

Massively Parallel Bacterial and Yeast Suspension Culture on a Chip

Mingzhe Gan, Yunfang Tang, Yiwei Shu, Hongkai Wu, and Liwei Chen*

Micro-organisms are of major economic, environmental, and social importance. They have been employed for producing valuable chemical feedstock, energy sources, enzymes, pharmaceuticals, and providing vital services such as waste treatment and pollution control. Strain improvement is a crucial part of process development in microbial industries.^[1] Selection of improved strains requires massively parallel culture of micro-organisms and efficient screening of microbial species and/or culture conditions. Typically the selection processes are carried out in numerous shaking flasks or test tubes. Such procedures have enjoyed a long history of success, but are highly repetitive and labor-intensive.^[2] Whitesides and co-workers envisioned that miniaturization of microbial growth vessels through microfabrication technologies may enhance the number of parallel experiments while reducing the materials consumption. The operation of microfabricated microbial reactors may also be automated via computer control and thus the efficiency of microbial selection process can be greatly improved.^[3] The potentials of microfabrication technologies in system integration and automation meet the demands of high-throughput screening in microbial strain improvement and bring new capabilities to basic research.

A unique feature of microbial culture is that it requires active solid-liquid mixing.^[4] Reports on microfabricated chips for parallel microbial culture with active mixing mechanisms have been rather limited so far. Szita and co-workers constructed a suspension culture system with 4 integrated growth chambers of 150- μ L working volume each.^[5] Balagadde and co-workers reported another chip with six suspension culture loops.^[6] A chip with eight microwells of 100- μ L working volume was also fabricated for *Escherichia coli* (*E. coli*) suspension culture.^[7] Although these pioneering works demonstrated important functions, the number of integrated culture units is inadequate for high-throughput microbial screening. A major reason for the low degree of integration is that the fabrication of active mixing devices often involves delicate

and complicated steps. In addition, most of the pioneering works have focused on the suspension cultivation of *E. coli* as a proof-of-concept.^[4] Considering the wide application of various microbes in industry, it is desirable to demonstrate microbial culture chips that provide sufficient solid-liquid mixing for cultivating a wide range of micro-organisms in massively parallel fashion.

We recently reported a multilayered bacterial culture chip with up to 32 identical culture chamber loops using serial peristaltic pumps for active mixing.^[8] A further increase in the degree of integration is limited by the necessary void area for the alignment of multiple layers across the chip. While several types of bacteria have been cultured, the maximum achievable flow rate in the chip was not high enough to suspend and culture yeast cells.

In this paper, we report a new microfluidic chip for massively parallel microbial suspension culture. A high degree of integration of 120 culture chambers (50 nL each) in a 7.5 cm \times 5 cm chip is achieved. A fast-circulating flow rate of 170 μ m s⁻¹ enables the suspension culture of various microbial strains with industrial values including bacteria *Escherichia coli*, *Pseudomonas stutzeri*, *Zymomonas mobilis*, *Bacillus subtilis* as well as yeast *Saccharomyces cerevisiae*.

Figure 1 shows a photograph and schematic illustration of the microfluidic suspension culture chip. The 7.5 cm \times 5 cm chip is fabricated using polydimethylsiloxane (PDMS) with 120 culture chamber loops arranged in three columns. Each rectangular culture chamber loop consists of a main channel of 100 μ m in width, and for a short section of the loop, the main channel bifurcates into two narrow branch channels of 50 μ m in width each (see Figure 1b). A pneumatic control layer is fabricated on top of the culture chamber layer with a 30- μ m thin PDMS membrane in the middle. The control layer is aligned so that two straight pneumatic control channels cross with both the main culture channel and the branched culture channel. For each culture loop, the pneumatic channels form three two-stage peristaltic pumps: one controlling the main channel and the others controlling the two branched channels. When the pumps are in actuation, the fluid in the main channel is driven along one direction while the fluid in the branched channels flow along the opposite direction. But since the efficacy of peristaltic pumps are non-linearly dependent on the width of the fluid channels, the pump on the main channel is much more powerful than the two pumps at the branch channel together. Therefore, the peristaltic pump at the main channel dominates the net flow

Dr. M. Gan, Y. Tang, Prof. L. Chen
Suzhou Institute of Nano-Tech and Nano-Bionics
Ruoshui Road 398, Suzhou 215123, P. R. China
E-mail: lwchen2008@sinano.ac.cn

Y. Shu, Prof. H. Wu
Department of Chemistry
Hong Kong University of Science and Technology
Clear Water Bay, Kowloon, Hong Kong, P. R. China



