A novel wide-range microfluidic dilution device for drug screening

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ABSTRACT

Microfluidic dilution chip is a crucial approach to perform gradient dilution of experimental samples in many biological investigations. In this study, we developed two serial wide-range dilution chips with dilution rates of 1:1 and 1:4 on the basis of the microfluidic oscillator by designing a series chamber, which was similar to a series circuit. The size of this chamber was adjusted and mixed with the neighboring air chamber to form dilution rates by oscillatory methods. We applied this microfluidic oscillator to estimate cellular kinetics and perform an acute oxidative stress test on *Caenorhabditis elegans* (*C. elegans*) in order to further validate their effectiveness. We estimated the kinetic parameters of β -galactosidase, the biocatalyst responsible for the hydrolysis of lactose, and found out that K_m was $602 \pm 73 \mu\text{M}$ and k_{cat} was $72 \pm 12/\text{s}$. In addition, our result of the study on acute oxidative stress of *C. elegans* using this novel chip was consistent with the result using 96-well plates. Overall, we believe that this novel chip can be applied to enzymatic reaction kinetics to evaluate accurately drug screening in bio-nematode models such as *C. elegans*. In summary, we have provided a novel microfluidic dilution chip that can form a wide range of sample concentration gradients. Our chip may facilitate drug screening, drug toxicology, and environmental toxicology.

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I. INTRODUCTION

Microfluidic chips are becoming increasingly more popular in studies relevant to environmental toxicology, drug screening, and continuous processing of biomolecules.^{1,2} Microfluidic chips cut off related reagent consumption so that it protracts reaction time. This method relies greatly on manual operation.^{3–5} The first study on microfluidic chips appeared in 2001, in which a passive sample diluent chip was proposed.^{6,7} This structure was improved later to a passive grade-by-grade dilution micro-flow control chip so that it could be used to study the toxicological effects of different concentrations of drugs on cells such as human embryonic kidney

cells.^{8–11} Although this type of diluent chip production was easy to operate, it was not able to generate arbitrary linearity or the logarithmic density gradient, which was required in some research studies. Moreover, this complicated process may result in unstable liquid density, thus limiting its use in drug screening.¹²

Besides the passive sample dilution chip, another method of sample dilution is to quantify the liquid through the geometry of the tube and to mix the sample with the diluent through a plurality of membrane valves, which interact with each other to produce a peristaltic pump. For example, a ring-shaped linear dilution microfluidic chip based on a membrane valve had been reported.¹³

It comprised of a five-valve peristaltic pump and could dilute a 400 nl sample four times in a ratio of 1:4 within 22 s. Compared to the traditional structure that needs to be designed with high precision, a ring-shaped linear dilution microfluidic chip can not only accurately quantify samples but also easily adjust the position of the film change the dilution ratio. Therefore, this design is more flexible (from 1:24 to 1:4) for researchers. Building upon the basic idea of this method, Jensen *et al.* developed an 8×8 micro-flow control digital platforms with 64 precision control valves, which can be applied by researchers at 1:1 or 1:3 ratio of the sample grade-by-grade dilution and complete multi-step reaction.^{14,15} It had been suggested that a linear trapezoidal dilution chip could accomplish multi-step dilution of the levels while keeping the product of every dilution step.¹⁶ However, the chip based on the thin film microvalve diluted sample requires a very large number of valves (three microvalves are required for each additional sample dilution), and each valve is independently controlled. Therefore, the control device also has more requirements. In order to improve the peristaltic pump, the chip used glass as the main material in their production, which increased the cost of the chip and the complexity of the process.

As a typical enzyme, β -galactosidase is a large protein (1024 amino acids) that forms a tetramer. The enzyme's function in the cell is to cleave lactose to glucose and galactose so that they can be used as carbon/energy sources,¹⁷ and a high β -gal activity has also been reported in several malignant tumors.¹⁸ The nematode *Caenorhabditis elegans* (*C. elegans*) was chosen to be the animal model for our study since its pattern had been thoroughly studied¹⁹ and utilized in a great number of investigations of toxicology (neurotoxicity), biochemical (ROS accumulation, the relative contents of TPH, and serotonin),²⁰ and molecular (stress-related gene expressions and serotonin-related gene expressions) responses.²¹ Hydrogen peroxide (H_2O_2) aqueous solution, also known as hydrogen peroxide, is a strong oxidant and is commonly used in oxidative stress testing of nematodes.²²

A high-throughput enzyme assay is a crucial step in drug discovery research and it speeds up the drug screening process.²³ This study developed a new automated drug screening chip based on the microfluidic oscillator, which had been described in our previous paper.²⁴ We then conducted a test that evaluates this wide-range dilution gradient chip.

