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3 **Microbial microdroplet culture system (MMC): an integrated platform for**

4 **automated, high-throughput microbial cultivation and adaptive evolution**

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1 Running title: MMC for microbial cultivation and adaptive evolution

2 **Abstract**

3 Conventional microbial cell cultivation techniques are typically labor intensive, low
4 throughput, and poor parallelization, rendering them inefficient. The development of
5 automated, modular microbial cell micro-cultivation systems, particularly those
6 employing droplet microfluidics, has gained attention for their high-throughput,
7 parallel and highly efficient cultivation capabilities. Here, we report the development
8 of a microbial microdroplet culture system (MMC), which is an integrated platform
9 for automated, high-throughput cultivation and adaptive evolution of microorganisms.
10 We demonstrated that the MMC yielded both accurate and reproducible results for the
11 manipulation and detection of droplets. The superior performance of MMC for
12 microbial cell cultivation was validated by comparing the growth curves of six
13 microbial strains grown in MMC, conventional shake flasks or well plates. The
14 highest incipient growth rate for all six microbial cell lines was achieved using MMC.
15 We also conducted an 18-day process of adaptive evolution of a methanol-essential
16 *Escherichia coli* strain in MMC and obtained two strains exhibiting higher growth
17 rates compared with the parent strain. Our study demonstrates the power of MMC to
18 provide an efficient and reliable approach for automated, high-throughput microbial
19 cultivation and adaptive evolution.

20

21 **Keywords:** microbial microdroplet culture system, automated operations, high-
22 throughput, microbial cultivation, laboratory adaptive evolution

1 INTRODUCTION

2 The cultivation of microbes is the foundation of microbiological research and is
3 absolutely necessary for microbial isolation (Kim et al., 2019; Maymo-Gatell et al.,
4 1997), identification (Wadlin et al., 1999), screening (Lu et al., 2018; Nautiyal 1999)
5 and evolution (Cubas-Cano et al., 2019; Dunham et al., 2002; Gao et al., 2017).
6 Conventional, reliable laboratory cultivation techniques include shake flasks, well
7 plates and solid medium. However, technological advances in applied microbiology,
8 for instance, using the metabolic capacity of microbes to produce chemicals (Nielsen
9 et al., 2013), fuels (Hong et al., 2012), pharmaceuticals (Ferrer-Miralles et al., 2009),
10 and enzymes (Li et al., 2015), necessitate the development of cultivation methods that
11 provide more information on strain and process properties and higher throughput than
12 conventional methods (Hemmerich et al., 2018). Improved cultivation methods would
13 also address limitations of conventional methods, including throughput, reagent and
14 labor cost, and cultivation properties such as mixing and parallelization.

15
16 Recent advances in miniaturization and parallelization of microbial cultivation
17 systems have translated into advances in the capabilities of microbioreactors, which
18 are now used in studies of microbial antibiotic resistance and evolution (Kaminski et
19 al., 2016; Toprak et al., 2012 & 2013), high-throughput analysis and sorting of
20 microbes (Agresti et al., 2010; Beneyton et al., 2017; Sjoström et al., 2014), microbial
21 enrichment (Akselband et al., 2006; Bachmann et al., 2013), and discovery of new
22 microorganisms (Nichols et al., 2010; Park et al., 2011). Precise control of cultivation
23 parameters (e.g., temperature, pH and nutrient concentrations) and methods of
24 detection (e.g., optical density, fluorescence intensity, pH and dissolved oxygen) has
25 been achieved by implementing novel analytical technologies (Faassen & Hitzmann,

1 2015; Marose et al., 1999). In addition, automated operations have reduced human
2 error in experiments, and parallelization has been achieved through miniaturization
3 and the use of independent control systems for each microbioreactor.