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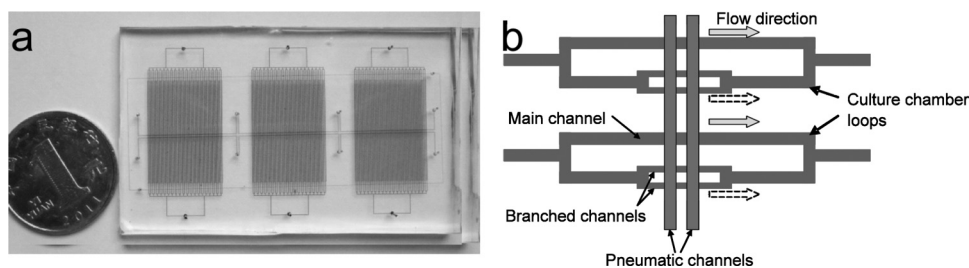


Figure 1. a) Photograph of a microfluidic suspension culture chip integrated with 120 culture loops in a chip area of 7.5 cm × 5 cm. b) Schematic diagram of culture loops (the orientation is rotated 90 degrees from that in the photograph a).

in the culture chamber loop. The advantage of this new chip design is that the alignment between the control layer and the culture chamber layer is easily achieved, which leads to a much simpler chip fabrication process and more integrated culture chamber loops than that in our previous report.

Actuation pressure and pumping frequency are the two critical parameters in flow rate control. The impact of actuation pressure on flow rate is shown in **Figure 2a**. The highest flow rate occurs at an actuation pressure of 80 kPa. Higher pressure in the pneumatic channel may cause the pump membrane to partially stick to culture channel wall and thus lead to lower flow rate. Figure 2b shows the effect of pumping frequency on flow rates. The highest flow rate of approximately $170 \mu\text{m s}^{-1}$ occurs at pumping frequency between 2.5 and 5 Hz. Higher pumping frequency results in rapid decrease of flow rate. To fully disperse the micro-organisms in medium, high flow rate is preferred to prevent clustering of cells and clogging of channels. Compared with the top flow rate of $110 \mu\text{m s}^{-1}$ in a previous report,^[8] the high flow rate of $170 \mu\text{m s}^{-1}$ achievable with this design allows the suspension of larger cells and breaking of cell chains, which helps to extend the ranges of micro-organisms culturable on the chip. The actuation pressure of 80 kPa and pumping frequency of 2.5 Hz are adopted for all microbial culture experiments. For parallel culture, the same flow rate in all chamber loops is required for the same growth environment. We measure the flow rates in all 40 culture chamber loops in one column as shown in Figure 2c. The average flow rates show good consistency among all channel loops with slight variations. The variation is not correlated with the position of the culture channel with respect to pneumatic channel inlets. Relay inlets of the pneumatic channel are placed between culture chamber loop columns to ensure the same actuation pressure in different loop columns.

To verify practical application on microbial suspension culture, four typical bacterial strains are cultured on the chip (**Figure 3a-d** and video documents in the Supporting Information). *E. coli* is commonly used in modern biological engineering and microbial industry. It is a facultative anaerobic bacterium usually existing in single cells or in pairs. *E. coli* is well dispersed in culture chamber loops with most cells circulating in medium after 6 h cultivation (Figure 3a). The growth of *E. coli* on chip is much faster than in shaking flasks, with a doubling time of 1.3 h. The cell concentration in chip suspension culture after 6 h is five times higher than that in conventional flask (Figure 3e). The enhanced *E. coli* growth rate is consistent with our previous report.^[9]

