A novel dual-well array chip for efficiently trapping single-cell in large isolated micro-well without complicated accessory equipment

Chenyu Wang,^{1,2} Wenwen Liu,^{1,2} Qingquan Wei,^{1,2} Lufeng Ren,^{1,2}
Manqing Tan,^{1,2} and Yude Yu^{1,2,a)}

¹State Key Laboratory on Integrated Optoelectronics, Institute of Semiconductors,
Chinese Academy of Sciences, Beijing 100083, China

²College of Materials Science and Opto-Electronic Technology, University of Chinese
Academy of Sciences, Beijing 100049, China

(Received 20 March 2018; accepted 20 April 2018; published online 7 May 2018)

Conventional cell-sized well arrays have advantages of high occupancy, simple operation, and low cost for capturing single-cells. However, they have insufficient space for including reagents required for cell treatment or analysis, which restricts the wide application of cell-sized well arrays as a single-cell research tool alone. Here, we present a novel dual-well array chip, which integrates capture-wells (20 μm in diameter) with reaction-wells (100 μm in diameter) and describe a flow method for convenient single-cell analysis requiring neither complicated infrastructure nor high expenditure, while enabling highly efficient single cell trapping (75.8%) with only 11.3% multi-cells. Briefly, the cells are first loaded into the dual-wells by gravity and then multi-cells in the reaction-wells are washed out by phosphate buffer saline. Next, biochemical reagents are loaded into reaction-wells using the scraping method and the chip is packed as a sandwich structure. We thereby successfully measured intracellular β -galactosidase activity of K562 cells at the single-cell level. We also used computational simulations to illustrate the working principle of dual-well structure and found out a relationship between the wall shear stress distribution and the aspect ratio of the dual-well array chip which provides theoretical guidance for designing multi-wells chip for convenient single-cell analysis. Our work produced the first dual-well chip that can simultaneously provide a high occupancy rate for single cells and sufficient space for reagents, as well as being low in cost and simple to operate. We believe that the feasibility and convenience of our method will enhance its use as a practical single-cell research tool. Published by AIP Publishing. <https://doi.org/10.1063/1.5030203>

I. INTRODUCTION

Cell-sized well array systems have been used in the last decades as a very attractive tool for single-cell-level biology research, thanks to their simple structure, convenience of operation, and high single-cell occupancy.^{1–4} The cell-sized well used in a single-cell array is based on a straightforward concept: an individual cell will be physically isolated in a micro-well whose space is little larger than one cell and smaller than two cells.⁵ Unlike other methods, for example, the water-in-oil micro-droplets generated by T-junctions,^{6–8} the percentage of cell-sized wells that contain only one cell reaches a level of almost 92% (Ref. 2) with no multi-cells because this technique overcomes the Poisson restriction principle.² Although many researchers utilize hydrodynamic mechanism to trap single cell by a fine structure manufactured in micro-channel with high throughput and occupancy, the reaction micro-environment containing an individual cell is hard to be isolated.^{9–12} Furthermore, the cells drop into the cell-sized wells by

^{a)} Author to whom correspondence should be addressed: yudeyu@semi.ac.cn. Tel.: 86-10-82304979.

gravity. This passive confinement¹³ of cells in micro-wells does not require complicated equipment or chemical modification.^{14,15} In addition, the structure of cell-sized wells is particularly simple and is easy to manufacture on silicon,¹⁶ glass,¹⁷ or polymer,^{18,19} to form an array for allowing high-throughput assays, which significantly decrease the cost and manpower of cell biology analytical research and medical diagnostic tests.¹

However, the obvious disadvantage of the cell-sized well array is that the volume of a standard cell-sized well is too small to accommodate sufficient liquid for subsequent experiments,^{20,21} such as single-cell reverse transcription polymerase chain reaction, which requires sample dilution of the cell suspension²² for reliable analysis. There are three strategies available to solve this problem. (1) Increasing the size of the wells *in situ*. This strategy obviously sacrifices the single-cell occupancy for providing sufficient space for reagents. If the size of the micro-wells are made much larger than one cell, the percentage of cells in one well will match the Poisson distribution, resulting in a low number of single-cell events and a high number of multiple or empty cell events.^{2,23} (2) Conveying a droplet of reagent into cells. With the help of other outside equipment, the required reagents could be conveyed into each well. Our group has invented an open-pattern droplet-in-oil planar array system, which adapted inkjet printing technology to distribute reagent droplets with a precision at the picolitre level to lyse cells in the cell-sized wells and simultaneously accommodate a high occupancy rate of single cells and sequential addition of reagents.²⁴ Fang *et al.*^{25,26} have also developed an automated sequential operation droplet array system for repeatedly adding reagents to a target location. Although some biological applications have been successfully conducted by these systems,^{24,27,28} the expensive optical devices for the alignment and delivery processes limit their developments. (3) Fabricating dual-wells. Lin *et al.*²⁹ first trapped single-cells using cell-sized well arrays and then transferred the captured cells into another chip with large micro-wells. This method requires the experimenter to carry out delicate operations. What is more, the electroactive double-well array utilizes an electric field to trap single cells in a large micro-well, while the others were washed out by a fluid.^{29,30} However, the external electric field could damage the cells and the cost for micromachining the necessary complicated chip structure^{31–33} is high.

