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Automated Microbial Cultivation and Adaptive Evolution using Microbial Microdroplet Culture System (MMC)

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TITLE:

Automated Microbial Cultivation and Adaptive Evolution using Microbial Microdroplet Culture System (MMC)

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SUMMARY:

This protocol describes how to use the Microbial Microdroplet Culture system (MMC) to conduct automated microbial cultivation and adaptive evolution. MMC can cultivate and sub-cultivate microorganisms automatically and continuously and monitor online their growth with relatively high throughput and good parallelization, reducing labor and reagent consumption.

ABSTRACT:

Conventional microbial cultivation methods usually have cumbersome operations, low throughput, low efficiency, and large consumption of labor and reagents. Moreover, microplate-based high-throughput cultivation methods developed in recent years have poor microbial growth status and experiment parallelization because of their low dissolved oxygen, poor mixture, and severe evaporation and thermal effect. Due to many advantages of micro-droplets, such as small volume, high throughput, and strong controllability, the droplet-based microfluidic technology can overcome these problems, which has been used in many kinds of research of high-throughput microbial cultivation, screening, and evolution. However, existing research mostly stays at the stage of laboratory construction and application. Some key issues, such as high operational requirements, high construction difficulty, and lack of automated integration technology, restrict the wide application of droplet microfluidic technology in microbial research. Here, an automatic Microbial Microdroplet Culture system (MMC) was successfully developed based on droplet microfluidic technology, achieving the integration of functions such as inoculation, cultivation, online monitoring, sub-cultivation, sorting, and sampling required by the process of microbial droplet cultivation. In this protocol, wild-type *Escherichia coli* (*E. coli*) MG1655 and a methanol-essential *E. coli* strain (MeSV2.2) were taken as examples to introduce how to use the MMC to conduct automated and relatively high-throughput microbial cultivation and adaptive evolution in detail. This method is easy to operate, consumes less labor and reagents, and has high experimental throughput and good data parallelism, which has great advantages compared with conventional cultivation methods. It provides a low-cost, operation-friendly, and result-reliable experimental platform for scientific researchers to conduct related microbial research.

INTRODUCTION:

Microbial cultivation is an important foundation for microbiological scientific research and industrial applications, which is widely used in the isolation, identification, reconstruction, screening, and evolution of microorganisms[1–3]. Conventional microbial cultivation methods mainly use test tubes, shake flasks, and solid plates as cultivation containers, combined with shaking incubators, spectrophotometers, microplate readers, and other equipment for microbial cultivation, detection, and screening. However, these methods have many problems, such as cumbersome operations, low throughput, low efficiency, and large consumption of labor and reagents. The high-throughput cultivation methods developed in recent years are mainly based on the microplate. But the microplate has a low level of dissolved oxygen, poor mixing property, and severe evaporation effect and thermal effect, which often lead to poor growth status and experiment parallelization of microorganisms[4–7]; on the other hand, it needs to be equipped with expensive equipment, such as liquid-handling workstations and microplate readers, to achieve automated cultivation and process detection[8,9].

As an important branch of microfluidic technology, droplet microfluidics has been developed in recent years based on traditional continuous-flow microfluidic systems. It is a discontinuous flow microfluidic technology that uses two immiscible liquid phases (usually oil-water) to generate dispersed micro-droplets and operate on them[10]. Because micro-droplets have the characteristics

of small volume, large specific surface area, high inside mass transfer rate, and no cross-contamination caused by compartmentalization, and the advantages of strong controllability and high throughput of droplets, there have been many kinds of research applying droplet microfluidic technology in high-throughput cultivation, screening, and evolution of microorganisms[11]. However, making droplet microfluidic technology popularized and widely applied still has a series of key issues. Firstly, the operation of droplet microfluidics is cumbersome and intricate, resulting in high technical requirements for operators. Secondly, droplet microfluidic technology combines optical, mechanical, and electrical components and needs to be associated with biotechnology application scenarios. It is difficult for a single laboratory or team to build efficient droplet microfluidic control systems if there is no cross-disciplinary collaboration. Thirdly, on account of the small volume of micro-droplet (from picoliter (pL) to microliter (nL)), it takes much difficulty to realize the precise automated control and real-time online detection of droplets for some basic microbial operations such as sub-cultivation, sorting, and sampling, and it is also difficult to construct an integrated equipment system[12].

