Liquid gradient in two-dimensional matrix for high throughput screening

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Based on the ingenious combination of two different gradient generation mechanisms, this work reports a novel approach for a high throughput linear liquid gradient in a two-dimensional (2D) matrix. Specifically, a typical Christmas Tree structure with two inlets was designed as the first mixture gradient generator, upon which the second diffusion gradient generator was coupled to produce the desired concentration series on the basis of the distance difference. Rather than a simple 1D line, the integration of the two generators would result in an innovative 2D matrix of reservoirs, which was then characterized both theoretically and experimentally. Theoretically, calculation of fluid field demonstrated the formation of a concentration gradient, which was then confirmed by the dye solution visualization analysis. For high throughput screening application, doxorubicin (Dox) was then selected as model medicine to treat the acute myeloblastic leukemia (HL-60) cells. Cell viability displayed that cell death rate enhanced with the increase of drug concentration, and this result was higher than that on a 96-well plate, and the corresponding mechanism was properly discussed. Subsequently, Dox and quercetin were employed simultaneously to generate an overlapping gradient and its effect on HL-60 cells was investigated. Due to the automatic formation of concentration gradient that could improve the work efficiency, this work provides a promising tool for future high throughput drug screening. © 2013 AIP Publishing LLC. [<http://dx.doi.org/10.1063/1.4847815>]

I. INTRODUCTION

Recently, microfluidic technology has drawn much research efforts to solve major problems in cell-based assays for drug screening.¹ Specifically, microchips for drug screening possesses the advantages of high throughput, little sample consumption, and good consistency, which greatly improve the researches in pharmacology, efficacy, and dose.^{2,3} Besides, they give a better prediction of how drugs or compounds would behave *in vivo* than traditional drug assays, since micro environment on chip, if well designed, can be closer to environment *in vivo* than that on plate.⁴ Therefore, microchips for drug screening are attracting paramount attentions in drug researches. To date, hundreds of microfluidic devices have been proposed for cell-based assays and biochemical screenings.⁴⁻⁶

Gradient generation is thought to be one of the key techniques for on chip high throughput screening. Over years, numerous concentration gradient generators have been proposed.⁴⁻⁶ Basically, these generators could be classified into two main kinds, based on the use of static or dynamic methods. To obtain a stable and static gradient, the first kind commonly utilizes static effects, such as selectively adsorbing substances^{7,8} or free diffusion processes. A diffusion based gradient generator utilizes concentration difference between two points to form a linear gradient.⁹ This kind of gradient is suitable for cell-based assays, since they do not introduce extra shear force on cells. However, pure diffusive gradient lacks of solution refreshment and

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the concentration gradient could not be modulated once generated. To obtain dynamic and tunable gradient, the second kind generators capitalize on two types of fluid mixers, i.e., the Parallel-flow Gradient Generator^{8,10} or Premixer Gradient Generators (Christmas Tree Structure).^{8,11} Dynamic generators could produce a steady gradient, and most of them can be modified dynamically via flow velocity control. However, such gradients only exist under constant fluid flow, which makes them solution consumptive. Besides, dynamic flow could cause shear force and drag force on cells, which could greatly influence the behaviour of cells. Using these above-mentioned generators, many protocols have been proposed for drug screening application. For high throughput, the traditional one dimension (1D) gradient suffers badly to the finite number of available data points, due to the limitation of chip size and pressure. As comparison, 2D matrix has the advantages of higher gradient content under the same pressure condition.^{12,13} Moreover, the combination of two gradient generators gives us the opportunity to overcome their respective defects. Previously, we fabricated a cell culture and screening chip, which only adopted the Christmas tree structure for gradient generation, leading to relatively limited content of the concentration gradient.¹⁴

Herein, we report a 2D liquid gradient chip using a three layer structure, with each reservoir corresponding to a specific concentration. Liquid channels on the top layer permit the fluid delivery and gradient formation. A 5*5 matrix of reservoirs in the middle layer links not only the top layer but also the small microchannels array (SMA) on bottom layer. The resulted 2D gradient generation chip can generate N^2 points, than N points on normal gradient chips, and hence, more suitable for concentration screening. For drug screening applications, the concentration gradient of model medicine doxorubicin (Dox) was generated on this platform for acute myeloblastic leukemia (HL-60) cells treatment. It is observed that cell viability related intimately with the Dox concentration. Dox and quercetin (Que) were then employed simultaneously to generate an overlapping gradient, and its effect on HL-60 cells was further explored. The proposed high throughput 2D gradient generation chip could provide a new approach for drug screening research.