Although the cell-sized well array possesses many advantages, such as high-throughput, high single-cell occupancy, low cost, and ease of operation for capturing single-cells, the follow-up cell treatment and analysis are hard to conduct; thus far, no appropriate solution has been found. Therefore, it is necessary to develop the cell-sized array into a workable single-cell analysis tool. In the current study, we present a novel dual-well structure, as shown in Figs. 1(j) and 1(k), which consists of a capture-well and a reaction-well; the capture-well is manufactured at the centre and base of the reaction-well and utilizes the flow fluid characteristics in it to conveniently trap a single-cell in a large micro-well. Specifically, the scale of the capture-well is similar to the standard cell-sized well (20 μm in diameter and 20 μm in depth) for trapping single cells, while the reaction-wells (100 μm in diameter and 20 μm in depth) are large enough to store reagents. Cells introduced into the dual-well by gravity and cells in the reaction-wells will be washed out by the phosphate buffer saline (PBS) flow and ultimately form a single-cell array. We also used hydrodynamic simulations to determine the contours of the flow velocity and wall shear stress distribution characteristics in the dual-well that illustrates the working principle of our dual-well array chip. Finally, we manufactured the dual-well array and successfully applied it to measure the intracellular β -gal activity in single K562 cells. This is the first presentation of a simple single-cell analysis tool based on cell-sized wells that satisfies the requirements for high single-cell occupancy, convenient operation, and an integrated large reactor, without the need for complex supplementary equipment.

II. MATERIALS AND METHODS

A. Cells and reagents

The K562 cells were purchased from China Infrastructure of Cell Line Resources. The cells were cultured in RPMI 1640 (Wisent, Canada) medium supplemented with foetal bovine serum (10%, Gibco, USA) and a humidified incubator at the temperature of 37 °C and an atmosphere

FIG. 1. Schematic representation of the dual-well array structure and working principles. (a)–(i) Schematic representation of single-cell analysis by the dual-well array chip. (a) PBS buffer was first added to the dual-well and then the bubbles in the micro-wells were degassed by vacuum chamber (b). Cell suspension solution (PBS as solvent) was added to the dual-well (c). PBS buffer was used to wash out the cells outside the capture-cells (d) and finally forming single-cell array (e). (f) Drying out the array by nitrogen. (g) The prepared reagents were transferred onto one end of the dual-well array by a pipette. (h) The blade was dragged slowly across the chip in a smooth motion to load reagent and then sealing oil was added to cover the micro-wells. (i) Packing the chip as sandwich structure. (j) Schematic diagram of the movement of cells in the dual-well according to (d). Cells were loaded into both reaction-wells and capture-wells in the dual well array. Cells that are outside the capture-well are cleared away by the momentum of the liquid. (k) A 3D microscopic image of an individual dual-well.

of 5% CO_2 . Hoechst 33342 solution (Hoechst, Life Technologies, USA) was used to stain the cells for identifying one cell under a fluorescence microscope. An enzymatic assay for intracellular β -gal was carried out using a fluorogenic β -gal substrate, fluorescein-di- β -D-galactopyranoside (FDG, AAT Bioquest, USA).

B. Dual-well array chip design and fabrication

The dual-well array chip was fabricated using standard soft lithography techniques.^{34–36} Briefly, we adapted micro-fabrication technologies to make a positive model (Fig. S1, [supplementary material](#)), on which a 10:1 mixture of Polydimethylsiloxane (PDMS) pre-polymer and curing agent (Sylgard 184, Dow Corning Corporation) was poured and allowed to cure in an oven at 80 °C for 2 h. Finally, the cured PDMS replicas were peeled from off model. We set the size of capture-well (20 μm in diameter and 20 μm in depth) to match that of one cell ($\sim 15 \mu\text{m}$ in diameter). The scale of the reaction-well could be flexibly designed according to special requirements. In this paper, the reaction-well (100 μm in diameter and 20 μm in depth, $\sim 157 \text{ pl}$) was designed over a 20×25 array on a $20 \times 20 \text{ mm}$ PDMS wafer spaced with a 200- μm distance between the centres of each dual-well for single-cell enzymatic assay.

C. Experimental setup for dual-well array chip

A single-cell analysis experiment based on our dual-well array chip is presented schematically in Figs. 1(a)–1(i). Prior to the experiment, cells were centrifuged at 800 rpm for 2 min and the culture medium was removed. Then, the PBS buffer was added to re-suspend cells at a concentration of 2500–4500 cells/ml. In order to clearly identify cells in the well, they were pre-stained with Hoechst 33342 for 10 min. Before loading cells into the dual-well chip, PBS buffer were introduced into the dual-well structure [Fig. 1(a)]. Then, a vacuum chamber was used to degas the bubbles in the micro-wells [Fig. 1(b)]. After the preparatory work, cell suspension solution was introduced into the dual-well array chip; taking advantage of gravity,²⁴ the cells sunk into both the capture-wells and reaction-wells [Fig. 1(c)]. Next, we drew the PBS buffer by pipette and jetted it onto the surface of the array in multiple directions [Fig. 1(d)]. As shown in Fig. 1(j), the cells in the reaction-well or on the surface of chip were forced by fluid. After washing several times, the cells that were in the reaction-wells would have fallen out of the wafer, while those cells in the capture-wells would be still inside [Fig. 1(e)]. At this moment, a single-cell array was formed. We used nitrogen with air pressure about 0.02 MPa for 10 s to carefully dry out the buffer in the dual-well chip [Fig. 1(f)] and adapted the scraping liquid method to load the biochemical reagent [Fig. 1(g)]. A piece of soft silica gel (5 mm in