In order to solve the above problems, an automatic Microbial Microdroplet Culture system (MMC) was successfully developed based on droplet microfluidic technology[13]. The MMC consists of four functional modules: a droplet recognition module, a droplet spectrum detection module, a microfluidic chip module, and a sampling module. Through the system integration and control of all the modules, automated operation system including the generation, cultivation, measurement (optical density (OD) and fluorescence), splitting, fusion, sorting of droplets is accurately established, achieving the integration of functions such as inoculation, cultivation, monitoring, sub-cultivation, sorting and sampling required by the process of microbial droplet cultivation. MMC can hold up to 200 replicate droplet cultivation units of 2–3 µL volume, which is equivalent to 200 shake flask cultivation units. The micro-droplet cultivation system can satisfy the requirements of non-contamination, dissolved oxygen, mixing, and mass-energy exchange during the growth of microorganisms, and meet the various needs of microbial research through multiple integrated functions, for instance, growth curve measurement, adaptive evolution, single factor multi-level analysis, and metabolite research and analysis (based on fluorescence detection)[13,14].

Here, the protocol introduces how to use the MMC to conduct automated and microbial cultivation and adaptive evolution in detail (**Figure 1**). We took wild-type *Escherichia coli* (*E. coli*) MG1655 as an example to demonstrate the growth curve measurement and a methanol-essential *E. coli* strain MeSV2.2[15] to demonstrate the adaptive evolution in MMC. An operation software for MMC was developed, which makes the operation very simple and clear. In the whole process, the user only needs to prepare the initial bacteria solution, set the conditions of the MMC, and then inject the bacteria solution and related reagents into the MMC. Subsequently, the MMC will automatically perform operations such as droplet generation, recognition and numbering, cultivation, and adaptive evolution. It also will perform the detection (OD and fluorescence) of the droplets with high time resolution online and display the related data (which can be exported) in the software. The operator can stop the cultivation process at any time according to the results and extract the target droplets for subsequent experiments. The MMC is easy to operate, consumes less labor and reagents, and has relatively high experimental throughput and good data

parallelism, which has significant advantages compared with conventional cultivation methods. It provides a low-cost, operation-friendly, and result-reliable experimental platform for researchers to conduct related microbial research.

PROTOCOL:

1. Instrument and software installation

1.1. Choose a clean and sterile environment (such as a clean bench) as a dedicated permanent space for MMC. Install the MMC steadily in the space.

NOTE: Keep the MMC away from the interference of strong electric fields, magnetic fields, and strong heat radiation sources. Avoid severe vibration from affecting the optical detection components. Provide the power supply of AC220 V, 50 HZ to the MMC. For details on MMC refer to the **Table of Materials** and the website for MMC[16].

1.2. Install the operation software from the MMC.zip file

NOTE: Contact the authors for the MMC.zip file.

1.2.1. Create a dedicated folder and save the zip file in it.

1.2.2. Create another dedicated folder as the “Installation Directory”. Unzip the MMC.zip and save the files in the new folder.

NOTE: The computer configuration is best to meet: (1) Windows 7 64-bit operating system or above; (2) CPU: i5 or above; (3) memory: 4 G or above; (4) hard disk: 300 G or above (rotational speed greater than 7200 rpm or solid-state disk).

2. Preparations

2.1. Connect the syringe needle (inner diameter is 0.41 mm and outer diameter is 0.71 mm), quick connector A, and reagent bottle (**Figure 2C**), and autoclave them at 121 °C for 15 min.

NOTE: Unscrew the cap of the reagent bottle slightly during sterilization. A few more reagent bottles can be prepared each time for use.

2.2. Use a 0.22 μm Polyvinylidene Fluoride (PVDF) filter membrane to filter MMC oil. Put the microfluidic chip (**Figure 2B**) and MMC oil into the clean bench in advance and sterilize them by ultraviolet irradiation for 30 min before use.

NOTE: For the details of Quick connector A, reagent bottle, MMC oil and microfluidic chip refer to the **Table of Materials**.

2.3. Install the microfluidic chip

2.3.1. Open the door of the operation chamber (**Figure 2A**) and lift the optical fiber probe.

2.3.2. Align the electric field holes with the electric field needles and gently place the chip on the chip pedestal. Then insert the two positioning columns into the positioning holes and put down the optical fiber probe (**Figure 2D**).

