A nanoliter microfluidic serial dilution bioreactor

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Bacterial culture is a basic technique in both fundamental and applied microbiology. The excessive reagent consumption and laborious maintenance of bulk bioreactors for microbial culture have prompted the development of miniaturized on-chip bioreactors. With the minimal choice of two compartments ($N=2$) and discrete time, periodic dilution steps, we realize a microfluidic bioreactor that mimics macroscopic serial dilution transfer culture. This device supports automated, long-term microbial cultures with a nanoliter-scale working volume and real-time monitoring of microbial populations at single-cell resolution. Because of the high surface-to-volume ratio, the device also operates as an effective biofilm-flow reactor to support cogrowth of planktonic and biofilm populations. We expect that such devices will open opportunities in many fields of microbiology. © 2015 AIP Publishing LLC.

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I. INTRODUCTION

In 1949, Monod wrote,¹ “The study of the growth of bacterial culture does not constitute a specialized subject or a branch of research; it is the basic method of microbiology.” Traditionally, the two most common methodologies for bacterial culture have been serial dilution transfer² and the chemostat.^{3,4} In the serial dilution transfer, the microbial population is allowed to grow exponentially in a closed environment (e.g., a test tube) with a fixed amount of nutrient medium. Once a growth cycle is finished, a fraction of the microbial culture is removed and fresh medium is inoculated to permit another growth cycle. In the chemostat mode, the microbial population is diluted continually with fresh medium to maintain a nearly steady microbial population. Traditional culture with liquid broth and agar plates are both reagent and labor consuming and efforts have been made to develop automated miniaturized bioreactors with working volumes from the micro- and nanoliter scales down to the scale of single cells.^{5–13} Groisman *et al.* have developed a flow-based microchemostat and the device can be used to monitor cellular response under the influence of stimuli.⁷ Subsequently, using a similar concept, Dorfman’s group used devices with submicron growth channels to monitor microbial growth under dynamic changes in environment at the single cell level.^{8,9} Balagadde *et al.* implemented a nanoliter chemostat device with 16 compartments to mimic a chemostat with continuous dilution. They employed the device for long term monitoring of bacteria undergoing programmed population control with a synthetic genetic circuit through a feedback mechanism based on quorum sensing.^{10,11} More recently, Han’s group simplified the design and realized an array of microchemostats with quasi continuous dilution for study of yeast.¹²

Biofilms play important roles in microbiology in clinical settings and natural environments.¹³ For example, it is believed that more than 80% of clinical infections are caused by biofilms. Biofilms have been studied extensively using microfluidic devices.^{14,15} Such devices provide an ideal tool to study the interplay of various physical processes affecting the formation of biofilms because of their high throughput screening capabilities, high surface to volume ratio, and low reagent volume. Jayaraman’s group developed a microfluidic flow cell to investigate biofilm formation under the influence of different biofilm signals.¹⁶ They also developed a

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mathematical model to take into account biofilm growth under quorum sensing in the microfluidic chamber.¹⁷ Subsequently, they used such a device to prove that a biofilm engineered with a synthetic quorum-sensing circuit can be used to displace an initial colonizer biofilm and be removed afterwards.¹⁸ Lee's group developed a simple one pot assay to generate a gradient of antibiotics with a microfluidic system for antibiotic susceptibility measurement.¹⁹ Benoit *et al.* have also developed high throughput viability screening of flow biofilms by combining a microfluidic device and a distributed pneumatic pump.²⁰ This device permits fine control of continuous or intermittent fluid flow over a broad range of flow rates, and the use of a standard well plate format supports rapid screening based on the effects of antimicrobials on *Pseudomonas aeruginosa* PAO1 flow biofilms. Sun *et al.* have done very similar work by using a microfluidic device with seven parallel channels to study the effects of tetracycline and erythromycin on *Escherichia coli* cells.²¹