thickness) was used to scrape reagents into wells. It was held at a 40° – 60° angle, relative to the chip, $20\ \mu\text{l}$ of prepared reagent was transferred onto one edge of wafer, and the silica gel was smoothly scraped across to the other edge. After scraping, FC-40 oil [FC-40 Fluorinated (TM) Electronic Liquid, 3M, USA], which is biocompatible, was added to cover the dual-well to prevent evaporation [Fig. 1(h)]. A spacer was made using double-sided adhesive tape (3M) to provide a square-shaped wall and create a tablet-like $15\ \text{mm} \times 15\ \text{mm}$ chamber for the assay; the final step was to cover it by glass [Fig. 1(i)].

D. Intracellular enzymatic assay

The reaction reagent that contained FDG and TritonX-100, with PBS as a solvent should be fresh. The fluorogenic substrate FDG was stored at -18°C as a 20-mM stock solution in dimethylsulfoxide. Prior to the enzymatic assay, the solution was diluted with PBS reagent to a 100- μM solution, containing 0.1% TritonX-100 as a split whose concentration avoids decreased fluorescence responses.³⁷

E. Fluid mechanics simulation

Simulations were performed to observe the velocity field distribution inside the dual-well structure. Two-dimensional fluid simulations were conducted with the computational fluid dynamics package (Fluent, ANSYS, Inc.). The working fluid (PBS buffer) was assumed to be water [a incompressible, homogeneous, Newtonian fluid; density is $998.2\ \text{kg/m}^3$, dynamic viscosity is $0.001\ \text{kg/(m s)}$], and the flow was assumed to be laminar and steady. The computer models were converted into finite elements using a tetrahedral meshing strategy with a resolution of $2.5\ \mu\text{m}$ between node points. The fluidic region on the dual-well is set as $200\ \mu\text{m}$ up the surface of substrate. The independence verifications are presented in Fig. S2 of [supplementary material](#). All results presented in this section are computed values of velocity until they are stabilized.

III. RESULTS AND DISCUSSION

A. Working principle of dual-well structure

Streamlines penetrate the micro-well with a high aspect ratio, while they did not for a well with a moderate aspect ratio.^{33,38} Such huge variations in the distribution of the flow field for different aspect ratios of wells imply that the force exerted on the cells will depend on their location. We define the shape factor (γ) as the ratio of well diameter to depth. Then, we utilize this phenomenon to design a dual-well structure that consists of a capture-well with a moderate aspect ratio ($20\ \mu\text{m}$ in diameter, $20\ \mu\text{m}$ in depth, $\gamma=1$) and a reaction-well with a high aspect ratio ($100\ \mu\text{m}$ in diameter, $20\ \mu\text{m}$ in depth, $\gamma=5$); the capture-well is in the centre and at the bottom of the reaction-well. Therefore, two types of flow field are created in an individual dual-well. Cells in the reaction-wells, i.e., the upper part of the dual wells that has a high aspect ratio, are subject to higher pressure and are flushed away, while the cells in the capture-wells, i.e., the lower part of the dual wells that has a moderate aspect ratio, are not flushed away. Consequently, the cells in the upper or lower part of a dual-well could easily be separated by fluid flow alone. Because only one cell is trapped in the capture-well, a single-cell array could be generated using this method of a dual-well structure. What is more, for two integrated wells, the upper wells can be a reactor, providing space for reagents which solves the problem of insufficient space in a traditional cell-sized well.

B. Flow characteristics in the dual-well

Simulations were performed to observe velocity field contours and streamlines in dual-wells. For instance, Fig. 2(a) shows the velocity distribution of fluid flow. The initial velocity of fluid at the inlet is $0.1\ \text{m/s}$. The velocity profile of the flow inside the main channel flow is almost laminar, and near the wall of channel, it decreases to zero parallel to the Y-axis, which confirms observations made by other researches.^{32,39,40} When going through the region shown

FIG. 2. Velocity profile and streamlines of the dual-well structure. (a) Velocity profile of the model with an inlet velocity of 0.1 m/s. The highest velocity is at the middle of the main channel and it decreases to zero at the bottom of the capture-wells. The ordinate origin (X-axis) was set in the middle of model and the (Y-axis) on the basal surface of the channel, as shown in the picture. (b) The velocity profile and streamlines of an individual dual-well. (c) and (d) Graphs showing the magnitude of velocity at the location [line (O-N)] marked in (a) and [line (O-S)] and (points: O, P, Q, R, and S) marked in (b) with four inlet flow rates: 0.1, 0.05, 0.01, and 0.005 m/s.

in Fig. 2(b), the majority of streamlines penetrate the reaction-well but do not go into the capture-well because of its moderate aspect ratio. The simulated results fit the original design concept of two types of fluid flow field created in an individual dual-well.