4

5 Micro-cultivation systems can be classified into single-phase microfluidic cultivation
6 systems and droplet-based cultivation systems (Tan et al., 2019a). A single-phase
7 microfluidic cultivation system is a millimeter-scale, automatic, continuous
8 cultivation system that is, essentially a smaller version of a conventional continuous
9 cultivation system, e.g., a chemostat or turbidostat. These systems are usually modular
10 and open-sourced, enabling further modification based on experimental needs
11 (Takahashi et al., 2015; Wong et al., 2018). On the other hand, development of the
12 micro total analysis system (commonly known as μ TAS; Angell et al., 1983; Terry et
13 al., 1979) has enabled the microscale manipulation of fluids and provided several
14 advantages over microfluidic-based cultivation systems, e.g., high inside mass
15 transfer rate (Lee et al., 2011) and large specific surface area, facilitating gaseous
16 exchange (Li et al., 2012) and higher throughput. Although various microfluidic-
17 based cultivation systems have been constructed (Balagaddé et al., 2005; Lee et al.,
18 2011), single-phase microfluidic systems are prone to microbial contamination
19 (Zhang & Xing, 2007). Microdroplet technology effectively addresses this problem by
20 compartmentalization, i.e., by encapsulating microbial cells in droplets on the scale of
21 microliters, nanoliters, or even picoliters. By separating carrier fluid from the culture
22 medium, contamination is essentially eliminated. Microdroplet technology also has
23 the potential for very high-throughput cultivation, which can reach hundreds (Jakiela
24 et al., 2013; Wink et al., 2018), thousands (Baraban et al., 2011; Ota et al., 2019), or
25 even millions of droplets in every experiment (Kaushik et al., 2017). In addition,

1 further miniaturization of a cultivation system enhances parallelization of cultivation.
 2 For example, high-throughput, continuous cultivation of *Escherichia coli* and
 3 *Pseudomonas fluorescens* to a final cell density of 10^6 cells per droplet has been
 4 achieved in Teflon tubes using 60-nl droplets (Grodrian et al., 2004). In addition,
 5 continuous monitoring and cultivation, adaptive evolution of microbial cells, and
 6 screening for antibiotic toxicity have been achieved in automated microdroplet
 7 cultivation systems (Churski et al., 2012; Jakiela et al., 2013). In such systems,
 8 however, the cell suspension and reagents are handled by pumps and passed through
 9 the pumps directly, which not only introduces a significant risk of contamination by
 10 other microorganisms but also makes it difficult to clean the system. In addition, these
 11 systems don't integrate their parts and didn't demonstrate the ability to achieve
 12 sustained and reliable operation.

13

14 Here we report the development of an integrated platform referred to as a microbial
 15 microdroplet culture system (MMC), with which researchers can perform automated,
 16 high-throughput microbial cultivation and adaptive evolution in up to 200 replicate
 17 droplets of 2.00 μ l volume. We integrated all the parts into a single chamber (Figure
 18 S1f), which resulted in a miniaturized, more reliable system. We also developed a new
 19 method for sample injection, which utilizes custom-designed reagent bottles that
 20 prevent contamination by other microorganisms and are much easier to be cleaned. In
 21 addition, we employed two Teflon tubes with good gas permeability, which enhances
 22 the gaseous exchange between droplets and external environment, to store and
 23 incubate droplets, allowing the microorganisms in the droplets to grow in a more
 24 preferred condition (such as higher concentration of dissolved oxygen) compared with
 25 cultivation on a chip. MMC allows automated, high-throughput cultivation of a

1 variety of microorganisms with real-time monitoring of growth. With droplet
2 manipulation on a chip, MMC also allows for continuous sub-cultivation, enabling
3 the automated, high-throughput adaptive evolution of microorganisms to obtain
4 strains that exhibit improved growth characteristics under certain stresses. In summary,
5 MMC enables automated and sustained high-throughput cultivation and real-time
6 monitoring of various microbial cells in droplets, and the system can also be used for
7 adaptive evolution of microorganisms.

8

9 MATERIALS AND METHODS

10 2.1. Materials

11 For our experiments, seven species of microbial cells were used: *E. coli* MG1655,
12 *Lactobacillus plantarum* subsp. *plantarum* CICC 20418, *Corynebacterium*
13 *glutamicum* ATCC 13032, *Saccharomyces cerevisiae* BY4741, *Pichia pastoris* Ppink-
14 HC S-1, *Methylobacterium extroquens* AM1 (Liang et al., 2018), and a methanol-
15 essential *E. coli* strain version 2.2 (MeSV2.2) (Meyer et al., 2018). Table S1
16 summarizes the culture medium used for each strain. Each strain was maintained in a
17 strain-specific medium containing 20% glycerol (GENERAL-REAGENT, China)
18 and frozen as pelleted cells at -80°C. Each strain was streaked on an agar plate
19 prepared with the appropriate medium and incubated until single colonies appeared.
20 Single colonies were picked and used to inoculate the appropriate liquid medium.
21 Cells were then cultivated using the conditions shown in Table S2.

22

23 2.2. The working principles of MMC

1 MMC comprises four main parts: (1) pumps and valves, (2) reagent bottles, (3) a
2 fluidic chip for droplet manipulation, and (4) a microbial cell cultivation tube (Figure
3 1a, refer to Figure S1 for photographs of the MMC components).