II. MATERIALS AND METHODS

A. Materials

PDMS (polydimethylsiloxane, RTV 615) was purchased from Momentive Performance Materials, USA. Photoresist SU-8 3050 was bought from Microchem Corporation of the United States. Photoresist AZ 4903 was obtained from Merck Performance Materials, Germany. FITC (Fluorescein isothiocyanate, CAS: 3326-32-7) was purchased from Sigma-Aldrich, USA. We also obtained methyltrichlorosilane (CAS: 75-79-6) from Beijing Belling Technology Co., Ltd., and collected FC-40 (CAS: 51142-49-5) from Beijing Yinuokai Technology Co., Ltd.

β -Galactosidase (CAS: 9031-11-2), β -D-galactopyranoside (CAS:95079-19-9), Resorufin (CAS:635-78-9), and bovine serum albumin (BSA) (CAS:9048-46-8) were purchased from Sigma-Aldrich,

USA. Tris-HCl buffer (0.1 M, pH 7.8), tryptone, yeast powder, cholesterol, and Agar B were all purchased from Beijing Dingguochangsheng Biotechnology Co., Ltd. Other chemical reagents used in this experiment were purchased from China National Pharmaceutical Group Chemical Reagent Co., Ltd.

B. Fabrication

The fabrication of this chip required a two-layer PDMS valve process. We first designed the layout of the chip with AutoCAD software and printed out the mask. A positive photoresist (AZ-4903, height 20 μ m) was spin-coated on a 3-inch single crystal silicon wafer to constitute a valve layer of the mold and a flow channel. The silicon wafer was placed on a hot plate at 120 °C for 40 min to reflow the photoresist and was further heated to 190 °C for 1 h to harden the photoresist. After natural cooling, a layer of negative photoresist (SU-8 3050, height 100 μ m) was placed on the silicon wafer to constitute a chamber for the runner layer. The valve layer and the runner layer were treated with trichloromethyl silane for 5 min. We configured PDMS of 5:1 and 20:1 (monomer: curing agent) separately and stirred them evenly, and then extracted the bubbles inside. The mold of the flow channel layer adhered to the bottom of the disposable culture dish with double-sided tape, and a 5:1 PDMS was poured on the mold to a thickness of about 5 mm. The valve layer mold was placed on a rotary laminator, dropped 2 ml of 20:1 PDMS, pre-rotated at 400 rpm for 30 s, and spun at 1800 rpm for 1 min to obtain a PDMS film (approximately 40 μ m thickness). The mold of the flow path layer and the mold of the valve layer were heated at 75 °C for 37 min. The PDMS which had been solidified on the flow channel layer was cut out, aligned with the PDMS film on the valve layer under a stereo microscope, and baked at 75 °C overnight. We punched the entrance and outlet by a puncher with a 2 mm external diameter the next day. The PDMS chip was treated with oxygen plasma together with the clean glass slide for 40 s and then heated for 2 h at 80 °C for binding.

C. Fluorescent image processing and data analysis

An inverted fluorescence microscope (Eclipse Ti-E, Nikon, Japan) with a highly sensitive EMCCD (iXon, Andor, Britain) was used to collect the fluorescence data in all chambers in a short time. This microscope took photos of each chamber of the microfluidic chip and then constituted a complete large picture with 10 s. Signal processing for each chamber was carried out using MATLAB software (<https://www.mathworks.com/products/matlab.html>).

D. Nematodes culture conditions

M9 medium, LB medium, and NGM medium plates were prepared. Wild type N2 *C. elegans* was cultured in the dark at 20 °C. The culture medium was NGM medium plates or M9 liquid medium. *E. coli* OP50 line was used as food.

E. Nematodes synchronization

To collect nematodes that were in the same developmental period, we treated the nematodes, which were in different development stages, with alkaline lysate. The eggs were obtained and cultured in 20 °C NGM for 24 h. Then, we inoculated about 100 μ l of

the obtained L1 larvae on NGM, around the OP50 microsphere. The synchronized growth nematodes climbed into the fungus circle by themselves. The reason for nematode synchronization was that animals in the different developmental period have different stress responses.