2.3.3. Connect the quick connector A on the chip to the corresponding port of the MMC according to the position number (C5—O5, C4—O4, C6—O6, C2—O2, CF—OF, C1—O1, C3—O3). Then close the door of the operation chamber.

2.4. Replenish the MMC oil (to about 80 mL) in the oil bottle and empty the waste liquid in the waste bottle before use.

NOTE: Pour the waste liquid into the organic waste liquid tank.

3. Growth curve measurement in MMC

3.1. Preparation for initial bacterial solution

3.1.1. Follow the related standard regulations to prepare Luria-Bertani (LB) medium and autoclave at 121°C for 15 min.

NOTE: Components of LB medium: NaCl (10 g/L), yeast extract (5 g/L) and tryptone (10 g/L).

3.1.2. Take out the *E. coli* MG1655 strain from glycerol stock and cultivate it in a 50 mL shake flask with 10 mL of LB medium in a shaking incubator (200 rpm) at 37 °C for 5–8 h.

NOTE: The cultivation time depends on the specific strains. It is optimal to cultivate the strain to the logarithmic period/phase.

3.1.3. Dilute the cultured *E. coli* MG1655 solution with the medium to an OD_{600} of 0.05–0.1 to obtain an initial bacteria solution (prepare about 10 mL).

3.2. Click on **Initialization** to initialize the MMC. After the initialization interface appears, set the cultivation temperature as 37 °C and the photoelectric signal value as 0.6 (**Figure 3A**). Initialization will take about 20 min.

3.3. Turn on the UV lamp (wavelength 254 nm) during initialization.

3.4. Inject the initial bacteria solution and MMC oil into the reagent bottle.

3.4.1. Take out a sterilized reagent bottle on the clean bench and tighten the cap.

3.4.2. Use a 10 mL sterile syringe to inject 3–5 mL of MMC oil from the syringe needle of the side tube. Tilt and rotate the reagent bottle slowly to make the oil fully infiltrate the inner wall.

3.4.3. Inject about 5 mL of initial bacteria solution, and then fill the reagent bottle by injecting 5–7 mL of the oil again.

3.4.4. Pull out the independent quick connector A, and insert the quick connector A of the reagent bottle into its quick connector B to complete the sample injection operation (**Figure 4A**).

3.5. Wait for the initialization to end and then turn off the UV lamp (wavelength 254 nm).

3.6. Open the door of the operation chamber, and put the reagent bottle into the metal bath.

3.7. Pull out the C2 connector of the chip and the quick connector A of the reagent bottle. Connect the side tube connector of the reagent bottle to the C2 connector and the top tube connector to the O2 connector. Then close the door of the operation chamber.

3.8. Click on **Growth Curve** to choose the function of growth curve measurement (**Figure 3A**). In the parameter setting interface, input the **Number** as 15, turn on the **OD detection** switch and set the **Wavelength** as 600 nm. Click on **Start** to start droplet generation. It will take about 10 min.

NOTE: Here, **Number** refers to the number of droplets to be generated. **Wavelength** refers to the wavelength of the OD to be detected. Set the **Number** (maximum 200) and **Wavelength** (350–800 nm) according to the experiment requirements.

3.9. When a pop-up window appears on the main interface prompting “Remove the reagent bottle between C2 and O2, then please click the OK button after completion”, open the door of the operation chamber to take out the reagent bottle and connect the C2 and O2 connectors.

3.10. Close the door, and click the **OK** button in the pop-up window to automatically cultivate the droplets and detect the OD values.

NOTE: The MMC detects the OD value when the droplet passes the optical fiber probe. Therefore, the detection period depends on the number of droplets generated.

3.11. When the growth curve reaches the stationary phase, click the **Data Export** button to export the OD data. Select the data save path and export the OD value recorded during the cultivation period in the .CVS format, which can be opened by appropriate software (e.g., Microsoft Excel). Then use a mapping software (e.g., EXCEL and Origin 9.0) to plot the growth curve.

NOTE: During the cultivation process, it is feasible to click on the **Data Export** at any time to export the OD data of all the current droplets.

4. Adaptive evolution in MMC

4.1. Preparation for initial bacterial solution

4.1.1. Follow the related standard regulations to prepare the special liquid medium and solid plates for the MeSV2.2 and autoclave at 121 °C for 15 min.

NOTE: For the components of the special medium refer to **Table 1** and the **Table of Materials**.