4

5 **2.2.1. Pumps and Valves**

6 MMC uses six pumps (Cavro Xcalibur Pump, TECAN, Switzerland) and an
7 electromagnetic valve (Mrv-01-T02-K1.5-C-M01, Runze Fluid, China) controlled
8 by a program created in-house using the programming application LabView. Each
9 pump has three different connection states (Figure S2a) controlling sample injection
10 and fluid flow in the system, and each pump regulates a pipeline. All pipelines are
11 connected to an oil bottle at one end that supplies the carrier oil (mineral oil with 10
12 g/l Span 80, Sigma Aldrich, Germany) for the entire system. An electromagnetic valve
13 controls the pipeline of waste discharge.

14

15 **2.2.2. Reagent bottles**

16 Bespoke 15-ml bottles with a top tube and side tube were used as the reagent bottles
17 (Figure S2b). These tubes are important for sample injection to prevent sample
18 evaporation and contamination to achieve long-term automated continuous
19 cultivation. Autoclaved (121°C, 15 min) reagent bottles are filled with 8–12 ml of oil
20 via the side tube in a laminar flow cabinet. Then, 2–6 ml of sample is added via the
21 same tube. Oil is then added to fill the remaining space in the bottle. The top tube is
22 connected to a pump, and the side tube is connected to the chip. The sample, which
23 occupies the lower part of the reagent bottle owing to its greater density, is pushed
24 into the chip via the side tube when oil is pumped into the reagent bottle (Figure 1b).

25

1   **2.2.3. Droplet–manipulation fluidic chip**

2   The droplet–manipulation fluidic chip, which is made of poly(methyl methacrylate),
 3   contains a channel that has an inner diameter of 1 mm. The chip has seven branches,
 4   each being connected to a pump, valve, reagent bottle, or microbial cell cultivation
 5   tube by an acrylonitrile butadiene styrene quick connector (Figure 1e).

6
 7   The fluidic chip contains a recognition site of droplets, a detection site of droplets,
 8   and two electrodes for electrocoalescence. The recognition site of droplets consists of
 9   a laser (620 nm), photoelectric sensor (TSL12T, TAOS, USA), mechanical supports,
 10   and related circuits (Figure 1c). Each droplet passing through this site is numbered in
 11   sequence by the program for traceability. In addition, the distance between two
 12   adjacent droplets is adjusted and maintained by replenishing or draining the oil
 13   between them to prevent the fusion of droplets, ensuring the stability of the droplet
 14   sequence in the channel.

15
 16   The detection site of droplets contains a halogen lamp (HL–2000, Choptics, China),
 17   optical fiber (Choptics), and a spectrometer (EQ–2000, Choptics) to monitor
 18   microbial growth in real time (Figure 1d). For our experiments, the system was set to
 19   measure the optical density at 600 nm (OD₆₀₀) of each droplet over a 1–mm optical
 20   path when it passed through the detection site.

21
 22   Finally, the pair of electrodes provide an alternating current electric field (1600V, 60
 23   kHz) for electrocoalescence in which droplets are fused (Szymborski, et al., 2011).

24

25   **2.2.4. Microbial cell cultivation tube**

1 Teflon microbial cell cultivation tubes (AF-2400, O.D. 1.67 mm, I.D. 1.07 mm,
2 length 1.5 m) were used to store and incubate droplets. The high total surface area to
3 volume ratio of the fluid increases the rate of mass transfer in the droplets, while the
4 movement enhances convection, which in turn facilitates mixing in the droplets
5 (Kaminski et al., 2016). In addition, gaseous exchange (Figure 1e) is enhanced by the
6 gas permeability (Zhang et al., 2017a) of the Teflon AF-2400 tubes used for microbial
7 cell cultivation and internal flow of droplets (Li et al., 2012).

8

9 **2.3. On-chip droplets manipulation**

10 Droplets can be manipulated in five different ways on the chip: (1) generation, (2)
11 incubation, (3) splitting, (4) fusion, and (5) sorting (Figure 2).

12

13 The MMC generates water-in-oil droplets, with their volume determined by the
14 pumps and valves (Cedillo-Alcantar et al., 2019; Jakiela et al., 2013). For our
15 experiments, the volume of each droplet generated was 2.00 μ l, and the maximum
16 number of droplets in each set of experiments was 200. The droplets are numbered
17 using the program as they pass through the recognition site located after the droplet-
18 generation site (Figure 2a, Video S1).

19

20 For incubation, the droplets are circulated in the droplet-manipulation fluidic chip and
21 the microbial cell cultivation tube. The OD₆₀₀ of droplets is measured as they pass
22 through the detection site. The channel also contains some right-angled curves to
23 enhance mixing by convection (Song et al., 2003; Song et al., 2006) (Figure 2b, Video
24 S2).