F. Nematodes' behavior observation and image processing

A video of the nematodes in each chamber was taken every 10 min using an inverted fluorescence microscope (IX73, Olympus, Japan) equipped with a CCD (DP80, 15 fps, Olympus, Japan). The frequency of the nematodes was also recorded.

III. RESULTS AND DISCUSSION

A. A novel wide-range dilution chip

1. A wide range of gradient dilution chip design

Stepwise dilution is a common method to form a large-scale concentration dilution (>1000 times) in conventional biochemistry experiments. This method results in a large-scale sample dilution by taking multiple dilutions (<10 times) of the sample. However, large-scale concentration gradient dilution is still a problem in microfluidic systems.

Here, we described a stepwise dilution method using a novel microfluidic chip that covered a wide detection range. As shown in Fig. 1, the chip consisted of five interrelated dilution units. Each time the dilution was completed, part of the diluted product would start the next dilution as the sample to be diluted in the next dilution unit. For each dilution unit, the dilution ratio could be adjusted by setting the volume ratio of the sample chamber to the dilution chamber (Fig. 1), and a part of each diluted product remained in the chamber for the next biological reaction or dilution. Compared to the earlier large-scale dilution chip, the novel gradient dilution chip has several advantages. By designing chambers of different sizes, the dilution ratio can be precisely controlled

FIG. 1. A simplified conceptual schematic of the 1:1 serial dilution microfluidic chip. (a) Dilution unit (green), priming valves, and oscillating valves (red) are shown. The priming valves and oscillating valves are marked. (b) The size of the chip compared with a Chinese coin.

to obtain any desired concentration gradient, and the experimental results are reproducible. The number of valves used was small; only one control valve is needed for each additional dilution. Based on our designed microfluidic oscillator, only 2 min is required for each dilution (1 min for mixing, 1 min for exhausting). The stepwise dilution of the six rounds of samples can be completed in 12 min, which significantly shortens the operation time.

2. Operating processes and working principle

This linear diluted microfluidic chip used pressure to push the liquid and controlled the flow of the various valves in the chip through a self-made valve control device. The operating process is shown here. We have poured the sample to be diluted (red dye), the diluent [phosphate buffered saline (PBS) buffer], and oil into the corresponding chamber [Fig. 2(a)]. We opened the isolation valve between the chambers and applied a 3 psi external pressure through the oil phase to push the mixture of the sample into the air chamber [Fig. 2(b)].

When the external pressure was removed, the compressed gas in the air chamber pushed the liquid back into place [Fig. 2(c)]. By periodically applying and withdrawing external pressure, the sample and diluent were rapidly oscillated and mixed in the chamber to complete a 50% dilution. This was the main working principle of our microfluidic oscillator. Each dilution process takes a total of 2 min, mixing the liquid in 1 min and removing the liquid in 1 min. When the mixing was completed, the external pressure was raised to 9 psi, and the partially mixed liquid was pushed into the sample chamber of the next dilution unit by the vent injection [Fig. 2(d)].

This dilution process was repeated to obtain a 25% dilution ratio of the product. Similarly, the sample was diluted to 1/32 of the original sample by five rounds of such dilution. All intermediate process liquids were retained, forming a logarithmic dilution gradient of the sample [Fig. 2(e)].

3. Oil optimization

In this design, an oil phase was used to quantify the liquid, transfer pressure, and stabilize the liquid interface. The oil satisfying the experimental conditions had the following characteristics: the interface between the oil and water was clear and stable; the oil did not wrap the water phase to form droplets when pushing the water; the viscosity of the oil was small and would be driven by the rapid flow.

In order to find a suitable oil phase, a three-chamber microfluidic test chip [Fig. 3(a)] was fabricated. Two chambers were filled with oil and water, respectively, while the last chamber was filled with air. We then tested the effect of different oils (chili oil, liquid paraffin, and FC-40) on water by venting the oil and water into the final air chamber.

The results suggested that the pepper oil exceeded the water phase and eventually formed a droplet during the advancement of the pepper oil push.²⁵ When liquid paraffin and FC-40 were used as the oil phase to push the water column, the interface between water and oil was relatively stable and the oil phase did not exceed the water phase. The reason for this phenomenon was that the water phase did not completely immerse the entire pipe in a square pipe.