4.1.2. Cultivate the MeSV2.2 using the solid plate (diameter = 90 mm) in a 37 °C constant temperature incubator for 72 h. Then pick an independent colony and cultivate it in a 50 mL shake flask with 10 mL of the special liquid medium in a shaking incubator (200 rpm) at 37 °C for 72 h.

4.1.3. Dilute the cultured MeSV2.2 solution with the medium to an OD_{600} of 0.1–0.2 (ensure that the total volume is not less than 10 mL) and continue cultivating it in the shake flask for 5 h to obtain the initial bacteria solution.

NOTE: The MeSV2.2 is a methanol-essential *E. coli* strain. The special liquid medium contains 500 mmol/L methanol, which is a strong stress for MeSV2.2, resulting in very slow growth. Note that obtaining the initial bacteria solution here is different from that described in step 3.1.

4.2. Initialize the MMC as explained in steps 3.2, 3.3, and 3.5.

4.3. Take out two sterilized reagent bottles, one of which is for the initial bacteria solution and the other is for the fresh medium. Inject the initial bacteria solution (5 mL), fresh medium (12 – 15 mL), and MMC oil into the reagent bottles as explained in step 3.4.

NOTE: Because adaptive evolution is a long-term process involving multiple sub-cultivations, store as much fresh medium as possible in MMC. The medium cannot be replenished during the experiment running.

4.4. Install the two reagent bottles into MMC as explained in step 3.6. Install the one for the initial bacteria solution between the C2 and O2 connector and the other for the fresh medium between the C4 and O4 connector.

4.5. Click on **ALE** to choose the function of adaptive evolution (**Figure 3B**). In the parameter setting interface, turn on the **OD Detection** switch.

4.6. Set the **Number** as 50, **Wavelength** as 600 nm, **Concentration** as 0%, **Type** as Time, **Parameter** as 30 h, and **Repetitions** as 99. Click on **Start** to start droplet generation. It will take about 25 min.

NOTE: Here, “Concentration” refers to the maximum concentration of chemical factors for

adaptive evolution. For different droplets, it is realizable in MMC to introduce different concentrations of chemical factors to provide different growth conditions. Calculate the introduced concentrations using the following equation:

$$C = a + \frac{b-a}{7} \times i, \quad i = 1, 2, \dots, 7$$

Here "*C*" refers to the concentration of chemical factors introduced into droplets; "*a*" refers to the concentration of chemical factors in the reagent bottles between the C4 and O4 connector; "*b*" refers to the concentration of chemical factors in the reagent bottles between the C6 and O6 connector; and "*i*" refers to the available concentration. There are eight concentrations for selection in MMC. Since the chemical factor here has a single concentration (500 mmol/L methanol) and it is one of the ingredients of the medium, only one reagent bottle containing the chemical factor is installed here, and the **Concentration** is set as 0%. **Type** refers to the mode of sub-cultivation, which is divided into three types: time mode, OD value mode, and fluorescence mode. The former means to cultivate the droplets for a fixed time and then sub-cultivate, while the latter two means to cultivate the droplets to a set OD value/fluorescence intensity and then sub-cultivate. **Parameter** refers to the related parameter required when choosing a mode of sub-cultivation. **Repetitions** refers to the number of sub-cultivations.

4.7. Remove the reagent bottle placed between the C2 and O2 connector as explained in step 3.8.

4.8. Observe whether the maximum OD values of the droplets during each sub-cultivation period have increased significantly. If the increase occurs and meets the experiment requirements, click on the **Data Export** button to export the OD data as explained in step 3.9.

NOTE: Here, the sub-cultivation period is related to the **Parameter**. For example, when setting the **Type** as Time and **Parameter** as 30 h, the sub-cultivation period is 30 h. During each sub-cultivation period, there are the maximum OD values of the droplets. Estimate whether the adaptive evolution meets the experiment requirements by the increase of maximum OD values (The increase depends on the actual cultivation process of the strain, for example, increased by more than 20%).

CAUTION: Pay attention to whether the stored fresh medium is exhausted. If the significant increase has not occurred even after the medium is exhausted, extract the better-growing droplets and carry out a new round of adaptive evolution.

4.9. Extract the target droplets from the MMC.

4.9.1. Click on the **Screening** button to choose the function of droplet extraction (**Figure 3C**). Choose the **Collect** option, click the numbers of target droplets, and then click on **OK**.