In order to look into the velocity distribution of flow, we plotted the velocity magnitude along the line (O-N) which runs through the centre of an individual dual-well as shown in Fig. 2(a). We also mark five points (O, P, Q, R, and S) to determine flow characteristics inside the dual-well as shown Fig. 2(b). Four different initial flow velocities at the inlet were simulated and the velocity magnitude distribution was plotted according position. Figure 2(c) shows the velocity at the line (O-N), which appears to be similar to a parabolic distribution, even at different initial velocities. However, the presence of the dual-well makes the curve abnormal near point (O), as the box marked in Fig. 2(c), where a length of curve remains close zero, which differs from the other end, near point (N), where the curve still appears to follow a parabolic trend. We then considered the velocity magnitude of flow near the point (O). Figure 2(d) shows that even with an increase in initial velocity, the velocity at point (P) is almost zero, in the middle of capture-well. This result indicates that little disturbance is produced in the capture-well by the flow passing through the reaction well, no matter how strong the flow. However, the velocity at point (R) is larger than at point (P). The purple curve in Fig. 2(d), when then inlet velocity was set as 0.1 m/s, shows that the velocity at point (R), which represents the reaction-well, was $V_R = 0.01036$ m/s, whereas the corresponding velocity at point (P) was only $V_P = 0.000243$ m/s. Their velocity ratio (V_R/V_P at an inlet velocity $v = 0.1$ m/s) is 42.6, which indicates that the cells in reaction-wells experienced a thrust force 42.6 times greater than that in the capture-wells, due to the fluid flow. The results also reveal that even with different initial velocities, the ratio of forces is very similar: V_R/V_P ($v = 0.05$ m/s) = 44.4, V_R/V_P ($v = 0.01$ m/s) = 45.3, and V_R/V_P ($v = 0.005$ m/s) = 45.5, which are shown in Fig. 2(d). Therefore, the cells in the reaction-wells are more unstable than those in the capture-well, being affected by the flow disturbance for a wide range inlet flow velocities.

C. Motion of cells in the dual-well

We then modelled the cells in the dual-well shown in Fig. 3(a). After the capture, cells would be able to stay in locations such as those marked by points (A–F), where the Y-value of

FIG. 3. (a) Simulated contours of the flow velocity and streamlines of dual-well and schematic representation of multi-cell in wells. The points (A–F) represent the locations where cell may stay. (b) Graph indicates the X-axis velocity at the cells location with inlet flow rate of 0.1 m/s.

line (A–E) is $-10\ \mu\text{m}$ and line (F) is $-30\ \mu\text{m}$. Figure 3(b) shows the velocity magnitude of points (A–F) parallel to the X-axis. The profile shows that the X-axis velocity magnitude of flow at the points (A–E) is larger than that at point (F). When the inlet velocity was set as 0.1 m/s, we calculated that the velocity ratios were $V_{Ax}/V_{Fx} = 6.8$, $V_{Bx}/V_{Fx} = 36.7$, $V_{Cx}/V_{Fx} = 49.9$, $V_{Dx}/V_{Fx} = 45.7$, and $V_{Ex}/V_{Fx} = 9.9$. These results showed that cells at the points where in the reaction-wells, points (A–E), would suffer at least 6.8 times the pressure due to fluid flow parallel to the X-axis, than those in capture-wells, point (F), at an inlet velocity of 0.1 m/s. Cells at the points (B–D), in particular, would be most likely to move with the fluid, for the velocity ratio was at least 36.7.

D. Wall shear stress of dual-well structure

Next, we considered the shear stress on the basal surface of the dual-well structure. The detachment of cells is tied to the shear stress occurring at the walls of wells when fluid is flowing through. The greater the wall shear stress, where the cells were located, the more likely they were to break away from the walls. According to the design of our dual-well, the reaction-wells are regions where shear stresses are large enough to eject cells by fluid flow, while the capture-wells serve as sanctuaries for single cells.

The average wall shear stress resulting from steady laminar flow of a Newtonian fluid between two parallel plates with a smooth surface can be estimated using the following equation: $\tau = 6Q\mu/w h^2$, where τ is shear stress, Q is flow rate, μ is the viscosity of the fluid, w is width of the parallel plates, and h is the vertical separation of the parallel plates.^{39,41,42} The equation can also be converted to $\tau = 6V\mu/h$ where V is velocity of the inlet flow. According to our computer model, V is 0.1 m/s, h is $200\ \mu\text{m}$, and μ is $0.001\ \text{kg}/(\text{m s})$ (using water as the medium). We obtain a shear stress, τ , of $30\ \text{dyn}/\text{cm}^2$.