FIG. 2. The operating processes and working principle of the gradient dilution chip. (a) In priming mode, sample (red), diluent (white), and oil (light blue) were loaded with priming valves opened and oscillating valves closed. All valves opened are priming valves, and all valves closed are oscillating valves. (b) and (c) In oscillating mode, the mixture of sample and diluent was oscillated by adding or removing the pushing pressure at a frequency of 1 Hz in 30 s. $P_{push} = 3$ psi. At this time, priming valves were closed and oscillating valves were opened. After the oscillating-flow mixing was completed, half of the mixture was transported into the next sample chamber in pushing mode. $P_{push} = 9$ psi. (d) Oscillating mode and pushing mode were repeated to form a five-stage series of 1:1 dilutions, as shown in red. (e) The sample was diluted to 1/32 of the original sample by five rounds of such dilution. All intermediate process liquids were retained, forming a logarithmic dilution gradient of the sample.

FIG. 3. Schematic of oil phase testing chip. (a) The results of pushing water into the air chamber by capsicol (b), liquid Vaseline (c), and FC-40 (d).

Instead, a “gutter” was formed at the four edges of the pipe.²⁵ More pro-face may quickly exceed the water phase from the ditch and wrap it around.^{7,26}

Based on the obtained results, as shown above, we considered both liquid paraffin and FC-40 as alternatives to the oil phase. However, the flow rate was slow during the oscillation process since the viscosity of liquid paraffin (100-145 counts/s) was larger than FC-40 (4.1 counts/s). Therefore, FC-40 was eventually selected as the alternative to the oil phase.

4. Result of linear dilution

Two kinds of stepwise dilution chips of 1:1 and 1:4 were designed by adjusting the volume ratio of the sample chamber and the diluent chamber. To test the effectiveness of these two chips, red dye was used as the sample and water was used as the diluent. The test results are shown in Fig. 4.

FIG. 4. The effectiveness testing for 1:1 series dilution chip and the 1:4 series dilution chip. (a) The results of a 5-stage 1:1 serial gradient dilution chip using red dyes. (c) The $\log_2$ plot of fluorescence intensity of 1:1 serial gradient dilution chip, this orders of magnitude concentration variation on 2 to 32. $R^2 = 0.997$. (b) The results of a 5-stage 1:4 serial dilution chip using red dyes; (d) the $\log_5$ plot of fluorescence intensity of 1:4 serial dilution chip, this orders of magnitude concentration variation on 5 to 3125. $R^2 = 0.987$. T^* means dilution times.

To verify the accuracy of the linear dilution concentration gradient, the $100\ \mu\text{M}$ fluorescent dye (FITC) was used as the sample and phosphate buffered saline (PBS) was used as the diluent. A 1:1 ratio and a 1:4 ratio were used to serially dilute the sample. Each dilution with the ratio of interest was repeated 10 times. According to the optimized conditions in the previous test, 100 nl of liquid was evenly mixed by shaking at a frequency of 3 psi for 20 s at 1 Hz. Therefore, each sample dilution, with a pressure of 3 psi, oscillates at 1 Hz for 1 min to ensure homogeneous mixing of the sample and diluent. As shown in Figs. 4(a) and 4(b), an image of each fluorescent dye in the chamber (16-bit image) were taken with a fluorescence microscope and EMCCD (iXon, Andor). The image was then converted to a gray value using Image J. We used this method to detect multiple dilutions that the chip can reach.²⁷ The principle of our detection method is continuous sample removal from the first chamber and not so much from the last chamber in the array. To quantify a practical bound on dilution, we generated an array containing a uniform composition of a fluorescent dye, diluted it with 4 water plugs of the same volume, and monitored the reduction in fluorescence of the first chamber in the array. The linear gradient dilution chip can complete five sample dilutions in a ratio of 1:4, and the maximum dilutions is 3125 [Fig. 4(d)]. We then took $\log_2$ or $\log_5$ of the fluorescence intensity to draw the curve. As shown in Figs. 4(c) and 4(d), these orders of magnitude concentration variation on 2 to 3125 illustrate the power of the method for screening applications. The results of stepwise dilution of 1:1 and 1:4 ratio was in agreement with the expected value and had great repeatability. The standard deviation of

the 10 experiments was less than 0.3. Therefore, the stepwise linear dilution chip can be used to form a wide range of concentration gradients. This new step-by-step dilution chip was designed to be more flexible and could obtain any desired concentration gradient compared to the passive dilution chip. In addition, this step-by-step dilution chip required a small number of valves.²⁸ We needed to add only one valve for each additional dilution. In comparison, the trapezoidal linear dilution chip required a large number of valves²⁹ for fluid mixing, and each additional dilution required to increase membrane microvalves.¹⁶