NOTE: Droplet screening includes "Collect", "Discard" and "Extract seed solution". "Extract seed solution" means to collect the remaining droplets[13] after the sub-cultivation operation.

4.9.2. Wait for the pop-up window to prompt, "Please pull out the CF quick connector and put it into the EP tube". Put the CF quick connector into the microcentrifuge tube for collection according to the software prompt and then click on **OK** (**Figure 4D**).

4.9.3. After 1–2 min, the software interface will pop up a new window prompting, "Please insert the connector back and click OK if finished". Then, insert the CF quick connector back and click on **OK** to make MMC continue to run (**Figure 4D**). When the next target droplet reaches the droplet recognition site, repeat 4.9.2–4.9.3 to collect it.

NOTE: After all the target droplets are collected, the MMC will continue cultivating the remaining droplets. If the cultivation is not necessary, click on **Stop** to directly terminate the operation.

4.9.4. Take out the droplet using a 2.5 µL pipette, drop it on the solid plate with a diameter of 90 mm, and spread it evenly with a glass triangular coated rod with a side length of 3 cm. Then cultivate it in a 37 °C constant temperature incubator for 72 h.

4.9.5. Pick 3–5 independent colonies and separately cultivate them in the 50 mL shake flasks with 10 mL of fresh medium in a shaking incubator (200 rpm) at 37 °C for 48–72 h. Follow the related standard regulations to store the cultured bacteria solution in the glycerol tube for subsequent experiments.

5. Clean of the MMC

5.1. After completion of the experiment, click on **Stop** to stop all the operations. Then click on **Clean** to clean the chip and tubes. It will take about 15 min.

REPRESENTATIVE RESULTS:

This protocol uses *E. coli* MG1655 and a MeSV2.2 strain as examples to demonstrate the microbial cultivation and methanol-essential adaptive evolution with an automated and relatively high-throughput strategy in MMC. The growth curve measurement was mainly used to characterize microbial cultivation. The adaptive evolution was conducted by automated continuous sub-cultivation and adding a high concentration of methanol as the selective pressure during each sub-cultivation. Whether adaptive evolution had been realized was estimated through the variation trend of the maximum OD value of the droplets during each sub-cultivation period. The tunable parameters and accuracy parameters of MMC are shown in **Table 2**.

Results of growth curve measurement

The OD_{600} values of the 15 droplets detected during the cultivation process were exported from the MMC after cultivating for about 20 h (**Figure 5A**). It can be seen that the detection was conducted approximately every 14 min. This detection period depends on the number of droplets generated because the droplets are cycled back and forth in the tubes for cultivation, and the MMC only detects the OD values (the detection and calculation of OD value are shown in **Supplementary Figure 1**) when the droplets pass the optical fiber probe. Therefore, the 14 min is a very short detection period, providing a high time resolution detection process to reflect the

growth of microorganisms more accurately.

According to the exported data, the average OD_{600} values and standard deviation (SD) of 15 droplets at each time point were calculated, and the growth curve of *E. coli* MG1655 was plotted (**Figure 5B**). The results show that the growth curve presents an "S" shape, including lag phase, logarithmic phase, and stationary phase, which is very consistent with the classic microbial growth model. At the same time, the standard deviations of 15 droplets are very small, indicating that the growth consistency and parallelism are good. Thus, it fully demonstrates the good microbial cultivation and detection performance of MMC. Moreover, it was also verified that there is little crosstalk between droplets during cultivation (**Supplementary Figure 2** and **Supplementary Table 1**).

Results of adaptive evolution

We have performed a long-term adaptive evolution of MeSV2.2 in MMC. On the 18th day, according to the increasing trend of the maximum OD_{600} values of the droplets during each sub-cultivation period from the growth curves displayed on the software interface, we believed that a good adaptive evolution was achieved in the 50 droplets. The OD_{600} data was exported and 8 droplets (including droplet 6) with relatively good growth performance were extracted[13]. **Figure 6A** shows the growth curves of 50 droplets in the whole adaptive evolution process. In 18 days, MMC automatically carried out 13 sub-cultivation operations. It can be seen from **Figure 6A** that MeSV2.2 grows slowly first and fast afterward, which indicates the track of adaptive evolution in MeSV2.2. To supply a selection pressure, the methanol was added to the MeSV2.2 medium. Initially, methanol inhibited cell growth. After the adaptive evolution, the enriched cells adapted to methanol had a higher growth rate. The growth curve of droplet 6 in the whole adaptive evolution process was plotted separately (**Figure 6B**). The maximum OD_{600} values in the first generation and last sub-cultivation period were 0.37 and 0.58, respectively, with a 56.8% increase. It indicates that the strain in droplet 6 has realized an obvious adaptive evolution.