For the micro-well array structure, the wall shear stress inside the wells can be calculated with the equation: $\tau = -\mu dv_x/d_y$ from the velocity profile.^{38,43} We calculated three kinds of micro-well structure to examine the distribution law of wall shear stress. Figures 4(a) and 4(b) show the velocity profile and shear stress profile, respectively, of the micro-wells with a high aspect ratio ($100\ \mu\text{m}$ in diameter, $20\ \mu\text{m}$ in depth, $\gamma = 5$). The shear stress curve is smooth along the original substrate while rising at the edge of well first and then steeply dropping inside the wells. This distribution was also observed in the micro-well with a moderate aspect ratio ($20\ \mu\text{m}$ in diameter, $20\ \mu\text{m}$ in depth, $\gamma = 1$), as shown in Figs. 4(c) and 4(d). The result indicated that the shear stress creates discrete regions that coincide with where micro-wells are generated in the substrate. We then validated this distribution behaviour in our dual-well structure [Fig. 4(e)] and found that two discrete regions were created, as shown in Fig. 4(f).

The different aspect ratios of the micro-wells will affect the shear stress values at key points. The wall shear at original substrate for three structures is $32.7\ \text{dyn}/\text{cm}^2$, $29.9\ \text{dyn}/\text{cm}^2$, and $32.7\ \text{dyn}/\text{cm}^2$ which is close to the average value of $30\ \text{dyn}/\text{cm}^2$. However, for example, Fig. 4(d) shows the micro-well ($\gamma = 5$) generating the maximum value of shear stress is

FIG. 4. (a) and (b) demonstrate the velocity profile and wall shear stress for micro-well with $100\ \mu\text{m}$ in diameter and $20\ \mu\text{m}$ in deepness, respectively. (c) and (d) are for micro-well with $20\ \mu\text{m}$ in diameter and $20\ \mu\text{m}$ in deepness. (e) and (f) are for dual-well consists of a capture-well ($20\ \mu\text{m}$ in diameter and $20\ \mu\text{m}$ in deepness) and a reaction-well ($100\ \mu\text{m}$ in diameter and $20\ \mu\text{m}$ in deepness). All simulated processes were carried out at inlet flow rate of $0.1\ \text{m/s}$.

$60.7\ \text{dyn/cm}^2$ while that for the well with ($\gamma = 1$) is only $38.4\ \text{dyn/cm}^2$. What is more, the value of shear stress increased first and then decreased in the bottom of a micro-well with a high aspect ratio, while it did not in one with a moderate aspect ratio. We calculated the micro-well model with different values of γ and found that for the same initial flow velocity, the micro-wells with a high γ would generate larger shear stresses, both at the edge and bottom of well, than those which with a low γ (Fig. S3, [supplementary material](#)). These phenomena indicate that the value of discrete shear stress changes depends on the shape factor (γ). For this relationship, the capture-well with a low γ would generate the smallest wall shear stress in the dual-well structure; the calculated wall shear stress profile of our dual-well structure [Fig. 4(f)], which is shown in Fig. 4(f), verified this hypothesis. Excepting the corners of the reaction-well, the wall shear stress in the capture-well is smaller than that in reaction-well. Therefore, the cells trapped in the capture-well would have suffered the smallest wall shear when fluid flow through the dual-well structure.

In summary, our simulation work revealed the distribution of the shear stress of a micro-well array structure, demonstrating that discrete stress regions would be created and that the smaller shape factor (γ) is correlated with lower wall shear stress. These relationships were also verified for other multi-well structures. We designed two kinds of tri-wells and the simulation results showed that discrete stress regions would be created where micro-wells existed, and wells with a smaller shape factor (γ) generated smaller wall shear stress inside the wells (Fig. S3, [supplementary material](#)). Based on these findings, our dual-well structure integrated a reaction-well, with high γ , where cells would be washed out by the flow and a capture-well with low γ , promoting their retention.

E. Dual-well array for high single-cell capture efficiency

Figure 5(a) shows the 3D image of a positive model of our dual-cell array. The height of reaction-well-pillar is $22\text{ }\mu\text{m}$ and the diameter is $95\text{ }\mu\text{m}$. The height of capture-well-pillar is $20\text{ }\mu\text{m}$ and the diameter is $18\text{ }\mu\text{m}$. Figure 5(b) is the real product chip made by PDMS material. We then use this chip to generated single-cell array. Figures 5(c) and 5(d) show the K562 cells trapped in the dual-well before and after the washing step. As per the design, cells were first loaded into the dual-well and occupied both the reaction-wells and capture-wells [Fig. 5(c)]. Each bright blue spot in the fluorescence images represented an individual cell. After washing, most of the cells in the reaction-wells had been washed out [Fig. 5(d)]. Figure 5(e) show bright-field and corresponding fluorescence images of cells in individual dual-well before wash step.

Fluorescence images in Fig. 5(f) show the two cases of single-cell in an individual dual-well. In most situations, one cell was isolated in the capture-well without other cells in the reaction-well. However, one cell in the reaction-well with empty capture-well would form single-cell case as well. As for multi-cell, two or more cells in the dual-well are showed in Fig. 5(g). We estimated an average single-cell occupancy of approximately 75.8% and 11.3% multi-cell occupancy (Fig. S4, [supplementary material](#)) from three single-cell capturing experiments. The results show that the dual-well chip satisfied the requirement for high single-cell occupancy with low incidence of multi-cell occupancy.

