microwells and flow parameters of our microfluidic device should be modified in further study. Despite this, our current model could be used as an effective and high-throughput cell-trapping device for various applications, for example an investigation of instantaneous cellular responses and reactions requiring single-cell capture and culture.

Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2023.057.

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Appendix A. Supplementary data

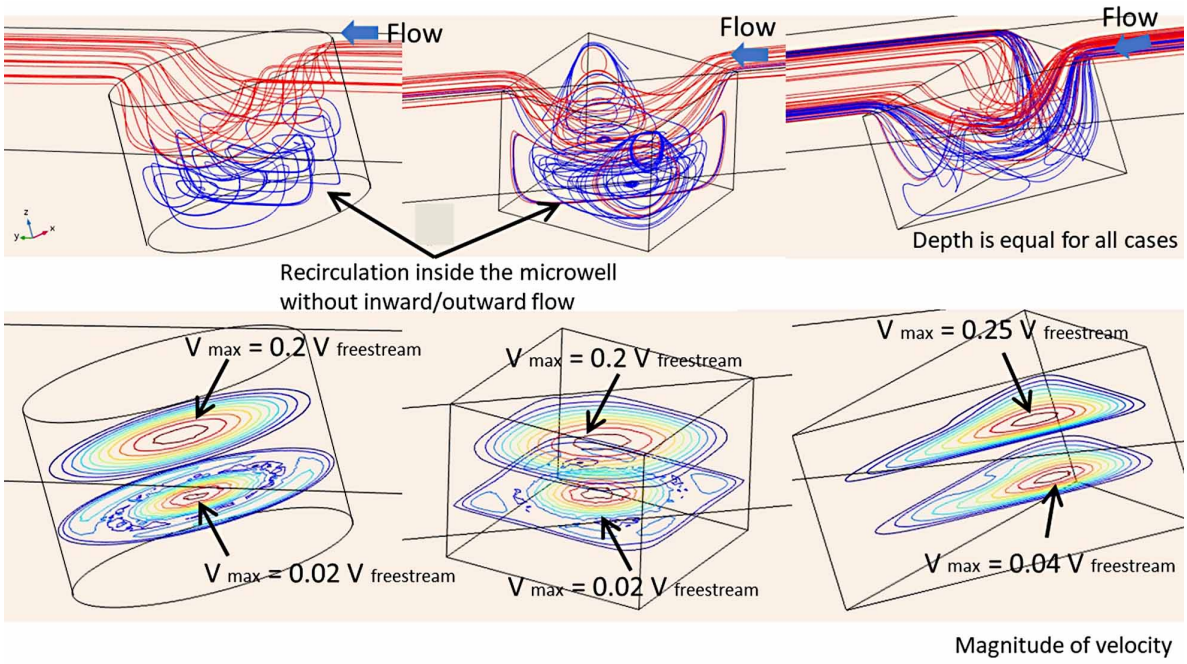

Fig. S1 Streamline and velocity contours for circular, square and triangular microwells of comparable sizes.