Recent advances in large-scale integrated (LSI) microfluidics allow researchers to design a chip with almost arbitrarily complex plumbing in a fashion similar to the design of electronic integrated circuits.^{22–24} In this work, we focus on seeking the most economical plumbing design for serial dilution bacterial culture at the nanoliter scale. This design should minimize the use of active/passive components, such as valves and inlets, while retaining the essential mechanistic functions of a serial dilution transfer. We consider a class of bioreactors in which the growth chamber consists of N compartments and the dilution procedure is repeated M times in discrete time steps T_j . For simplicity, we assume a periodic operation, i.e., $T_j = jT$ ($j = 0, 1, 2, \dots$). After the j th growth cycle is completed, a fraction F of the microbial population is removed and the remaining fraction $\eta = 1 - F$ is refilled with fresh medium. Herein, T , η , and F are referred to as growth cycle time, dilution ratio, and removal fraction, respectively. For our serial dilution bioreactor in a typical biofilm-free operation, we choose $N = 2$ and $M = 2$. The plumbing design is shown in Figs. 1(a) and 1(c). A similar device to ours but on the macroscale (volumetrically 10^6 times larger) was developed by de Crecy-Lagard *et al.*^{25,26} All compartments are cleaned for biofilm prevention before a new growth cycle begins. As a result of this biofilm-free constraint, 2^2 -fold dilution in the microbial population is executed, thus the dilution ratio $\eta = \frac{1}{4}$ and the removal fraction $F = \frac{3}{4}$ for each growth cycle. We also study one variant in which the biofilm-free constraint is removed to serve as a biofilm-flow reactor. One of the two compartments ($N = 2$) is left uncleaned to permit biofilm growth and there are two ($M = 2$; Fig. 1(b)) dilution steps.

II. EXPERIMENTAL SETUP

A. Cell culture

We used the *E. coli* strain MG 1655 for our study. In each experiment, we used growth cycle time $T = 10$ h or 5 h at 30°C with Luria-Bertani (LB) medium (10 g/l tryptophan, 10 g/l yeast extract, and 5 g/l NaCl). The bacteria were stored at -80°C with glycerol. Prior to loading into the microfluidic devices, the bacteria were thawed and allowed to grow at 37°C in LB medium in a shaken incubator. After cell loading, an on-chip dilution of the microbe was carried out and the microbe was allowed to grow for 10 h for the initial inoculation. All the microbial growth data shown here included such an inoculation cycle. Subsequently, the microbe was allowed to grow with either $T = 10$ h or $T = 5$ h with periodic dilution steps.

B. Device design and fabrication

The core of the plumbing consists of a bi-segmented ring-shaped growth chamber furnished with a three-phase peristaltic pump and inlets to load the cell, supply nutrient medium, and flush the chamber, as shown in Fig. 2. The dimensions of the growth chamber in all the chips are $d_{\text{in}} = 1$ mm (inner diameter), $d_{\text{out}} = 1.2$ mm (outer diameter), and $h = 8\ \mu\text{m}$ (height), and the volume is $V = (\pi/4)h(d_{\text{out}}^2 - d_{\text{in}}^2) = 2.8$ nl. Our microfluidic device was fabricated by multilayer soft lithography from polydimethylsiloxane (PDMS).^{22,23} The control layer was a few millimeters thick and made by casting an RTV 615 mixture (with cross linking ratio 5:1) on a SU-8 2015 photoresist mold. The flow layer was made by spin coating an RTV 615 mixture (with

FIG. 1. Operation of serial dilution bioreactors. (a) A serial dilution bioreactor with $N=2$ and $M=2$. Two compartments are alternatively cleaned and refilled with fresh medium for biofilm-free operation. (b) A serial dilution bioreactor as a biofilm-flow reactor with $N=2$ and $M=2$. Only one compartment (left) is cleaned and the other compartment (right) is used to seed the biofilm (gray area) on the wall. (c) Plumbing sequence for four-fold dilution of a serial dilution bioreactor with $N=2$ and $M=2$ (Fig. 1(a)). The upper segment is cleaned and the nutrient (yellow) is refilled and mixed with microbial solution (blue) in the first dilution. Subsequently, the lower segment is cleaned and the nutrient is refilled and mixed with the remaining microbial solution (dark green). (d) Plumbing sequence for four-fold dilution of a serial dilution bioreactor with $N=2$ and $M=2$ (Fig. 1(b)). Only the lower segment is cleaned.