F. Intracellular enzymatic assay at the single-cell level

We applied our dual-well array chip to measure the intracellular β -gal activity at a single-cell level. Figure 5(a) states that nonfluorescent di- β -D-galactopyranoside (FDG) is hydrolysed by the enzyme, β -galactosidase, which would be released from the K562 cells, to the highly fluorescent fluorescein.^{44–46} By long-term and *in situ* monitoring the fluorescence intensity from the micro-well reactor trapping only one cell, the intracellular β -galactosidase kinetics could be researched. In this paper, for each enzymatic assay experiment, the diluted K562 cells were stained with Hoechst dye and introduced into the dual-well array. We use a pipette to draw PBS buffer and jet it onto the surface of the array in multiple directions to achieve the maximum possible wash out of cells from the reaction-well. Nitrogen was used to dry the PBS buffer in the wells slowly. Then, a single-cell array was generated. Next, the reaction reagent ($20\text{ }\mu\text{l}$) that contained FDG ($100\text{-}\mu\text{M}$) and TritonX-100 (0.1%) with PBS as a solvent was

FIG. 5. (a) 3D image of a positive model made of silicon material. (b) Dual-well array made of PDMS material. (c) Cells introduced into a dual-well and (d) cells in the dual-wells after the washing step. Bright-field and corresponding fluorescence images (e) of multiple cells in an individual dual-well before washing step. Fluorescence images show single-cell (f) and multi-cell (g) in an individual dual-well after washing step.

FIG. 6. (a) The detection principle of intracellular enzymatic assay for β -galactosidase. (b) Merged photo of bright-field image of the enzymatic assay and fluorescence images of the single-cell array. The numbers point out the dual-well which has trapped cells. (c) Kinetics of the average fluorescence intensity from the wells containing a cell. (d)–(g) Post-lysis fluorescence images of the wells for monitoring the presence of the fluorescent product (fluorescein) from FDG hydrolysis by intracellular β -gal.

loaded into the dual-well array and the retained reagent between the wells was scraped, as shown in Fig. 1(h). Finally, the enzymatic assay was quickly sealed by FC-40 oil and packed as a sandwich structure. The bright-field image of the enzymatic assay and fluorescence images of the single-cell array are merged together and the dual-wells including cells were marked by circles, shown in Fig. 6(b).

We then collected fluorescence images over the course of a 15-min period using a colour CCD camera with a filter set of 490/20 nm for fluorescence excitation and 514/30 nm for emission. Using the ImageJ software, we split fluorescence images to obtain 8-bit grayscale images of green fluorescence and extracted the mean fluorescence intensity of each well-point; these images (only over the course of a 30 min period) are shown as in Figs. 6(d)–6(g). For the empty dual-wells (denoted as 5, 7, 8, 12, 16, and 18), there was no increase in fluorescence intensity inside and no fluorescein observed outside the wells. These experimental results indicate that the intracellular materials from single cells were efficiently isolated. No cross contamination was detected between two dual-wells. As a based demand of single cell analysis, each reactor, each dual-well of our chip, should be segregated from each other to ensure the accuracy of the experiment results.

We also found that the fluorescence intensity increased as the time for catalytic reaction was extended and at the same time points, signal values arising from different dual-wells differ. The values are plotted in Fig. 6(c). The slope of the plot for each individual cell (exclude plot 20) was distinct. However, the variation trend for all plots is same. The variation trend is also same as the multi-cell situation of plot 20. Although the K562 cells were cultured under the same conditions, the large cell-to-cell variations in intracellular β -gal activity were released when studied on single-cell level. This phenomenon has also been reported in the previous studies.^{24,46} Therefore, our dual-well array chip allowed highly sensitive measurement of cellular heterogeneity.

IV. CONCLUSIONS

We have presented a dual-well array based on a cell-sized well and have used it successfully to measure the intracellular β -gal activity in single K562 cells and reveal the inter-cellular

heterogeneity. Our dual-well chip satisfies the requirement for high single-cell occupancy, by overcoming the Poisson limit, at the same time, as satisfying the requirement for large reaction-wells that can contain reagents concurrently. What is more, compared with the reported micro-well array chip, we used our micro-well array to do single-cell research without complex operational procedures and auxiliary equipment. We just employed a simple washing step to wash out the cells in the reaction-wells, while isolating single-cells in the capture-well with an average single-cell occupancy of approximately 75.8% and only 11.3% multi-cell occupancy.