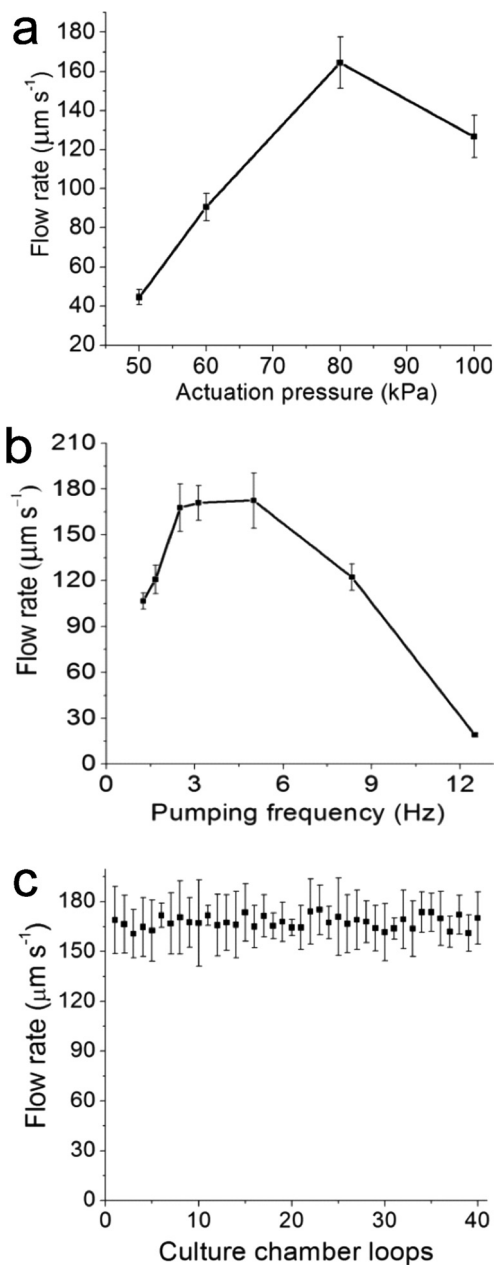


Figure 2. a) Flow rates at different actuation pressures with pumping frequency at 2.5 Hz. b) Flow rates at different pumping frequencies with actuation pressure at 80 kPa. c) Flow rate of each culture loop in one column at pumping frequency of 2.5 Hz and actuation pressure of 80 kPa.

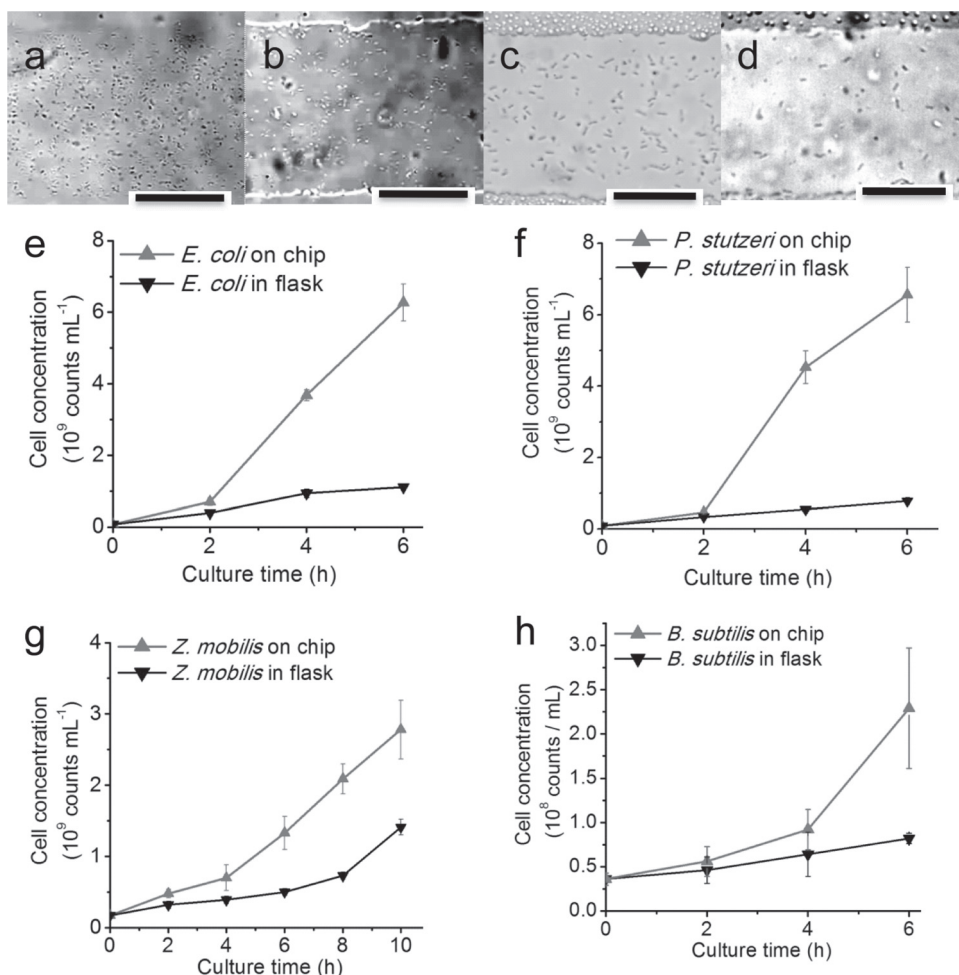


Figure 3. a–d) Optical micrographs of the suspension culture of bacteria on the chip: a) *E. coli*; b) *P. stutzeri*; c) *Z. mobilis* and d) *B. subtilis*. Scale bars are 50 μm . e–h) Growth curves of the bacteria on chip and in shaking flask: e) *E. coli*; f) *P. stutzeri*; g) *Z. mobilis*. and h) *B. subtilis*.