cross linking ratio 20:1) with an AZ 4620 photoresist mold. The two layers were then baked, aligned under a microscope, and bonded together by curing at 80 °C. Subsequently, inlet holes were punched with 20-gauge needles and the entire elastomer was bonded to a glass slide coated with a blank PDMS layer by air plasma treatment (Harrick Plasma, Inc., USA). Finally, we carried out extensive baking for 36 h at 80 °C to reduce cytotoxic effects from PDMS.²⁷

FIG. 2. Magnified view of the chip design. In a typical device, a ring-shaped growth chamber is bi-segmented and connected to multiple inlets. In this version, three labeled inlets are used for cell loading, nutrient medium, and de-ionized water flushing. All the control lines (red) are connected to external solenoid valves (not shown here) for pneumatic actuation. The chip is colored with food dye for illustration.

C. Microfluidic device operation

The microfluidic device was placed on a temperature-controlled microscope incubator (Haison, Inc., Taiwan) custom made for the inverted microscope (Leica DMI-LED) at 30 °C. A custom fixture was made to house the microfluidic device with a Tygon tube control line. The plumbing was controlled pneumatically via solenoid valves (Pneumadyne, Inc., USA). The technical details can be found in the literature.^{22,23} Plumbing functions such as mixing, dilution, and nutrient delivery were executed via micromechanical membrane valves. See supplementary material for a representative plumbing sequence with cell loading and dilution steps.²⁸ The device operates in three modes: dilution mixing of the microbe and substrate, circulation mixing during the growth cycle, and randomization mixing for cell counting.^{10,11} Dilution mixing is performed between growth cycles to dilute the population density and replenish the nutrient medium. During the growth cycle, the fluid is continuously circulated by the peristaltic pump connected to the growth chamber to prevent bacterial aggregation. For cell counting, a brief randomization mixing is executed to distribute the cells and minimize statistical errors in cell counting. The cell number is monitored in real time with single-cell resolution by processing the optical microscope image with a Matlab program.²⁸

For biofilm-free operation, we employed the cleaning protocol depicted in Figs. 1(a) and 1(c), in which de-ionized water was used to flush and expel the cells, including any wall-adhering cells. In addition, surfactant (0.006% Tween 20) was added into the nutrient medium to prevent bacterial adhesion to the channel wall. This addition of surfactant is very critical and we followed Hasty group's protocol because they used Tween 20 for observation of a synthetic genetic oscillation in *E. coli* based on quorum sensing.²⁹ For the device to operate as a biofilm-flow reactor, the growth of green fluorescent protein (GFP) expressing *E. coli* was monitored via a fluorescent microscope (Dino Lite Premier AM4113T-GFBW). The plumbing sequence was the same as that in the biofilm-free mode except that the upper compartment was left uncleaned, as shown in Figs. 1(b) and 1(d). LB medium was used as the nutrient medium and de-ionized water with 0.006% Tween 20 was used to flush and clean the lower compartment. Fluorescent images were taken every 10 min, post processed to extract the fluorescence intensity, and compiled into a movie. Integrated intensity F_{upper} and F_{lower} for the upper segment and lower segment were separately obtained with the region of interest shown in Fig. 4. Intensity for planktonic population equals the intensity of the lower segment, i.e., $F_{\text{planktonic}} = F_{\text{lower}}$. Intensity for the biofilm was obtained by subtraction of the intensity of the lower segment from the intensity of the upper segment, i.e., $F_{\text{biofilm}} = F_{\text{upper}} - F_{\text{lower}}$. Continuous circulation ensured that the planktonic population was the same in both the upper and lower compartments.

D. Description of the biofilm-flow reactor model

We have developed the biofilm-flow reactor model taking into account the growth of both planktonic and biofilm populations, adsorption and detachment processes, and periodic serial dilution, which we consider relevant.²⁸ The model deals with the co-growth of biofilm and planktonic populations in a fashion similar to the earlier work by Pylyugin and Waltman.³⁰ Hsu and Yang have incorporated the serial dilution step and analyzed the global behavior of growth dynamics of biofilm and planktonic populations.^{31–33} For the coexistence of a planktonic population and a biofilm population with a single nutrient source, S , we can write a set of differential equations:

$$\begin{aligned}\frac{dS}{dt} &= -\frac{1}{\gamma}f_u(S)u - \frac{\delta}{\gamma}f_w(S)w \\ \frac{du}{dt} &= (f_u(S) - \alpha)u + \delta\beta w \\ \frac{dw}{dt} &= (f_w(S) - \beta)w + \frac{\alpha u}{\delta},\end{aligned}\tag{1}$$