B. Determination of β -galactosidase enzyme kinetic parameters

1. Enzyme kinetic parameter determination chip design

K_m and k_{cat} are the enzyme kinetic parameters that serve as enzyme function tags and they were usually measured by the Lineweaver-Burk Plot.³⁰ To determine the value of K_m and k_{cat} , we first calculated the required initial rate of product formation under different substrate concentrations by the Mie equation, so that the chip can form a concentration gradient of the substrate and allow different concentrations of the substrate to react with the enzyme.

The enzyme kinetic parameter determination chip consisted of a 1:1 ratio linear dilution module (Fig. 5) and an additional set of air oscillating chambers (Fig. 5). Each chamber in the linear dilution module had a length of $850\ \mu\text{m}$, a width of $500\ \mu\text{m}$, a

FIG. 5. Schematic of the novel microfluidic chip to estimate enzyme kinetic parameters. (a) The enzyme kinetic parameter determination chip consisted of a linear dilution module (red frame) and an additional set of air oscillating chambers (yellow frame). (b) The real size of the chip compared with a Chinese coin.

height of $80\ \mu\text{m}$, a total volume of $30\ \text{nL}$, an additional air oscillating cavity length of $1600\ \mu\text{m}$, a width of $500\ \mu\text{m}$, a height of $80\ \mu\text{m}$, and a total volume of $60\ \text{nL}$. All corners of the chamber were chamfered to reduce liquid residue during the oscillation process.

2. Enzyme kinetic parameter determination chip operation flow

We added the original concentration of the substrate (red dye) and the reaction buffer (colorless water) to the chip [Fig. 6(a)]. Because the chambers of the first row were to be reused in subsequent experiments, the substrate was first diluted with air to oscillate the liquid. The time required for each dilution was $2\ \text{min}$ ($1\ \text{min}$ mixing, $1\ \text{min}$ exhaust). The time to complete 6 dilutions was $12\ \text{min}$. The concentrations of the substrate after dilution were $1/2$, $1/4$, $1/8$, $1/16$, $1/32$, and $1/64$ of the original concentration, respectively [Fig. 6(b)]. We rinsed the diluent chamber with de-ionized water, added the enzyme reaction solution (green dye), and then closed the valve between the chambers to form 6 independent reaction units [Figs. 6(c) and 6(d)]. We added the oil phase (FC-40) at the top of the inlet and used an additional set of air oscillating chambers to oscillate the liquid with the oil phase to rapidly mix the enzyme and substrate [Figs. 6(e) and 6(f)] and pass the fluorescence microscope every minute. The fluorescence intensity of the liquid in all chambers was scanned once by an automated fluorescent scanning platform.

Compared to the enzyme kinetic parameter determination chip that had been reported, this novel chip was shown to be more flexible, has better adaptability, and was able to generate a larger concentration gradient range at one time.

3. Standard curve drawing

β -galactosidase is a hydrolase in the cell lysosome that hydrolyzes its substrate, Resorufin β -D-galactopyranoside. Resorufin is a commonly used fluorescent dye that emits orange light ($585\ \text{nm}$)

under excitation of $540\ \text{nm}$ and captures fluorescent images by CCD. Since the fluorescence intensity emitted by resorufin has a corresponding relationship with its concentration, the standard curve of resorufin can be drawn first, and then the concentration of resorufin generated in the enzymatic reaction can be obtained through the standard curve, and the initial rate of the reaction can be further calculated.

To plot the standard curve, the reaction buffer was first configured as the following: $2.0\ \text{mM}$ KCl, $0.01\ \text{mM}$ MgCl_2 , 0.1% BSA, and 0.05% Tween-20 dissolved in $0.1\ \text{M}$ Tris-HCl buffer, pH 7.8. Resorufin powder was then dissolved in the reaction buffer to make a $32\ \mu\text{M}$ original sample. We added BSA and Tween-20 to the device made of PDMS to block the adsorption before the sample dilution. Since PDMS chip adsorbs proteins, it will affect the accuracy of the concentration gradient. The original sample was added to the first sample chamber of the chip by venting. The spirulina sample and the reaction buffer were diluted five times using a 1:1 ratio by the operation procedure that had been described above so that we obtain $1/2$, $1/4$, $1/8$, $1/16$, and $1/32$ times dilution product.