Pseudomonas stutzeri (*P. stutzeri*) is an obligate aerobe in nitrate absent environment. In particular, it has received attention in environmental applications for the ability of denitrification, degradation of aromatic compounds, and nitrogen fixation.^[10] *Pseudomonas* species tend to adhere to substrates and form biofilms due to a range of adhesion proteins.^[10,11] Thus *P. stutzeri* may adhere to the channel wall and block the microchannels. Some reports have revealed that surface modification of PDMS with a surfactant polyethylene oxide–polypropylene oxide–polyethylene oxide tri-block copolymer (PEO/PPO/PEO) could prevent cell adhesion without significantly affecting bacterial growth.^[12,13] Therefore we modified our PDMS chips with PEO/PPO/PEO before *P. stutzeri* culture. It is found that more *P. stutzeri* cells adhere to the channel wall than *E. coli*. Nevertheless, most cells are suspended and circulate in loops during on-chip cultivation. The *P. stutzeri* concentration on chip after 6 h is 8.4 times of that in conventional shaking flask (Figure 3f), indicating the applicability of our chip for adherent bacterial suspension culture.

Gas molecules such as carbon dioxide are common microbial metabolic products. While gas bubbles are naturally or mechanically broken in conventional fermentation, gas bubbles in microfluidic channels are hard to eliminate and may

block the channels. To verify the applicability of the chip on aerogenic bacterial suspension culture, *Zymomonas mobilis* (*Z. mobilis*) was selected as an example for aerogenic fermentation on chip. *Z. mobilis* is a promising biofuel industrial producer because it can rapidly and efficiently produce ethanol from glucose.^[14] Two moles of CO_2 and two moles of ethanol are produced from one mole of glucose metabolized by *Z. mobilis*. Unlike that in conventional vessels, gas bubbles were not observed in culture chamber loops during on-chip suspension cultivation. Shorter lag phase and higher cell concentration are found on chip culture compared with those in shaking flask (see Figure 3g). The permeability of PDMS and the high surface-to-volume ratio of microchannels facilitate the discharge of gaseous products and prevent the formation of gas bubbles. Thus, the chip can be used for aerogenic bacterial suspension culture.

Bacillus subtilis (*B. subtilis*) is a well-known microorganism in industry for enzyme production, and wastewater treatment. Unlike the strains described above, *B. subtilis* has the tendency to grow in chains.^[15] Chains of *B. subtilis* can reach several tens of micrometers in length and form clusters, which could clog microfluidic channels. We find that a high flow rate of about $170 \mu\text{m s}^{-1}$ in the culture loop is necessary

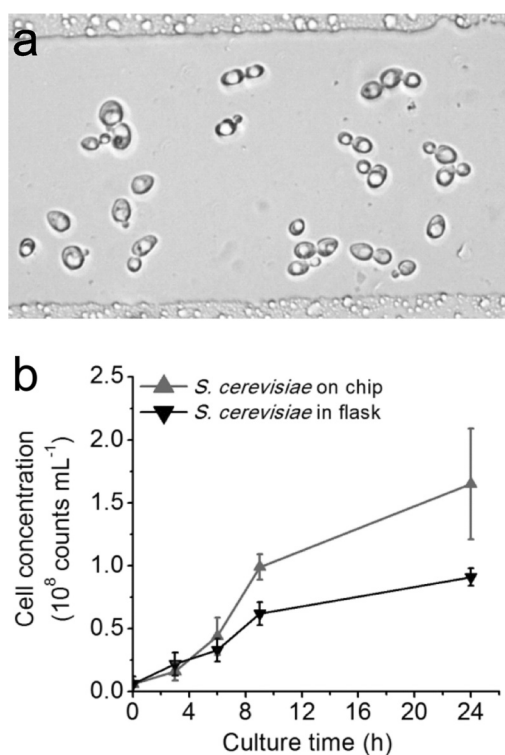


Figure 4. a) Optical micrograph of the suspension culture of *S. cerevisiae* on chip. b) Growth curve of *S. cerevisiae* on chip and in shaking flask.

to prevent the cells from chaining. Interestingly, cell chaining still happens at the bifurcation points of the branched channels due to disturbed flow and blocks the branch channels within a few minutes. This problem is solved by a new pumping sequence that reverses the flow direction once every few minutes. The reversal of flow flushes the clustered cells out of the branched channels and thus prevents them from forming long chains. As a result, *B. subtilis* can grow in suspension culture for 6 h with a growth rate higher than the ordinary culture in shaking flasks. (Figure 3h)