1 Droplets splitting and fusion are two important aspects necessary to achieve
2 continuous cultivation, which can conduct the operation of cell passage by splitting
3 the droplets containing cells into two parts and then merging one of them with the
4 droplets containing fresh medium (Figure 2c and 2d, Video S3). The fraction of
5 splitting, f_{sp} is defined as

$$6 \quad f_{sp} = \frac{V_{re}}{V_0} \times 100\%$$

7 where V_{re} is the volume of the remaining droplet after being split and V_0 is the initial
8 volume of the droplet. By maintaining the volume of the droplet after fusion the same
9 as the initial volume of droplet, the fraction of splitting, f_{sp} , determines the
10 concentration of the microbial cells present in the droplet after fusion, which can help
11 us determine the concentration of inoculum when sub-cultivating cells. The fraction
12 of splitting can be manually defined for the particular needs of each experiment.
13 Droplets can be sorted and extracted using a droplet-numbering system in MMC
14 (Figure 2e, Video S4).

15

16 **2.4. Calibration of the measurement of OD₆₀₀ in MMC and well plate**

17 For our experiments, OD₆₀₀ was used to determine the density of microbial cells in
18 liquid culture medium. However, the Beer–Lambert law relating optical density to
19 sample concentration (or cell density) is only applicable when the optical density of
20 the sample solution falls within the linear range of the optical density vs.
21 concentration calibration curve (Begot et al., 1996). Generally, before the OD₆₀₀ of a
22 microbial culture is measured, the culture is diluted to yield measurements that fall
23 within the linear range of the spectrophotometer. However, manual dilution for
24 spectrophotometer measurement is difficult in both the well plate and MMC formats.
25 Therefore, it was necessary to plot calibration curves correlating OD₆₀₀ values for

each of these different formats to OD₆₀₀ values obtained using a standard method. To generate these calibration curves, the OD₆₀₀ values for various dilutions of each microbial species were first measured with a UV–Vis spectrophotometer (Ultrospec 3100 pro, GE, USA; linear range: 0.1–0.8). For high–density microbial cell suspensions, the culture was concentrated by centrifugation. To create high–density microbial cell suspensions, the cells were cultivated in a 100–ml shake flask using 20 ml of the appropriate liquid medium for a long enough time to reach the maximum cell density (for the time, refer to the growth curves of the shake flask in Figure 5). Then the cell suspension was transferred to a centrifuge tube and spun at 5000 rpm for 5 min at 4 °C. After centrifugation was completed, discarded a part of supernatant, and then resuspended the cells to obtain high–density microbial cell suspensions. Microbial cell suspensions of the same densities used for the UV–Vis measurements were then analyzed with a microplate reader (infinite M200 PRO, TECAN) and in the MMC system. Each measurement was performed in triplicate. The calibration curves for each strain were constructed by plotting the mean OD₆₀₀ measured with the UV–Vis spectrophotometer against the mean OD₆₀₀ measured with the microplate reader or in the MMC system (Figure 3 and S3).

2.5. Adaptive evolution of MeSV2.2 in MMC

MeSV2.2 was cultivated in a shake flask for 72 h using lysogeny broth culture medium. The culture medium was inoculated with MeSV2.2 suspension (2% of the total volume, Table S1) and cultivated in a shake flask for 5 h before seeding into MMC. Fifty droplets were generated, and the concentration of inoculum was set to 15% in each sub–cultivation. The duration of each sub–cultivation was 30 h. The culture medium was then replaced with fresh medium by droplet splitting and fusion.

1

2 **RESULTS**

3 **3.1. Calibration curve of OD₆₀₀ values for MMC**

4 We constructed OD₆₀₀ calibration curves for six different microbial strains by plotting
 5 the OD₆₀₀ values measured by UV–Vis spectrophotometry against OD₆₀₀ values
 6 measured in both well–plate and MMC systems (Figure S3). Comparison among the
 7 calibration curves (Figure 3) revealed only a small effect of species difference on OD
 8 measurement in both well–plate and MMC formats, as the deviation among curves
 9 was minor. Notably, however, the shapes of all strains used for our experiment were
 10 rod–like or spherical, so this result might not be applicable to microbes of other
 11 shapes.