Subsequently, droplet 6 strain and the initial strain in shake flasks were cultivated, and their growth curves were compared (**Figure 6C**). According to the methods given in the literature[17,18], the maximum specific growth rates (μ_{max}) of the droplet 6 strain and the initial strain were calculated, which were 0.096 h^{-1} and 0.072 $h^{-1,}$ respectively. **Figure 6C** reveals that the droplet 6 strain exhibited a higher maximum specific growth rate (increasing by 54.8%) and had a higher cell concentration in the stationary phase (increasing by 20.0%) than the initial strain when cultivated in shake flasks, which further proved that the adaptive evolution in MeSV2.2 has realized.

FIGURE AND TABLE LEGENDS:

Figure 1: Overall workflow of growth curve measurement and adaptive evolution in MMC. (**A**) Growth curve measurement in MMC. Firstly, cultivate the strain in shake flask to prepare the initial bacterial solution. Then, inject the initial bacteria solution into the reagent bottle. Next, generate the droplets in MMC. MMC makes the droplets cycle back and forth in the microfluidic chip and tubes to cultivate them. When droplets pass the detection site, the OD data will be detected and recorded. Finally, export the data for analysis. (**B**) Adaptive evolution in MMC. Pick

a single colony from the solid plate and cultivate it in a shake flask to prepare the initial bacterial solution. After injecting the initial bacteria solution into the reagent bottle, conduct the adaptive evolution in MMC. Adaptive evolution involves continuous sub-cultivation, which can be automatically operated through droplet splitting and fusion. After the adaptive evolution, export the data for analysis. Target droplets can be extracted and then spread on the plate to obtain single colonies.

Figure 2: Structure and essential tools of MMC. (**A**) External and operation chamber of MMC. (**B**) The microfluidic chip of MMC. The chip has seven channels (C1–C6 and CF). (**C**) Reagent bottle. It has a top tube and a side tube. Before injecting the sample into the reagent bottle, it needs to connect a syringe needle to a quick connector A first and then connect the quick connector A to the side tube. **(D**) Installation of the microfluidic chip. The microfluidic chip is installed on the pedestal. Then the seven channels (C1–C6 and CF) are respectively connected to the corresponding ports of MMC (O1–O6, and OF).
1 — Operation chamber of MMC.
2 — Oil bottle containing the MMC oil.
3 — Waste bottle for collecting waste liquid.
4 — UV lamp (wavelength 254 nm) for sterilization. This lamp can be turned on in advance to sterilize the chip and tubes.
5 — Laser (620 nm) for droplet recognition. The point where the laser is irradiated on the chip is the droplet recognition site.
6 — Temperature probe to measure the temperature inside the operation chamber.
7 — Heater for the operation chamber. It can be used to maintain the temperature of microbial cultivation. The range of temperature that can be set is 25 ± 0.5 °C to 40 ± 0.5 °C.
8 — Optical fiber probe to measure the OD or fluorescence of droplets.
9 — Chip pedestal to install the Microfluidic chip.
10 — Metal bath to fix the reagent bottles and heat them to quickly raise the temperature of a reagent to the temperature of microbial cultivation.
11 — Ports for the microfluidic chip (O1–O6, and OF). The microfluidic chip is connected to the MMC through these ports.
12 — Tubes for droplet storage and cultivation.
13 — Magnet blocks to quickly locate the microfluidic chip during installation.
14 — Syringe needle to inject the samples into the reagent bottles. Its inner diameter is 0.41 mm, and its outer diameter is 0.71 mm.
15 — Quick connector A. Connect with quick connector B.
16 — Quick connector B.