As a typical species of yeast and a eukaryotic model organism, *Saccharomyces cerevisiae* (*S. cerevisiae*) plays an important role in biological research and fermentation industry. However, *S. cerevisiae* cultivation on chip is yet to be demonstrated due to its larger size and quicker sedimentation than bacteria. In our previously reported multilayer chip, the maximum attainable flow rate of about 100 $\mu\text{m s}^{-1}$ was not enough to suspend *S. cerevisiae*. The significantly improved flow rate of about 170 $\mu\text{m s}^{-1}$ in the present chip keeps *S. cerevisiae* suspending and circulating in culture chamber loops for 24 h without clogging. The yeast cells are observed to exist in singles, pairs and tetrads, and circulate along with the fluidic flow (Figure 4a and video documents in Supporting Information). While some cells are found to stick on the pumping membrane in several tens of minutes, they can be easily flushed back into suspension by periodically reversing the flow direction. The cell concentration after 6 h is observed to be higher than conventional culture (Figure 4b), suggesting that the peristaltic pumps have little adverse effect on the *S. cerevisiae* growth.

In summary, we present a microfluidic chip that integrates 120 independent and identical channel loops with a volume of 50 nL each. The maximum achievable flow rate in culture channel loop is 170 $\mu\text{m s}^{-1}$. The flow rate in each culture channel loop is nearly equal, which ensures identical conditions for parallel cultures. Massively parallel suspension cultures of bacteria *Escherichia coli*, *Pseudomonas stutzeri*, *Zymomonas mobilis*, *Bacillus subtilis* and yeast *Saccharomyces cerevisiae* has been successfully demonstrated. Growth rates of micro-organisms on chip are higher than those in conventional shaking flasks. With the high degree of integration and simple fabrication process, this chip could be a central component for future high-throughput microbial screening and selection systems.

Experimental Section

Chip fabrication: The microfluidic chip was made of three layers of PDMS (Dow Corning, Sylgard 184) using soft lithography.^[16,17] The master moulds were made from photoresist AZ4620 (~12 μm thick) on glass. PDMS base, mixed at a 10: 1 (w/w) ratio with a curing agent was poured onto the master mould of control layer and cured at 90 °C for 20 min and peeled. A thin layer of PDMS prepolymer (~40 μm thick) was spin-coated onto the mould of culture layer at 3500 rpm for 60 s and cured at 80 °C for 10 min. The control layer was aligned onto the culture layer and cured at 90 °C for 20 min to bond together. Then the cured PDMS was peeled from master mould. All inlets and outlets were punched by 23 gauge flat needle. Finally the cured PDMS containing control layer and culture layer was bonded with a flat cured PDMS slab to form the whole chip. The chip was baked at 90 °C overnight for tight seal.

Operation procedures and measurements: Before culture experiment, the chip was incubated with 2% Pluronic F127 (PEO/PPO/PEO copolymer) in 1 h for surface modification and autoclaved at 121 °C for 20 min. All micro-organisms were suspended in culture medium separately and then injected into chips. The inlets and outlets were sealed by epoxy. The pneumatic channels were filled with water as pressure transferring medium and the inlets were connected with compressed air. The chip was placed into water bath at growth temperature. The actuation process of two-stage peristaltic pump was followed as previous report.^[18] The flow direction reversed every 10 min if required. The flow rate in culture loop was determined by measuring the time of *E. coli* traveling a known distance under a microscope (Nikon AZ100).^[8] The cell concentration was measured by counting cell numbers in a known volume under a microscope.

Biological experiments: *E. coli* TOP10 was kindly provided by Prof. Qiangbin Wang, Suzhou Institute of Nano Tech and Nano Bionics, Chinese Academy of Sciences. *Bacillus subtilis* CICC 23591, *Pseudomonas stutzeri* CICC 31616 and *Zymomonas mobilis* CICC 10232 were obtained from China Center of Industrial Culture Collection. *Saccharomyces cerevisiae* was obtained from Angel Yeast Co., Ltd. The medium for bacterial culture consisted of 0.5% peptone, 0.5% yeast extracts, 1% beef extracts, 5% glucose and 0.5% NaCl in tap water with pH 7.4. The medium for yeast culture consisted of 0.9% yeast extracts, 0.1% (NH₄)₂SO₄, 5% glucose in tap water with pH 6.0. The growth temperatures were 37 °C for *E. coli* and 30 °C for other strains. In a conventional shaking culture, the agitation speed was 150 rpm.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

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