The diluted chip was placed on a fluorescence microscope. A fluorescent image of the liquid in each chamber was taken by a CCD. The average fluorescence intensity of the liquid in each chamber, which was used as a standard curve, was then analyzed using ImageJ software. As shown in Fig. 7, the linearity of the standard curve was clearly shown ($R^2 = 0.996$) and the fluorescence intensity after dilution was consistent with the expected value. In the subsequent enzymatic reaction, the concentration of the product resorufin produced during the reaction was obtained from a standard curve and the initial rate of the reaction was calculated.

4. Enzyme concentration optimization

In order to measure the initial rate v_0 of the reaction at different substrate concentrations, a plot of the concentration of the product over time was drawn. We calculated the value of initial rate

FIG. 6. Workflow of the dilution chip in estimation of enzyme kinetic parameters. (a) The substrate (dye with red color) and buffer solution (water) were loaded onto the chip; (b) forming a concentration gradient of the substrate; (c) and (d) the enzyme was loaded onto the chip; (e) and (f) the enzyme was mixed with the substrate.

v_0 from the linear region of the curve. However, if the concentration of the enzyme was too high, the rate of product formation became too large, the reaction rate rapidly decreased, and the linear range of the curve became narrow. It was difficult to obtain a sufficient number of data points to calculate the initial reaction rate v_0 . Therefore, it is necessary to adjust the concentration $[E]$ of the enzyme to control the rate of product formation and to obtain a sufficiently long linear region.

Since the concentration of the enzyme was adjusted in a small range, this study designed a parallel dilution microfluidic chip. It formed a linear concentration gradient of 0.1–1 by adjusting the ratio of the sample chamber to the dilution chamber (Fig. 8) and reacted to different concentrations of the enzyme with the same concentration of substrate by fluid oscillation. We observed the fluorescence intensity of each chamber by a fluorescence microscope.

200 $\mu\text{g/l}$ galactosidase, reaction buffer, and 222 μM substrate were added to the inlet of the chip. The air bubbles in the chamber were excluded by the gas permeability of PDMS. After the injection was complete, the valves between each column of chambers were closed to form a separate reaction unit. The valve between the chambers in each unit was opened and oscillated with a 6-psi gas pressure and a 1 Hz frequency to allow the enzyme to mix well with the substrate. All air-oscillating chambers were then photographed every minute using an automated XY moving platform of a fluorescence microscope.

As shown in Fig. 9, the concentration of the product of different enzyme concentrations varied from reaction time was obtained. The experimental results showed that as the concentration of galactosidase increased, the reaction reached the plateau stage more quickly. When the concentration of the enzyme was 120 $\mu\text{g/l}$, the fluorescence intensity remained substantially linear during the reaction time of 20 min. The reaction did not reach the plateau stage in this condition. Therefore, 120 $\mu\text{g/l}$ was selected as the reaction concentration of the enzyme in the following experiments.

5. β -Galactosidase enzyme kinetic parameter determination results

We used a linear dilution module of the chip to generate a series of concentration gradients of resorufin- β -D-galactopyranoside (10–333 μM) and reacted with 120 $\mu\text{g/l}$ galactosidase according to the Lineweaver-Burk double reciprocal mapping method. Figure 10(a) shows the plot of product formation over time for different substrate concentrations. It was observed that the plateau period was not reached within the reaction time of 20 min, and the fluorescence intensity increased with the increase of the substrate concentration.

In this experiment, the concentration of the product corresponding to different fluorescence intensities was obtained according to the standard curve. The initial rate v_0 of the reaction was calculated based on the slope of the linear region. The reciprocal of

FIG. 7. The standard curve of resorufin with fluorescence intensity. A standard curve showed that the fluorescent intensity was proportional to the resorufin concentration ($R^2 = 0.996$). The upper part is an image taken by a fluorescence microscope, and the following is a standard curve analyzed by ImageJ software.