II. MATERIALS AND METHODS

A. Cells and reagents

Sylgard 184 elastomer base and curing agent for polydimethylsiloxane (PDMS) were both purchased from Dow Corning (Midland, MI). SG-2506 borosilicate glass (with 145 nm thick chrome and 570 nm thick positive S-1805 type photoresist) was purchased from Shaoguang Chrome Blank Co. Ltd. (Changsha, China). Dox, Que, Acridine orange (AO), and Ethidium bromide (EB) were purchased from Sigma. Methylene blue was provided by Shanghai Reagent Co, Ltd. HL-60 cells were cultured and propagated in Dulbecco's modified eagle medium (DMEM) cell culture medium (Gibco) with 10% fetal bovine serum and 100 mg l⁻¹ penicillin and 100 mg l⁻¹ streptomycin at 37 °C under 5% CO₂ in standard incubator. 100 mM Phosphate buffered saline (PBS, pH 7.0) was purchased from Keygen Biotech Ltd. (Nanjing, China).

B. PDMS microchip design, fabrication, and assembly

This PDMS microfluidic device is composed of a three layer structure: Top layer with liquid channels, middle layer with gas channels at lower surface and through-holes as reservoirs, and bottom layer with SMAs. The three layers are assembled with the reservoirs connecting liquid channels with SMAs (Figure 1). The liquid channels on top layer act as gradient generators and liquid delivery system. The main liquid channels form a network structure. The widths of the liquid channels in x and y directions are 500 μ m and 700 μ m, respectively. The zigzag liquid mixer is 250 μ m for width. Channels on the top layer are all 60 μ m for depth. The zigzag liquid mixer forms a Christmas Tree Structure to generate the first gradient. Fluid from two inlets is split by Christmas Tree Structure and the resulting fluid is delivered into the network structure and then the reservoirs. Reservoirs are used for cell capturing, cell culture, and drug assay. Every reservoir is surrounded by a ring-shaped gas channel, with the width of 200 μ m

FIG. 1. 3D image of chip structure (a) the top layer, (b) the middle layer, (c) the bottom layer, and (d) a part of the top view to show the meaning of *a*, *b*, and *r*. (e) A 3D image of the total structure and zoom in for details.

and the depth of $60\text{ }\mu\text{m}$. These ring shaped channels are linked to a $500\text{ }\mu\text{m}$ wide main gas channel, which connects with the open air. Reservoirs are fabricated by Harris uni-core punch kit. There are two major differences with our previous work:¹⁴ The first one is that the main liquid channels are splitted into two at each Christmas tree outlets, so as to isolate every line of reservoirs; the second one is that 25 reservoirs formed a 2D diamond matrix, but not in square shape. Reservoirs in each column form a slash does not parallel with *y* direction. This arrangement was used to generate the second gradient. Reservoirs and ring-shaped channels are connected by SMA at the bottom layer. The SMA is composed of 9 parallel channels; each of them is 3.5 mm long, $8.2\text{ }\mu\text{m}$ deep, and $38\text{ }\mu\text{m}$ wide. Channels in an array are ranged at a distance of $100\text{ }\mu\text{m}$. During the liquid filling process, SMA acts as a gas outlet to lead fluid into reservoirs. After reservoirs filling by fluid, solution in reservoir changes from dynamic to static. Liquid will not flow out from SMA because of the strong surface tense in SMA.¹⁵