Figure 3: Operation software interface of MMC. (**A**) The main interface of the software. (1) Temperature in the operation chamber. (2) Photoelectric signal value of droplet recognition. When the droplet passes, the signal value is high (>2 V). When the oil passes, the signal value is low (<1 V). (3) Function selection. There are four functions to choose from: growth curve measurement (Growth Curve), adaptive laboratory evolution (ALE), single factor multi-level analysis (One-factor) and customizing the operations according to experimental needs (Customization). (4) Parameter setting interface. Set the corresponding experimental parameters

here after choosing one function. (5) Command run area. (6) Switch of camera. The camera is installed directly above the chip, which can online observe the droplets in the chip. (7) Process display area. Shows the running time, monitoring data, and the operation being executed. (**B**) The parameter setting interface of adaptive evolution. (**C**) The droplet screening interface. The MMC can automatically number the droplets. Here the target droplets can be selected and extracted from the MMC. (**D**) Camera observation interface.

Figure 4: Sample injection, droplet generation, and droplet extraction. (**A**) The reagent bottle after the injection of bacteria solution and MMC oil. Both the bacteria solution and MMC oil are injected from the side tube. The oil phase is in the upper layer and the bacteria solution is in the lower layer. After the injection, connect the quick connector A and B, and then install it into the MMC. (**B**) Droplet generation in the microfluidic chip. In order to facilitate the observation of droplets, a red pigment solution was used to demonstrate the process of droplet generation. (**C**) Droplet stored in the tube observed by microscope. Scale bar: 400 μm. (**D**) Pop-up window prompts and the corresponding operations. When the prompt " Please pull out the CF quick connector and put it into the EP tube" appears, pull out the CF connector and put it into the EP tube to collect the target droplet; when the prompt " Please insert the connector back" appears, the droplet collection is complete, insert the CF connector back into the OF port.

Figure 5: Data export and figure plotting of growth curve. (**A**) Screenshot of part of the exported data. The exported data include each detection time point of the 15 generated droplets and the corresponding OD_{600} values. (**B**) Growth curve of *E. coli* MG1655 plotted based on the exported data. Calculate the average OD_{600} values and standard deviation (SD) of 15 droplets at each time point and plot the growth curve. It is clear to see that this growth curve includes the lag phase, logarithmic phase, and stationary phase.

Figure 6: Results of the adaptive evolution of MeSV2.2 in MMC. (**A**) Growth curves of 50 droplets in the whole adaptive evolution process. The OD_{600} detection data of 50 droplets during the 18-day adaptive evolution process were exported from the MMC and plotted. On the 18th day, 8 droplets, including droplet 6 were extracted. (**B**) Growth curve of the droplet 6 in the whole adaptive evolution process. The maximum OD_{600} values in the first generation and last sub-cultivation period were 0.37 and 0.58, respectively, with a 56.8% increase. (**C**) Comparison of droplet 6 strain and the initial strain in the shake flask. The strain of droplet 6 and the initial strain were cultivated in shake flasks, and the growth curves (including SD, n = 3) were measured. This figure has been modified from Jian X. J. et al.[13].

Table 1: Components of the special medium for MeSV2.2.

Table 2: Tunable parameters and accuracy parameters of MMC. Tunable parameters refer to the parameters that can be adjusted according to the specific requirements of users; accuracy parameters refer to the parameters that reflect the accuracy and reproducibility of the different fluidic operations.

Supplementary Figure 1: Recognition and detection of droplets in MMC. (**A**) The waveform of a

droplet in MMC. This waveform comes from the raw spectral data of the MMC spectrometer. After processing the raw spectral data in the background, MMC will give the measured OD value. (**B**) OD calculation of droplets in MMC. In the waveform of the droplet, 'a' represents the maximum length of the droplet, 'c' represents the arc-shaped interface formed by oil phase and water phase, and 'b' represents the main part of the droplet. Based on the Lambert-Beer law, the OD value of the droplet is calculated using the following formula: OD value = lg(E /D) x 10. 'E' refers to the average spectral signal value of oil phase; 'D' refers to the average spectral signal value of the main part b of the droplet. It should be noted that the OD value measured by MMC is different from that measured by a spectrophotometer.

Supplementary Figure 2: **Test of crosstalk between the droplets.** To verify whether there is crosstalk between the droplets during the long-term cultivation, the E. coli MG1655 solution was diluted to a very low concentration (according to Poisson distribution, λ = 0.1), and then 200 droplets were generated and cultivated for 5 days. After measuring the OD, it was found that the E. coli MG1655 grew in a small number of droplets. And there was almost no bacterial growth in the droplets around these droplets. The result also preliminarily shows that there is little crosstalk between droplets.