12

13 On the other hand, the linear range of the calibration curve varied among different
 14 instruments, a result of a difference in path length. The cuvette we used for UV–Vis
 15 spectrophotometry had a path length of 10 mm, and the linear OD₆₀₀ range was 0.1–
 16 0.8. For the well plate, the path length was 6 mm (the same as the height of the cell
 17 suspension in the well plate), and the linear OD₆₀₀ range was 0–6. For MMC, the path
 18 length was 1 mm, and the linear OD₆₀₀ range was 0–11. According to the Beer–
 19 Lambert Law, an increase in optical path length will increase the spectral noise of the
 20 system exponentially as a result of increased intensity of the strong absorption band
 21 (Inagaki et al., 2017). In turn, this increase in spectral noise increases the standard
 22 deviation of absorbance measurements, thereby narrowing the linear range of the
 23 absorbance curve.

24

25 **3.2. Accuracy and reproducibility of inoculation**

Automated, continuous cultivation in MMC is achieved by splitting and fusing droplets. Therefore, it is important to monitor the accuracy and reproducibility of the MMC inoculation process to gauge the impact of inoculation on parallelization. For ease of measurement, purple pigment and 10% sodium chloride solution were used to simulate microbial cells and culture medium. A series of solutions were prepared containing 10% sodium chloride and seven different concentrations of purple pigment (13.1%, 21.7%, 32.0%, 41.8%, 51.9%, 64.8% and 75.2%). For each sample, 50 droplets were generated. After the droplet-splitting process, the remaining droplets were inoculated into fresh 10% sodium chloride solution without purple pigment. The absorbance of the initial droplets and the fused droplets was measured at 600 nm, and the fraction of absorbance relative to that of the initial droplets was defined as the concentration of inoculum. The coefficient of variation of the concentration of inoculum relative to the targeted concentration varied between 2.2% and 5.0% for each sample (Figure 4), indicating that the inoculation process in MMC is reproducible. When the targeted concentration was 13.1% or 21.7%, the coefficient of variation was 5.00% and 4.96%, respectively, which were the highest among all samples (Figure 4b). This result is attributable to the higher precision requirement for the pump, both when segregating droplets into smaller fragments and droplet fusion. Given that the precision is consistent, the coefficient of variation will increase as the targeted concentration is decreased.

3.3. Characterization of the performance of microbial cultivation

To evaluate the performance of MMC with respect to microbial cultivation, six microbial-cell species were cultivated in shake flasks, well plates, and MMC, and the growth curves were plotted (Figure 5). For each type of microbial cell, a single colony

growing on an agar plate was used to inoculate a 20-ml starter culture (see Table S1 for species-specific media and Table S2 for culture conditions) and cultivated in shake flasks. When the starter culture reached $OD_{600} \approx 0.1$, aliquots (20 ml for a shake flask, 200 μ l for a well and 2 μ l for a droplet) were used to inoculate the various cultivation systems.

6

At early stages of cultivation, the growth rate of all microbial cells was higher in MMC compared with both shake flask and well-plate culture. With a larger total surface area to volume ratio, the droplet cultivation system had a higher mass-transfer rate, which enabled rapid gas exchange compared with other cultivation systems and thus provided more oxygen to support the growth of microbial cells while rapidly removing intracellular carbon dioxide. This property resulted in a higher initial growth rate when excess nutrients were present. At the middle and later stages of cultivation, when compared with shake-flask cultivation, *E. coli* MG1655 cultivated in the MMC grew much faster. In addition, *E. coli* MG1655 cultivated in the MMC had a higher-OD stationary phase. The cultivation performance of both *L. plantarum* subsp. *plantarum* (CICC 20418) and *C. glutamicum* (ATCC 13032) in the MMC was similar to that in the shake flask. However, the cultivation performance of *S. cerevisiae* BY4741, *P. pastoris* Ppink-HC S-1, and *M. extroquens* AM1 in MMC was inferior to that in the shake flask. For these three strains, the growth rate generally slowed when the OD_{600} reached ~ 8 , presumably because the droplet form inhibits cell survival at a high concentration of cells (Figure S4). In theory, when the cell concentration in the droplet is too high, the growth of these cells is inhibited because of several factors, such as space and nutrient limits, along with the accumulation of secondary metabolites in the droplets. The disadvantage in material transfer and the

1 accumulation of secondary metabolites also explains the lower growth rate and final
2 microbial cell density in well plates compared to MMC.