The glass molds for top and middle layers are fabricated by typical microlithography technique.¹⁶ Briefly, designed pattern was printed on film to form a mask. Then the mask was pressed on the SG-2506 borosilicate glass. The area of the glass is 102 cm^2 . The hybrid was subject to 1 min of UV exposure, so that the pattern was transferred. Then NaOH solution soaking was applied for 1 min, followed by 1 min of soaking in chromium etchant. After washing with pure water, glass with pattern was baked at 80°C for 10 min. Then glass was exposed to HF etching solution (HF: HNO_3 : NH_4F (1.0M: 0.5M: 0.5M)) for 60 min, washed with water and dried for later application. The glass mold for bottom layer is fabricated by an improved technique.¹⁶ After UV exposure and NaOH soaking, chromium etchant was used to etch away chromium in exposed area, and then ethanol was employed to wash away the photoresist on top of the remaining chromium layer. Then followed the baking and HF etching steps same as described above. For PDMS soft lithography, PDMS precursor and curing agent were thoroughly mixed under the ratio of 10:1, poured to the glass molds, degassed, and cured at 80°C for 1 h. The weight for mixture poured onto the mold for the second layer was 10.5 g with the layer thickness of $600\text{ }\mu\text{m}$ all the time. The PDMS layers were then peeled off from the molds and stored in clean containers. After the reservoirs and connection holes were punched, the first PDMS layer and the top surface of the second PDMS layer were treated with air plasma and bonded together irreversibly. The bottom surface of the second PDMS layer and the third layer were kept hydrophobic and then bonded reversibly.

C. Two dimensional gradient generation

The two dimensional liquid gradient was generated during the same injection process. Liquid A and liquid B were injected from the two liquid inlets separately. The liquid flow

velocity was kept at 10 $\mu\text{l}/\text{min}$. To visualize the gradient, water was applied as liquid A, and blue dye solution (Methylene blue) was applied as liquid B.

D. Drug screening test

The HL-60 cells in the exponential growth phase were centrifuged and washed by PBS twice. Then the cells were resuspended by PBS.

In the liquid filling process, solution was injected through both of the liquid inlets at the same flow velocity of 1.2 ml/h, which continued until all the reservoirs were filled with fluid. Then the injection was stopped and cell suspension was replaced by PBS to wash away any untrapped cells. After the filling process, the flow velocity was changed to 0.2 ml/h. Then solutions A and B were injected from corresponding inlets. For Dox screening assays, 20 μM Dox solved in DMEM was applied as solution A and DMEM was applied as solution B. In Dox and Que assays, solution A was changed to 20 μM Dox in DMEM and B was 100 μM Que in DMEM.

The two-dimensional drug gradient was generated on chip, and different reservoirs were exposed to solutions in x channels above them. The novel structure of these reservoirs allowed easy solution replacement. Then cells were cultured in environment containing drugs at different concentrations at 37 °C with 5% CO_2 for 8 h.

For comparison, the same drug screening assay was also conducted on a 96-well plate. After cells were inoculated, different concentrations of Dox in DMEM solution were injected to wells, corresponding to each concentration in reservoirs on chip. Cells were cultured at 37 °C with 5% CO_2 for 8 h. A 24 h assay was also conducted.

E. EB/AO staining for cell state examination

In cell staining assay, we used PBS solution with 100 $\mu\text{g}/\text{ml}$ EB and 100 $\mu\text{g}/\text{ml}$ AO. Staining solution was injected through both of the liquid inlets at 2 ml/h for 5 min, and then PBS was injected to replace staining solution. The resulting fluorescence images of the cells were monitored by the Leica DMIRE2 microscope fluorescence analyzing system. Cell viability data were captured by Image-Pro Plus (IPP) program.

III. RESULTS AND DISCUSSION

A. Diffusion based linear concentration gradient

This microfluidic device generates a gradient in 2D form. Gradient in x direction is generated by mixing two streams in a standard Christmas Tree Structure. Then fluid runs into a network structure. To be brief, in this network structure, we name channels in x direction as x channels and channels in y direction as y channels. There is a gradient generated in each x channel, since they connect y channels with fluid in different concentrations.¹⁷ This gradient is generated by diffusion field and the concentrations at endpoints are determined by fluid in y channels. Running fluid provides a continuous solution supplement to make up for solution consumption during gradient generation, so concentration at every endpoint maintains constant. This helps the gradient to reach the steady state soon, and a series of x channels connecting the same two y channels share the same gradient. Reservoirs are ranged below x channels; gradient in y direction is generated by ranging reservoirs at different location of the x channels containing a continuously changed concentration gradient.^{18,19} Due to the existence of diffusion field, concentration of solution in reservoirs will soon reach equilibrium, with fluid right above the reservoir. The location of reservoirs between the same two y channels gradually changes from the left point to the right. This generates the gradient in y direction.