u is the volume density of the planktonic population and w is the surface density of the biofilm population. $\delta = A_{eff}/V$ is the surface to volume ratio and provides the proper scaling between u and w . (A_{eff} and V are the effective surface area for biofilm growth and the volume of the growth chamber, respectively.) α is the adsorption rate from the planktonic population to the biofilm population and β is the detachment rate from the biofilm to the planktonic population. We further assume an equal uptake function with logistic growth, in other words, $f_u(S) = f_w(S) = mS$, as our biofilm is well below maturation. Because of the serial dilution operation, we reset the initial condition at the end of each growth cycle, i.e., at $t = T_j = jT$

$$\begin{aligned} S(T_j^+) &= \eta S(T_j^-) + FS^{(0)} \\ u(T_j^+) &= \eta u(T_j^-) \\ w(T_j^+) &= \theta_w w(T_j^-), \end{aligned} \quad (2)$$

where θ_w characterizes the remaining fraction of wall adhering biofilm population during the dilution and cleaning steps. For $N=2$ and $M=2$; in other words, two two-fold dilution steps are executed as shown in Figs. 1(b) and 1(d) and no biofilm is removed during the dilution step, so $\eta = \frac{1}{4}$ and $\theta_w = 1$.

III. EXPERIMENTAL RESULTS

A. Biofilm-free bacterial culture in the microfluidic serial dilution bioreactor

We can operate the device in biofilm-free growth mode. Representative growth curves for five growth cycles with the four-fold dilution protocol ($\eta = \frac{1}{4}$ and $F = \frac{3}{4}$) for $N=2$ and $M=2$ are shown in Figs. 3(a) and 3(b). Figures 3(a) and 3(b) correspond to growth cycle $T=10$ h and $T=5$ h, respectively. In both cases, the microbial growth shows monotonic growth behavior with periodic dilution, consistent with the single strain dynamics described by Smith.³¹ At the end of the experiment, devices were inspected to confirm no biofilm growth even in the vicinity of the microvalves of the peristaltic pumps.

We can fit the growth curve with logistic growth curves to extract growth parameters, consistent with the previous finding.^{28,34} It is also interesting to quantitatively compare the growth rate data to other reported data. Using surfactant Tween 20 to prevent the microbial adhesion, the maximal growth rate reaches 0.85 h^{-1} in LB medium.²⁸ This growth rate is consistent with the value $\sim 0.8 \text{ h}^{-1}$ reported by Balagadde *et al.* in their microchemostat device^{10,11} but smaller than with the value $\sim 1.2 \text{ h}^{-1}$ reported by Mohan *et al.* for a chamber volume of ~ 3 nl in a chip growth experiment³⁴ and the maximal growth rate values of $\sim 1.4 \text{ h}^{-1}$ reported for bulk culture.³⁵ If we replace LB medium with M9 minimal medium with 0.2% glucose, the obtained growth rates ranges between 0.45 h^{-1} and 0.62 h^{-1} and therefore are consistently lower than those in LB medium. We also attempt to use only de-ionized water to flush without surfactant and achieve the growth rate $\sim 1.5 \text{ h}^{-1}$ but biofilm forms inevitably (data not shown). Using combination of lysis buffer (B-PER, Thermo Scientific) and de-ionized water flushing, we obtain similar growth rate $\sim 0.7 \text{ h}^{-1}$ but with excessive delay in growth curve (data not shown).

We also display the measured dilution ratio data in Fig. 3(c) and the data are compared with the ideal value, $\eta = \frac{1}{4}$. We find that the dilution ratio for $T=5$ h is very close to the ideal value. For $T=10$ h, the dilution ratio is lower than the ideal value. A plausible explanation is that certain planktonic population is adsorbed on the wall during the dilution step due to availability of fresh adsorption sites but we can rule out this possibility since the time elapse movie did not show significant wall adhesion right after the dilution step.²⁸ It is likely that the low dilution ratio results from incomplete mixing during the dilution step due to inhomogeneous population distribution when the microbe reaches the stationary phase.