We used hydrodynamic simulations to investigate the principles of our dual-well array. For flow characteristics in the dual-well, our calculated results show that the magnitude of the velocity at the mid-point of reaction-wells is 42.6 times greater than that of the capture-wells of our dual-well array chip, indicating that the cells in the reaction-wells experienced a force from the flowing water that was 42.6 times greater than that in the capture-wells. Notably, this force increases to almost 49.9 times in the X-axis direction. Second, we found that the smallest wall shear stresses, near to zero, were associated with capture-wells that promoted single cell retention, while larger shear stresses in the reaction-wells were good indicators of the effective removal of multi-cells during the fluid washing step. What is more, our simulated results also revealed the relationship of shear stress distribution in the multi-micro-well array structure that discrete shear stress regions are created where micro-wells are generated and the amplitude of variation is tied to the shape factor (γ) of wells. This dependency was verified in a tri-well array structure and will guide further research to design multi-well arrays.

In conclusion, the dual-well array for the first time allow the ability to simultaneously accommodate the high occupancy rate of single cells and sufficient space for reagents while has advantages as low cost and simple operation. Although in this paper, the washing step is purely manual processes which result that the flow boundary condition cannot fit perfectly with the simulation model, the single-cell capturing results has achieved the desired goal. We believe that once we solve the problems of automation and integration, our dual-well structure will likely constitute a new and effective biotechnology for single cell research.

SUPPLEMENTARY MATERIAL

See [supplementary material](#) for the manufacturing process flow diagram of the positive model, statistical data of the single-cell occupancy rate, and the detailed data of simulated results.

ACKNOWLEDGMENTS

This work was supported by grants from the National Natural Science of China (Grant No. 61334008) and the Instrument Developing Project of the Chinese Academy of Sciences (Grant No. YZ201402).

- ¹S. Lindström, R. Larsson, and H. A. Svahn, *Electrophoresis* **29**(6), 1219–1227 (2008).
- ²J. R. Rettig and A. Folch, *Anal. Chem.* **77**(17), 5628–5634 (2005).
- ³C. D. Di and L. P. Lee, *Anal. Chem.* **78**(23), 7918–7925 (2006).
- ⁴G. Agarwal and C. Livermore, *Lab Chip* **11**(13), 2204–2211 (2011).
- ⁵S. Lindström and H. Andersson-Svahn, *Biochim. Biophys. Acta* **1810**(3), 308–316 (2011).
- ⁶M. Lundqvist, P. Nygren, B. H. Jonsson, and K. Broo, *Angew. Chem.* **45**(48), 8169 (2006).
- ⁷P. Kumaresan, C. J. Yang, S. A. Cronier, R. G. Blazej, and R. A. Mathies, *Anal. Chem.* **80**(10), 3522–3529 (2008).
- ⁸E. Z. Macosko, A. Basu, R. Satija, J. Nemes, K. Shekhar, M. Goldman, I. Tirosh, A. R. Bialas, N. Kamitaki, and E. M. Martersteck, *Cell* **161**(5), 1202–1214 (2015).
- ⁹A. Karimi, S. Yazdi, and A. M. Ardekani, *Biomicrofluidics* **7**(2), 21501 (2013).
- ¹⁰D. Jin, B. Deng, J. X. Li, W. Cai, L. Tu, J. Chen, Q. Wu, and W. H. Wang, *Biomicrofluidics* **9**(1), 014101 (2015).
- ¹¹M. Dhar, J. Wong, A. Karimi, J. Che, C. Renier, M. Matsumoto, M. Triboulet, E. B. Garon, J. W. Goldman, and M. B. Rettig, *Biomicrofluidics* **9**(6), 064116 (2015).
- ¹²L. Mi, L. Huang, J. Li, G. Xu, Q. Wu, and W. Wang, *Lab Chip* **16**(23), 4507 (2016).
- ¹³I. Inoue, Y. Wakamoto, H. Moriguchi, K. Okano, and K. Yasuda, *Lab Chip* **1**(1), 50–55 (2001).
- ¹⁴A. Folch and M. Toner, *Annu. Rev. Biomed. Eng.* **2**, 227 (2000).
- ¹⁵M. Ochsner, M. R. Dusseiller, H. M. Grandin, S. Luna-Morris, M. Textor, V. Vogel, and M. L. Smith, *Lab Chip* **7**(8), 1074–1077 (2007).