3

4 Furthermore, it is important to note that, although there was no growth of
5 methanotrophic *M. extroquens* AM1 in the well–plate format, it exhibited a high
6 initial growth rate in MMC. As a compartmentalized cultivation system, carrier oil in
7 MMC insulates droplets from the environment, thus preventing the evaporation of any
8 volatile compounds in the droplet (data not shown). During the cultivation of *M.*
9 *extroquens* AM1, methanol was added to the culture medium to support growth.
10 Methanol evaporated rapidly in the well–plate system, resulting in loss of the carbon
11 source for *M. extroquens* AM1 and failure to grow. Although it is possible to add a
12 layer of oil to the well–plate system to reduce evaporation, this approach renders
13 further supplementation of methanol difficult and reduces oxygen diffusion into the
14 culture medium and thus reducing the growth rate of *M. extroquens* AM1. These
15 problems encountered in the well–plate system were not an issue with MMC.

16

17 **3.4. Adaptive evolution of MeSV2.2 in MMC**

18 Using microbial continuous sub–cultivation, we could conduct adaptive evolution to
19 obtain microbial strains with desired properties. The engineering of synthetic
20 methylotrophic microorganisms is of great significance for the conversion of one–
21 carbon compounds in an industrial setting (Whitaker et al., 2015; Zhang et al., 2017b).
22 Generally, engineering such synthetic microorganisms requires optimization in
23 laboratory–based adaptive evolution experiments (Meyer et al., 2018). Here, we used
24 MMC to perform adaptive evolution of methanotrophic MeSV2.2. As the highest
25 density of MeSV2.2 we achieved was $OD_{600} \approx 0.3$ after 40 h (Figure 6b and Figure

1 S5), a mutant with higher growth rate and higher final cell density was desired.
 2 Considering the high evaporation rate of volatile compounds in well plates and the
 3 low throughput of shake–flask culture, MMC was clearly the superior option for
 4 adaptive evolution of MeSV2.2.

5

6 MeSV2.2 was cultivated in MMC for 18 days, and a growth curve for each droplet
 7 was plotted (Figure 6a). The peak and valley in each curve indicated the process of
 8 adaptive evolution of MeSV2.2. In MeSV2.2 culture medium, methanol was
 9 supplemented as a selection pressure for MeSV2.2. In the growth curves, inhibition of
 10 cell growth during the adaptation phase is indicated by the valley, whereas the higher
 11 growth rate of enriched cells adapted to methanol is indicated by the peak.

12

13 Eight droplets with high OD₆₀₀ values after 18 days of cultivation were extracted for
 14 further investigation (droplets 2, 3, 6, 14, 20, 23, 28, 35). For the mutant strains in
 15 droplets 6 and 14, shake–flask cultivation yielded the highest growth rate (Figure S5).
 16 Furthermore, the growth rate of mutants in droplets 6 and 14 was compared with the
 17 parent strain in shake–flask and MMC cultivation (Figure 6b). The results revealed
 18 that the mutants in both droplets exhibited higher growth rates and had a higher cell
 19 density in the stationary phase when cultivated either in shake flasks or in MMC.

20

21 **DISCUSSION**

22 We report the development of an automated, high–throughput microbial cultivation
 23 and adaptive evolution integrated platform called MMC that consists of four main
 24 parts, namely pumps and valves, reagent bottles, a droplet–manipulation fluidic chips,
 25 and microbial cell cultivation tubes, with novel designs (Figure 1), i.e., the sample–

1 injection unit and quick connector. In similar work by Baraban et al. (2011) and
 2 Jakiela et al. (2013), the cell suspension and reagents flowed through the pumps
 3 directly and then were pushed into the chip. Because cleaning and sterilizing the
 4 pumps in this configuration is difficult, such a method not only renders the droplets
 5 highly susceptible to contamination by other microorganisms but also adds difficulty
 6 to system cleaning. In our novel method of sample injection, the cell suspension and
 7 reagents do not flow through the pumps, and the reagent bottles are easily cleaned and
 8 sterilized. The quick connectors connect modularized parts in the system, provide
 9 flexibility for parts replacement, and allow for straightforward system modification
 10 and customization.

11

12 The MMC has a large linear range of OD₆₀₀ measurement (0 to ~11), which
 13 eliminates the need for sample dilution prior to measurement via UV–Vis
 14 spectrophotometry. In addition, the small effect of different species on the OD₆₀₀
 15 calibration curves indicates the great reproducibility of the OD₆₀₀ measurement
 16 system in MMC (Figures 3 and S3). These advantages enable continuous and in situ
 17 monitoring of microbial growth in MMC. Furthermore, it is possible to add
 18 fluorescence intensity–detection devices to MMC, enabling it to be used for analysis
 19 and sorting of microorganisms (Shen et al., 2019).