v_0 was plotted on the Lineweaver-Burk reciprocal plot and the intercept of the horizontal axis ($-1/K_m$) and the intercept of the vertical axis ($1/V_{max}$) were obtained using linear regression analysis. As shown in Fig. 10(b), the average $K_m = 602 \pm 73 \mu\text{M}$ in three independent chip experiments and $k_{cat} = 72 \pm 12/\text{s}$. The test results were compared to the recorded value in previously reported enzyme kinetic parameters of galactosidase. The result of the chip measurement in earlier literature was $K_m = 450 \pm 200 \mu\text{M}$ and $k_{cat} = 54 \pm 20/\text{s}$.³¹ Such a difference may be caused by the usage of different substrates and detection methods. In this study, resorufin- β -D-galactopyranoside was used as a substrate detected by fluorescence, while o-nitrophenyl- β -D-galactopyranoside was used as a substrate in the literature detected by absorbance. Another possibility is that BSA and Tween-20 were added to the reaction system to reduce the non-specific adsorption of the enzyme by PDMS, which may affect the reaction between the enzyme and the substrate.

This large-scale dilution chip consisted of a plurality of inter-related dilution units. Part of the product of the previous dilution unit was also the sample to be diluted in the next unit, thereby completing the stepwise dilution of the original sample. For a single dilution unit, our study adjusted the dilution ratio by setting the volume of different chambers. In addition, the step-by-step dilution process could be quickly completed by fluidic oscillation. In this study, we demonstrated that the linear dilution chip could perform five sample dilutions in a ratio of 1:1 (dilution time is 2-32) and 1:4 (dilution time is 5-3125) and the dilution time was only 10 min. We showed that the dilution results of two methods were highly consistent, and the required number of valves used was less using the novel dilution microfluidic chip compared to the

(a)

(b)

FIG. 8. Parallel dilution microfluidic chip to measure enzyme reaction. (a) It formed a linear concentration gradient of 0.1–1 by adjusting the ratio of sample chamber to dilution chamber and then reacts with different concentrations of enzyme with the same substrate concentration by fluid oscillation. (b) The real size of the chip compared with a Chinese coin.

previously reported large-scale sample dilution chip. Only one control valve is added for each additional dilution. In theory, any concentration gradient could be designed. This novel large-scale gradient dilution chip could be customized according to the needs of the concentration gradient.

C. Acute oxidative stress test of *C. elegans*

The L1 larvae of wildtype N2 *C. elegans* and hydrogen peroxide aqueous solution were used as models of acute oxidative stress experiments. The 1:1 ratio logarithmic concentration gradient of hydrogen peroxide solution was formed by the chip. The behavior of nematodes exposed to different concentrations of hydrogen peroxide was recorded and analyzed.

1. Acute oxidative stress test of nematodes in a 96-well plate

In order to observe the behavior of nematodes exposed to different concentrations of hydrogen peroxide solution, we prepared

FIG. 9. Time course of the enzyme reaction. The concentration of the product at different galactosidase concentrations was varied over 20 min. When the concentration of the enzyme was 120 µg/L, the fluorescence intensity remained substantially linear during the reaction time of 20 min.

the M9 medium in concentrations of hydrogen peroxide and observed the movement state of nematodes in a 96-well plate.

We prepared a 300 mM hydrogen peroxide stock solution, which was further diluted with the M9 medium in order to perform the test with concentrations of 5 mM, 10 mM, and 15 mM. 100 µl of each concentration of the assay solution was added to a 96-well plate, and 100 µl of the M9 medium was used as a control. 10 to 20 L1 stage nematode larvae were added to each well and observed under a stereomicroscope for 120 min to record the movement status of the nematode.

FIG. 11. Different behaviors of worms. S-shape (a), C-shape (b), O-shape (c), and J-shape (d).

The experimental results showed that the movement state of the nematode was mainly S type and C type in control well [Figs. 11(a) and 11(b)]. The worm body was stretched and the swing frequency was very fast with an average of 3.3 Hz (Fig. 12). In the hydrogen peroxide group, the movement state of nematodes was either O-type (collapse) or J-type (worm body stiffness) [Figs. 11(c) and 11(d)]. The decrease in the swing frequency with increasing treatment time indicates that hydrogen peroxide did have an oxidative toxic effect on nematodes (Fig. 12).

In this experiment, L1 stage nematodes were added to a micro-pipe chip with a width of 150 µm and a height of 80 µm by pipetting. After incubation for 120 min in the chip, the average swing frequency of nematodes was found to be 3.0 Hz. It was not significantly different from the nematodes (3.3 Hz) cultured in 96-well plates. It suggested that the cultural environment of the chip did not cause obvious toxicity to the nematode and could be tested for a long period of time.