- ¹⁶Y. Tokimitsu, H. Kishi, S. Kondo, R. Honda, K. Tajiri, K. Motoki, T. Ozawa, S. Kadowaki, T. Obata, and S. Fujiki, *Cytometry, Part A* **71**(12), 1003–1010 (2007).
- ¹⁷M. Deutsch, A. Deutsch, O. Shirihai, I. Hurevich, E. Afrimzon, Y. Shafran, and N. Zurgil, *Lab Chip* **6**(8), 995 (2006).
- ¹⁸A. Khademhosseini, J. Yeh, S. Jon, G. Eng, K. Y. Suh, J. A. Burdick, and R. Langer, *Lab Chip* **4**(5), 425 (2004).
- ¹⁹D. B. Weibel, W. R. Diluzio, and G. M. Whitesides, *Nat. Rev. Microbiol.* **5**(3), 209 (2007).
- ²⁰S. Lindström, M. Eriksson, T. Vazin, J. Sandberg, J. Lundberg, J. Frisén, and H. Andersson-Svahn, *PLoS One* **4**(9), e6997 (2009).
- ²¹V. Lecault, M. Vaninsberghe, S. Sekulovic, D. J. Knapp, S. Wohrer, W. Bowden, F. Viel, T. McLaughlin, A. Jarandehi, and M. Miller, *Nat. Methods* **8**(7), 581 (2011).
- ²²A. K. White, M. Van Insberghe, O. I. Petriv, M. Hamidi, D. Sikorski, M. A. Marra, J. Piret, S. Aparicio, and C. L. Hansen, *Proc. Natl. Acad. Sci. U. S. A.* **108**(34), 13999 (2011).
- ²³M. C. Park, J. Y. Hur, H. S. Cho, S. H. Park, and K. Y. Suh, *Lab Chip* **11**(1), 79 (2011).
- ²⁴C. Wang, W. Liu, M. Tan, H. Sun, and Y. Yu, *Biomicrofluidics* **11**(4), 7918–7925 (2017).
- ²⁵Y. Zhu, Y. X. Zhang, W. W. Liu, Y. Ma, Q. Fang, and B. Yao, *Sci. Rep.* **5**, 9551 (2015).
- ²⁶Y. Zhu, L. N. Zhu, R. Guo, H. J. Cui, S. Ye, and Q. Fang, *Sci. Rep.* **4**(6186), 5046 (2014).
- ²⁷W. B. Du, Q. Fang, Q. H. He, and Z. L. Fang, *Anal. Chem.* **77**(5), 1330 (2005).
- ²⁸Y. Zhu and Q. Fang, *Anal. Chim. Acta* **787**(13), 24 (2013).
- ²⁹C. H. Lin, Y. H. Hsiao, H. C. Chang, C. F. Yeh, C. K. He, E. M. Salm, C. Chen, I. M. Chiu, and C. H. Hsu, *Lab Chip* **15**(14), 2928 (2015).
- ³⁰S. H. Kim, M. Antfolk, M. Kobayashi, S. Kaneda, T. Laurell, and T. Fujii, *Lab Chip* **15**(22), 4356 (2015).
- ³¹E. Bisceglia, M. Cubizolles, F. Mallard, F. Vinet, O. François, and P. B. Le, *Lab Chip* **13**(5), 901–909 (2013).
- ³²M. Bocchi, L. Rambelli, A. Faenza, L. Giulianelli, N. Pecorari, E. Duqi, J. C. Gallois, and R. Guerrieri, *Lab Chip* **12**(17), 3168–3176 (2012).
- ³³Y. Tian, Y. L. Zhang, J. F. Ku, Y. He, B. B. Xu, Q. D. Chen, H. Xia, and H. B. Sun, *Lab Chip* **10**(21), 2902–2905 (2010).
- ³⁴M. A. Unger, H. P. Chou, T. Thorsen, A. Scherer, and S. R. Quake, *Science* **288**(5463), 113 (2000).
- ³⁵D. A. Chang-Yen, R. K. Eich, and B. K. Gale, *J. Lightwave Technol.* **23**(6), 2088–2093 (2005).
- ³⁶M. P. Lutolf, R. Doyonnas, K. Havenstrite, K. Koleckar, and H. M. Blau, *Integr. Biol.* **1**(1), 59–69 (2009).
- ³⁷G. Ocvirk, H. Salimi-Moosavi, R. J. Szarka, and E. A. Arriaga, *Proc. IEEE* **92**(1), 115–125 (2015).
- ³⁸A. Manbachi, S. Shrivastava, M. Cioffi, B. G. Chung, M. Moretti, U. Demirci, M. Yliperttula, and A. Khademhosseini, *Lab Chip* **8**(5), 747–754 (2008).
- ³⁹J. Y. Park, M. Morgan, A. N. Sachs, J. Samorezov, R. Teller, Y. Shen, K. J. Pienta, and S. Takayama, *Microfluid. Nanofluid.* **8**(2), 263–268 (2010).
- ⁴⁰H. Somaweera, S. O. Haputhanthri, A. Ibragimov, and D. Pappas, *Analyst* **140**(15), 5029–5038 (2015).
- ⁴¹D. P. Gaver III and S. M. Kute, *Biophys. J.* **75**(2), 721–733 (1998).
- ⁴²S. Y. Hwang, K. W. Kwon, K. J. Jang, M. C. Park, J. S. Lee, and K. Y. Suh, *Anal. Chem.* **82**(7), 3016–3022 (2010).
- ⁴³N. Korin, A. Bransky, M. Khoury, U. Dinnar, and S. Levenberg, *Biotechnol. Bioeng.* **102**(4), 1222 (2009).
- ⁴⁴A. Plovins, A. M. Alvarez, M. Ibañez, M. Molina, and C. Nombela, *Appl. Environ. Microbiol.* **60**(12), 4638 (1994).
- ⁴⁵J. Hofmann and M. Sernetz, *Anal. Biochem.* **131**(1), 180–186 (1983).
- ⁴⁶S. Q. Gu, Y. X. Zhang, Y. Zhu, W. B. Du, B. Yao, Q. Fang, and A. Chem, *Anal. Chem.* **83**(19), 7570–7576 (2011).