B. Biofilm growth in the microfluidic serial dilution bioreactor

In our device, the upper compartment of the growth chamber serves effectively as a biofilm-flow reactor and the biofilm develops from wall-adhering cells supplied from the

FIG. 3. Representative growth curves of a planktonic population. (a) Growth curves for a serial dilution bioreactor for $T = 10$ h, operating with the biofilm-free plumbing protocol displayed in Fig. 1(c). (b) Growth curves for a serial dilution bioreactor as biofilm-flow reactor in the dilute biofilm limit. In both cases, the growth of the initial inoculation cycle for 10 h is also displayed. (c) Measured dilution ratios versus cycle number. Dilution ratio data are extracted from growth curves for $T = 10$ h and $T = 5$ h. The ideal dilution ratio, $\eta = 1/4$, is displayed as a dot line for comparison. Note that the data points of the first cycle are from the inoculation cycle.

cyclically growing planktonic population. We placed the GFP expressing strain *E. coli* MG1655 in the device and executed the protocol shown in Figs. 1(b) and 1(d) for growth cycle $T = 10$ h and $T = 5$ h, respectively. Figure 4 shows representative sequences of images for biofilm growth for growth cycles $T = 10$ h and $T = 5$ h, respectively.²⁸ We find that the biofilm formation always starts in the vicinity of the microvalves of the peristaltic pump. Figures 5(a) and 5(b) show representative growth curves of biofilm and planktonic populations, extracted from the fluorescence images in Fig. 4. In general, the biofilm population accumulates from cycle to cycle due to non-removal of the wall population. Within each cycle, the biofilm first shows an increase then a decrease because of the combination of microbial growth and adsorption and detachment processes. In contrast, the planktonic population shows monotonic increase but no accumulation from cycle to cycle. All of these features are faithfully reproduced in Figures 5(c)

FIG. 4. Biofilm formation in the bioreactor. (a) Successive fluorescent images of biofilm formation from fluorescent *E. coli* cells in the growth chamber for $T = 10$ h. The images at the end of each growth cycle are displayed and the leftmost image is at $T = 0$ h. Biofilm formation starts in the vicinity of the micromechanical valves of the peristaltic pump. (b) Same as (a) but with $T = 5$ h.

and 5(d) in the simulation of our model using plausible parameters. To the best of our knowledge, this is the first time the dynamics of coexisting biofilm and planktonic population has been measured with such temporal resolution to validate a companion mathematical model in the microfluidic devices. Biofilm formation under periodic nutrient supply is ubiquitous in animal guts and flow reactors.³⁶ Our device therefore serves as an *in vitro* model for such phenomena. While the biofilm in our device is below the maturation, experiments can be carried out in larger microfluidic device and for a more extended period for observation of mature biofilm. In this case, it is necessary to extend our simple model to a developmental model that incorporates nutrient diffusion and fluidic dynamics.³⁷

FIG. 5. (a) Integrated fluorescence intensity versus time for biofilm and planktonic populations for $T = 10$ h obtained from data as in Fig. 4. (b) Same as (a) but for $T = 5$ h. (c) Growth curve in the biofilm-flow reactor model with no biofilm removal ($\theta_w = 1$). In this figure, we use $T = 10$ h, $\alpha = 0.1 \text{ h}^{-1}$, $\beta = 0.05 \text{ h}^{-1}$, $m = 0.007 \text{ hr}^{-1}$, $\gamma = 1$, $S^{(0)} = 100$, and dilution ratio $\eta = 1/4$. (d) Same as (c) but with $T = 5$ h.

IV. CONCLUSION

We have demonstrated a serial dilution microfluidic bioreactor at nanoliter working volume. The device supports automated, long term bacterial culture with cell counting at single cell resolution. By changing the plumbing sequence, we are able to operate the device in either biofilm-free or biofilm-flow reactor mode. In the biofilm-flow reactor mode, our unique design enables the observation of co-growth of the planktonic and biofilm populations. While we operate the device in some representative serial dilution sequences, it is straightforward to generalize the design to execute higher order dilution, in other words, with higher N and M values. We believe our device is suitable for a range of applications such as microbial competition,³¹ characterization of genetic circuits for synthetic biology,³⁸ and biofilm growth.¹⁵

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