2. Procedure of nematodes acute oxidative stress in the chip

After measuring the response of *C. elegans* to hydrogen peroxide in a 96-well plate, we conducted an identical study as described

FIG. 10. (a) Fluorescent intensity of the product was proportional to the substrate concentration of β -galactosidase; (b) Lineweaver-Burk reciprocal plot.

FIG. 12. The plot of stroke frequency of N2 worms and incubation time after hydrogen peroxide treatments of different concentrations. The swing frequency of nematodes in the chip was not significantly different from the swing frequency of nematodes in 96-well plates.

above using the extensive dilution chip. The procedure is shown in Fig. 12. We used a large-scale dilution chip with 60 mM hydrogen peroxide solution as the sample to be diluted. A logarithmic concentration gradient was formed in a 1:1 ratio, and a valve isolated each chamber. The hydrogen peroxide concentrations in each chamber were (from left to right) 30 mM, 15 mM, 7.5 mM,

FIG. 14. The plot of swing frequency of N2 worms and incubation time after hydrogen peroxide treatment of different concentrations on the microfluidic chip.

3.75 mM, 1.88 mM, and 0.94 mM, respectively [Fig. 13(a)]. The L1 stage nematode larvae were added to the chip from the entrance of the lower row, and make sure about 1-3 nematodes in each chamber. We opened the valve between the second row and the third row and pushed the nematode and different concentrations of hydrogen peroxide solution into the first row of nematode culture chambers with FC-40 [Fig. 13(b)]. The motion state of the nematodes and the swing frequency was recorded every 10 min.

FIG. 13. Schematics of acute toxicological experiment of nematodes. (a) In priming mode, hydrogen peroxide solution, the diluent, and the nematodes were perfused. The hydrogen peroxide concentrations gradient was formed; (b) in oscillating mode, the external pressure was added and pushed nematodes into culture chamber. All the isolated valves were opened.

3. Results of nematodes acute oxidative stress in the chip

The test results using the chip are shown in Fig. 14. The swing frequency of the worm dropped significantly as the concentration of hydrogen peroxide increased. When the concentration of hydrogen peroxide measurement solution was the same, the swing frequency of the worm gradually decreased as the treatment time increased. These results were consistent with the results we obtained using the 96-well plate. This consistency of results using two methods demonstrated that the novel microfluidic system could be used to measure acute oxidative stress in nematodes.

IV. CONCLUSIONS

This paper mainly describes two microfluidic chips with different dilution ratios. The sample could be diluted quickly due to the microfluidic oscillator, and the required valves are less. In this study, we used galactosidase and its substrate resorufin- β -D-galactopyranoside as a biological model to evaluate the enzymatic reaction on the gradient dilution chip. We then applied a linear dilution module to generate a concentration gradient of resorufin- β -D-galactopyranoside. By measuring and calculating the change of the reaction rate, we found that the key enzyme kinetic parameters of galactosidase are $K_m = 603 \pm 73 \mu\text{M}$ and $k_{\text{cat}} = 72 \pm 12/\text{s}$. The test results were consistent with the recorded value in previously reported enzyme kinetic parameters of galactosidase. Also, we studied the acute oxidative stress response of nematodes exposed to different concentrations of hydrogen peroxide using L1 wild-type N2 *C. elegans* as our animal model. The results demonstrated that the movement pattern of nematode was either a collapsed O-type or a rigid J-type. In addition, the oscillation frequency increased as the concentration of hydrogen peroxide and the treatment time increased in a high concentration of hydrogen peroxide. The test results using our novel chip was consistent with the test the results obtained using the 96-well plate.

This novel drug screening microfluidic chips allowed us to detect a wide range of concentration gradients, with better flexibility and quicker speed, and more data can be obtained from a single experiment. The device could be utilized to estimate enzyme kinetic parameters. Moreover, it could be used to perform drug screening, biocatalyst development in enzyme engineering, model organism nematode reproduction, lifespan research, etc.

In sum, we successfully designed a microfluidic dilution chip that covered a large-scale concentration gradient using a microfluidic oscillator. This chip was able to estimate the enzyme kinetic parameters of galactosidase and the acute oxidative stress of nematodes based on the linear dilution module. This novel drug screening microfluidic system may also be used in future toxicological studies using model animals. We planned to replace hydrogen peroxide with other drugs and develop a nematode model using this chip in the future.

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