This mechanism is confirmed by theoretical calculations. Here, we suppose a matrix contains M rows and N columns. In y direction, we have M data points. The radius of reservoir is r , the length of line across reservoirs is b , and the projector distance in x direction of two adjacent reservoirs is set as a (Fig. 1(d)). In this chip, liquid channels, except line b , are not supposed to go across the reservoirs, so we could have

$$a \times (M - 1) + 2r \leq b. \quad (1)$$

Then a reaches maximum when $a \times (M - 1) + 2r = b$. According to the characterization above, the slope of this linear gradient is $-(a/b)$, and the intercept is $(b + a * M + a)/2b$.

We utilize a microfluidic chip containing only the second gradient generator to characterize this gradient. On this chip, we have 5 data points. So the value of M is 5, and we suppose the inlet concentrations are 1 and 0. Under the restriction of Eq. (1), we choose $r = 1.5$ mm, $a = 1.0$ mm, and $b = 8.0$ mm, so the concentration of reservoir x is

$$c(x) = \frac{7}{8} - \frac{1}{8}x. \quad (2)$$

As shown in Fig. 2(a), fluid field simulation results clearly show that solution concentration in reservoirs has a linear correlation with the distance between reservoirs and channels. Corresponding experimental results are shown in Fig. 2(b). In this arrangement, each reservoir has different proportion of water from inlet A and blue dye from inlet B. And in Fig. 2(c), the colour changes from transparent to blue, confirming the gradient was generated.

We do linear fitting about both theoretical prediction concentrations and experimental concentrations, respectively. As shown in Fig. 2(d), the curve linearity is good and they are consistent with each other.

B. Liquid gradient in 2D matrix

Combining the 1D diffusion gradient with the Christmas tree gradient generation mechanism in the 2nd dimension, a 2D gradient matrix can be formed.

We suppose a matrix contains M rows and N columns as mentioned above. If the concentrations in two inlets are set as A and B , the Christmas tree structure divides the concentration difference between two inlets into N parts equally, then followed the next generator to get desired concentration series. On the basis of previously deduced results, we can make a prediction of concentrations in reservoirs. If we name a reservoir as (x, y) according to its location in the matrix, the concentration of that reservoir is

$$c(x, y) = A - \frac{A - B}{N} \left[\frac{b - a * M - a}{2b} + \frac{a}{b} * x + (y - 1) \right]. \quad (3)$$

Here, in this 5×5 matrix, same as the previous chip, we still have $r = 1.5$ mm, $a = 1.0$ mm, and $b = 8.0$ mm. If we set concentration in inlet A as 1 and in inlet B as 0, Eq. (3) turns into

$$c(x, y) = \frac{39}{40} - \frac{x}{40} - \frac{y}{5}. \quad (4)$$

FIG. 2. Characterization of the diffusive-based gradient: (a) Concentration distribution of the gradient generator; (b) blue dye solution gradient results of this gradient; (c) a zoom in image of b; and (d) the linear fitting curve corrected by working curve (the blue line shows the theoretical curve).

The theoretical prediction for solvent concentration in each reservoir is shown in Fig. 3(a). We also take blue dye visualization analysis on this 2D gradient chip. The result is shown in Fig. 3(b). Each reservoir has different proportions of water from inlet A and blue dye from inlet B. The colour changes from transparent to blue, showing this gradient generated. This gradient in 2D matrix divides solution concentration difference into 25 shares. Two different generators lead to two different linear gradients.