20

21 The concentration of inoculum is a crucial parameter in each sub–cultivation. In
 22 MMC, this parameter is determined by splitting and fusing droplets (Figure 2). When
 23 the concentration of inoculum was between 13.1% and 75.2%, the coefficient of
 24 variation (n = 50) was between 2.2% and 5.0% (Figure 4), indicating reproducible
 25 droplet manipulation in the MMC system. This reproducibly droplet manipulation

1 system improves parallelization of each droplet, thereby maximizing the accuracy and
2 validity of the results obtained.

3 The performance of microbial cultivation in MMC was investigated by growing six
4 species of microbial strains and obtaining growth curves for each species in
5 microliter-scale droplets (Figure 5). There are few reports on the growth of
6 microorganisms in microscale droplets. Therefore, our results are important not only
7 with respect to demonstrating the capability of long-term automatic cultivation in
8 MMC but also for expanding the application of MMC, e.g., for laboratory-scale
9 adaptive evolution. With the drawbacks of conventional cultivation techniques, i.e., a
10 low-throughput and labor-intensive process, high reagent consumption of shake
11 flasks, and poor mixing properties of well plates, MMC represents a great advantage
12 in microbial cell cultivation. Use of a pump with greater power enabled manipulation
13 of more droplets. The small volume of the reaction and the automated and
14 reproducible operation of MMC greatly reduces reagent and labor costs and improves
15 the parallelization of experiments. In a prior study, the maximum OD₆₀₀ of the
16 microbial cell cultures was less than 1 owing to gas-impermeability of the tube
17 (Jakiela et al., 2013). The Teflon tube in MMC and the high mass transfer rate of the
18 droplet system has improved microbial cell cultivation. In the present study, all six
19 species of microbial strains were cultivated to high density (Figure 5). Although
20 certain microbial strains, i.e., *S. cerevisiae* BY4741, *P. pastoris* Ppink-HC S-1 and *M.*
21 *extroquens* AM1, had a lower growth rate in MMC compared to shake flask in the
22 middle and late stages of cultivation, these parameters do not affect adaptive evolution
23 of these strains in MMC as cultivation of microorganisms to a late stage is not
24 required for adaptive evolution. Furthermore, the cultivation environment of MMC
25 can be further improved by implementing a tube-in-tube reactor system (Zhang et al.,

1 2017a). We can customize the gas atmosphere around the droplets, enabling
2 microorganisms to grow under conditions of higher oxygen concentration or
3 anaerobiosis according to experimental needs.

4

5 We also demonstrated adaptive evolution in MMC using the methanol-essential *E.*
6 *coli* strain MeSV2.2 as a model. Generally, the equipment for maintaining continuous
7 cultivation to sustain continuous evolution includes shake flasks, chemostat, an in-
8 vial continuous cultivation system, a microfluidic-based continuous cultivation
9 system, and a droplet-based continuous cultivation system (Tan et al., 2019b). MMC
10 possesses many advantages when compared with such equipment, e.g., reduction in
11 reagent consumption, high-throughput cultivation, and automated operation and high
12 parallelization. In addition, modularization provides flexibility to the system. Owing
13 to the great reliability of MMC, it is possible to conduct adaptive evolution
14 experiments across several months.

15

16 Our results obtained with MMC suggests that this system can be adapted to many
17 more applications in the future, such as the determination of the minimal inhibitory
18 concentration of antibiotics (Andrews, 2001; Baraban et al., 2011; Kirchhoff et al.,
19 2018), multi-factor optimization of microbial cultivation conditions (Ainala et al.,
20 2016; Guo et al., 2009), and high-throughput cultivation and screening of single-cell
21 droplets (Beneyton et al., 2017; Brouzes et al., 2009; Gruenberger et al., 2014). We
22 expect that MMC will be used as a multi-functional integrated platform owing to its
23 high throughput and automated operations.