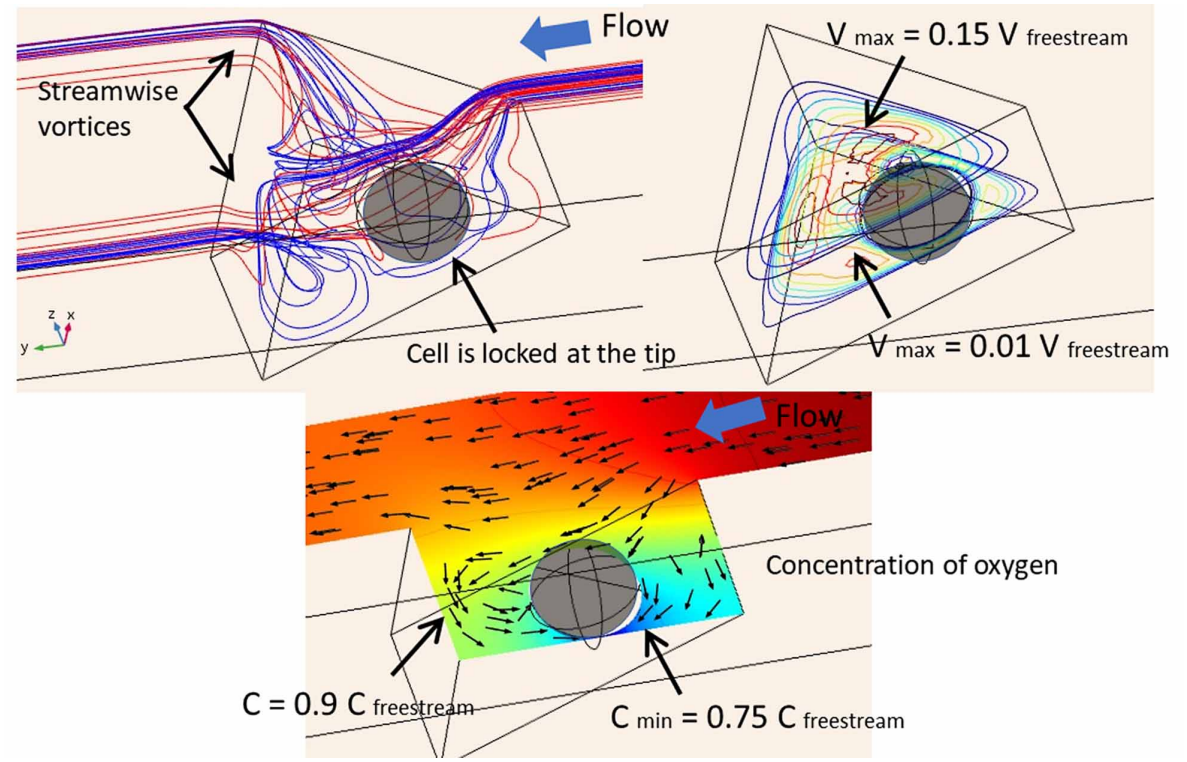

Fig. S2 Streamline, velocity, and oxygen contour inside a triangular microwell occupied by a single cell.

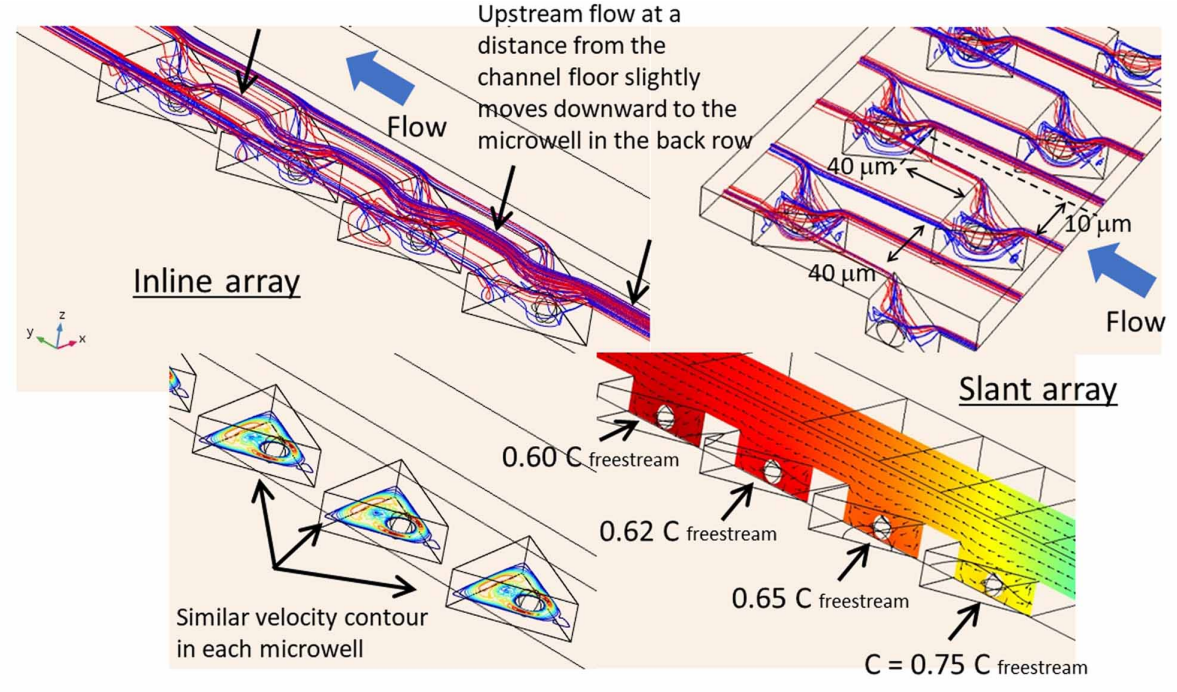

Fig. S3 Schematic pictures of the microfluidic chip comparing inline and slant arrays.