C. Drug screening on chip and on plate

As shown in our previous work,¹⁴ cells are captured during the restricted liquid filling process and then get protected from the fluid field. In this device, y channels do not run across reservoirs, while x channels with fluid velocity near zero go through reservoirs to delivery solution instead. This shields cells from shear force. And the high aspect ratio of the micro well also contributes to it.

With no shear force and continuous nutrient supplement, this culture system shows an excellent performance on cell culture. This ensures the quality of the result of cell viability in drug experiments. Cells are cultured for 8 h, 24 h, and 48 h on chip with a death rate of less than 5%.²⁰ We collected statistical data about the viability of cells cultured on chip for 8 h and made a column chart, according to the reservoir positions.²⁰ Cell viability in reservoirs in different positions remains high, so we draw the conclusion that culture situation is not affected by the position of reservoirs on chip.

Dox is one of the most commonly used chemotherapeutic drugs in the therapy of several kinds of cancers, such as human breast tumors,²¹ lymphoma,^{22,23} and esophagogastric cancer.²⁴ And in recent years, research on synergistic effect of Dox improved its drug efficiency greatly.^{25,26} So, we choose Dox as a model medicine for drug screening application. Cells are captured in reservoirs which contain drugs in 25 different concentrations. A high concentration range and a sharp gradient are chosen in order to show an obvious cell viability difference in a short time. As shown in Fig. 4(a), after EB/AO staining, fluorescence pictures of each reservoir are taken; a viability gradient from 100% to 0% is formed. Statistical results in Fig. 4(b) show the efficacy of Dox in each concentration more clearly. This shows the possibility of conducting 25 cell assays at different drug concentrations at one time.

Que is reported to have a synergistic effect with Dox in aggressive tumor cells. Here, we utilize Dox in solution A and Que in solution B to build the gradient. In this model, the total content of two compounds is fixed, but the ratio is different in gradient. As shown in Fig. 4(b), cell death rates after Dox and Que treatment are higher than those of Dox treatment at the same concentration, showing cells under combinatorial treatment. So, we draw the conclusion that these two gradients overlapped.

The drug screening assay is also conducted on 96-well plate repeatedly. The results are quite different from those obtained on chip. Cell death rates on plate are lower than on chip at the same concentration. After 8 h of drug treatment, even though Dox was found enriched in

FIG. 3. (a) Theoretical prediction concentration of each reservoir. (b) Blue dye solution visualization analysis.

FIG. 4. (a) Fluorescence pictures of every reservoir after drug assay and EB/AO staining. (b) Death rate statistical results of Dox drug screening assay (red) and Dox + Que (black) assay on chip.

FIG. 5. EB/AO staining Fluorescence pictures of cells on plate after 8 h (a) and 24 h (b) Dox treatment with different concentrations: (1) 0, (2) 4, (3) 8, (4) 12, (5) 16, and (6) 20 μM . (c) Statistical results of cell living rate for Dox assays conducted on plate: The red bars represent 8 h Dox treatment, and the grey bars represent 24 h Dox treatment.

cells,²⁰ it is still not enough to kill any cell. Cells, even in the highest concentration of Dox, still have a viability of nearly 100 as shown in Figs. 5(a) and 5(c), red bar. After 24 h of drug treatment, viability forms a gradient which quickly goes down to zero as shown in Figs. 5(b) and 5(c), grey bar. The discrepancies of results obtained between on chip and on plate may be due to the different drug exposure modes. For on chip assays, continuous perfusion supplies nutrients and drugs, drug concentrations do not change with time. The results reflect drug efficacy at fixed concentration. For on plate assays, they reflect drug efficacy with fixed quantity.

IV. CONCLUSIONS

We have developed a microfluidic cell array with a 2D liquid gradient consisting of 25 different concentrations that is suitable for cell culture and cell-based assays. For drug screening applications, a Dox concentration gradient was applied to HL-60 cells, and a clear cell viability gradient could be observed. Such results displayed a great difference as compared to the results on a 96-well plate due to the difference in drug exposure mode. Finally, the overlapping gradient of Dox and Que was generated for a combinatorial treatment. This work provides a promising tool for high throughput drug screening.

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