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25

1

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22

Figure Legends

Figure 1. Schematic depiction of the MMC system. (a) The integrated framework for MMC. In the group of pumps and valves, there are six pumps (P1–6) and an electromagnetic valve (V1), which control the seven pipelines linked to the chip for droplet manipulation. In the group of reagent bottles, there are three special reagent bottles (B2, B4 and B6) containing the cell suspension (B2), culture medium (B4), and chemical factor (B6). The chip for droplet manipulation carries out the generation, cultivation, splitting, fusion, and sorting of droplets, which are identified and numbered at the recognition site of droplet (red dot). The optical density (OD) of each droplet is measured in real time at the detection site of droplet (green dot). The electrodes (orange dots) cause droplets to merge using an electrocoalescence mechanism. The tube for microbial cultivation is made of Teflon, which has good gas permeability. At certain pipeline joints, a quick connector is used for quick and easy connection of different pipelines. (b) Method for sample injection. In state O, the pump suctions oil from the oil bottle. In state I, the pump pushes the oil out, so the sample in the reagent bottle is pushed into the chip via the side tube. (c) Schematic of the recognition of droplets. The detection laser and the plane of the chip are at an angle of 36° . Owing to the difference in refractive index, when the droplet passes, the laser deviates more after passing through the droplet, then the photoelectric sensor below the chip receives more light and outputs a higher value of signal (>2 V). When the droplet is absent, the laser deviates less, and thus the photoelectric sensor receives less light and outputs a lower signal (<1 V). The length of the droplet and the interval between two adjacent droplets can be determined by the duration time of the signal. (d) Schematic of the monitoring of microorganisms in droplets. The halogen lamp,

1 optical fiber, and spectrometer are arranged such that, when the droplet comes to the
 2 detection site, the OD (the wavelength range of detection is 350 nm ~ 800 nm) inside
 3 the droplets is measured in real time. **(e)** Schematic of the structure of the quick
 4 connector and the gas exchange in droplets. Left: structure of a quick connector. Right:
 5 gas exchange in droplets. The tube for microbial cultivation (Teflon with good gas
 6 permeability) enables the exchange of gases inside and outside the droplets, thereby
 7 promoting the growth of microorganisms in the droplets.

8

9 **Figure 2. Schematic of the operations used to manipulate droplets on the chip. (a)**

10 Generation of droplets. Cell suspension from the reagent bottle is sheared at the T–
 11 junction by the oil phase coming from the vertical direction to form droplets. **(b)**
 12 Cultivation of droplets. After droplets are formed, they are cycled back and forth in
 13 the tube for microbial cultivation. Right–angled pipelines on the chip enhance the
 14 mixing of substances inside the droplets. At the monitoring site of growth, the OD is
 15 measured to monitor cell growth in real time. **(c)** Splitting of droplets. Droplets are
 16 sheared at the T–junction by the oil phase coming from the vertical direction to be
 17 split into the remaining droplet and a waste droplet. **(d)** Fusion of droplets. The
 18 remaining droplet is fused with a droplet containing fresh culture medium and
 19 chemical factor using an electrocoalescence mechanism and becomes a new droplet.
 20 In this system, the volume of the new droplet is the same as the droplet before
 21 splitting. **(e)** Sorting of droplets. The selected droplet proceeds to the tube for
 22 microbial cultivation, and the unselected droplet goes to the waste.

23

Figure 3. Calibration curves for OD₆₀₀ measurements in well plates and MMC. In each situation, i.e., well plate (inset) or MMC, calibration curves for the various dilutions of six different strains are determined and plotted against measurements of the same dilutions made with a UV–Vis spectrophotometer. Each calibration curve is shown in Figure S3 in detail. The calibration curves of the six strains were quite similar for both the well plates and MMC. The linear range of the measurement of OD₆₀₀ in MMC (0–11) is much greater than that in a well plate (0–6).

Figure 4. Characterization of the accuracy and reproducibility of inoculation in MMC. (a) Plot illustrating the measurement of the concentration of inoculum from seven experimental groups. The concentrations of inoculum in each experimental group were 13.1%, 21.7%, 32.0%, 41.8%, 51.9%, 64.8% and 75.2%. Fifty droplets were formed in each experimental group. (b) The coefficient of variation (C.V.) of 50 droplets in each experimental group in (a). The range of C.V. was 2.2% to 5.0%.

Figure 5. Growth curves for six strains cultivated in shake flasks, well plates, or MMC. For shake flasks and well plates, there were three parallel experimental groups. For MMC, 15 droplets were formed for every strain. All the growth curves are calibrated. (a) Comparison of MMC (black line) and shake flask (purple line). (b) Comparison of MMC (black line) and well plate (blue line).

Figure 6. Results of the adaptive evolution of MeSV2.2 in MMC. (a) Growth curves for MeSV2.2 by continuous sub–cultivation. The highest points of the growth curves in each sub–cultivation first fell and then rose overall, resulting in the valley

- 1 and peak in the figure. **(b)** Comparison of two selected strains and the initial strain.
- 2 For the strains in droplets 6 and 14, whether they were cultivated in MMC or in shake
- 3 flask, they all grew faster than the initial strain and had higher-OD stationary phases.

(a) Generation of droplets

(b) Cultivation of droplets

(c) Splitting of droplets

(d) Fusion of droplets

(e) Sorting of droplets

(a)

(b)