Supplementary Table 1: Stability of droplet generation in MMC. As shown in **Supplementary Figure 1**, the droplet has a fixed waveform. The spectrometer of MMC generates a certain number of data points per second, so the number of data points of the droplet waveform can reflect the size of the droplet. The red dye solution was used to generate 397 droplets in the MMC, and the OD value was measured. The raw spectral data was exported, the data points of each droplet waveform were counted, and the coefficient of variation (C.V) of the droplet data points was calculated.

Supplementary Table 2: Droplet evaporation in MMC. Here the red dye solution was used to generate droplets in the MMC and the droplets were stored in the cultivation tube. The tube was then placed in a 37 °C constant temperature incubator for 30 days, and the droplet length was regularly measured (take photos under a microscope and measure the length with a scale bar). It shows that the volume of the droplet was reduced by about 12.3% after 30 days, which indicates that the evaporation of the droplet is very small in MMC.

DISCUSSION:

This protocol presents how to use the Microbial Microdroplet Culture system (MMC) to perform automated microbial cultivation and long-term adaptive evolution. MMC is a miniaturized, automated, and relatively high-throughput microbial cultivation system. Compared with conventional microbial high-throughput cultivation methods and instruments, MMC has many advantages such as low labor and reagent consumption, simple operation, online detection (OD and fluorescence), high-time-resolution data collection, and superior parallelization. MMC also has some special advantages different from the conventional droplet microfluidic technology, which usually uses the pL and nL droplets. Most previously reported systems that used pL and nL droplets have poor cultivation performance and few detectable parameters (usually only fluorescence)[18–21]. Although there have been some platforms that can achieve better cultivation

performance and multiple parameter detection, it is difficult and requires a lot of effort. For example, some researchers reported the OD detection of pL droplets. It is based on image recognition, which has not only false positives but also needs further verification of accuracy[22]. However, MMC can accomplish these things relatively simply. MMC uses microliter (μL) droplets that are rarely reported. The superior microbial cultivation performance of MMC has been verified, and it can also directly detect OD and fluorescence. Due to the large volume of the μL droplets, the droplet generation is less susceptible to interference, which has higher stability. Meanwhile, more diverse operations can be performed in the microliter droplets, conducive to the realization of automated operations. Furthermore, because the droplets are enclosure spaces, the volatility of the contents can be suppressed (**Supplementary Table 2**), conducive to performing the long-term microbial cultivation and adaptive evolution when volatile substances exist in the medium[14]. This is difficult to achieve in shake flasks and microplates.

However, certain critical points in the protocol are worth emphasizing. Firstly, it should be noted that the OD value measured by MMC is different from that of a spectrophotometer because their optical path lengths of OD measurement are different (1 mm and 10 mm, respectively). Therefore, when comparing the OD value of MMC with that of shake flask, it is necessary to measure the calibration curve[13]. Fortunately, the adaptive evolution process does not require calibration curves because we focus on the relative trends among the growth curves. Next, not all microorganisms can be cultivated using MMC. The droplets rely on the surface tension of the oil-water interface to maintain stability[23]. If the microorganisms produce certain substances that destroy the surface tension of the oil-water interface, such as some *Bacillus subtilis* strains producing surfactants[24], the droplets cannot maintain stability. Furthermore, if the medium itself is an obstacle to the generation of droplets, it is not viable to be used in MMC, for example, the medium is very viscous or contains large solids. At present, the species we have successfully cultivated in MMC includes *E. coli*, *Lactobacillus plantarum*, *Corynebacterium glutamicum*, yeasts, *Methylobacterium extorquens*, *Aspergillus oryzae*, microalgae and so on. It is recommended to cultivate the strain in MMC in advance for a test. Finally, connect the connectors and ports between the chip, the reagent bottle, and the MMC in strict accordance with the protocol; otherwise, the bacteria solution may flow into the MMC and contaminate the interior. Additionally, it must be pointed out that the current throughput of MMC is not very high (0–200). That is because if there were too many droplets, the present automated operations of sub-cultivation would take a long time. In the future, we will optimize the control software and the size of the chip to shorten the time and improve the throughput. Since MMC is a modular system, only related parts or software need to be replaced without the requirement of new equipment.

At present, MMC can not only conduct growth curve measurement, adaptive laboratory evolution, and single-factor multi-level analysis but also customize programming according to experimental needs to set up different droplet operation procedures. In the future, it is necessary to further enrich the application functions of the MMC system in response to the different needs of microbial research, such as conducting the multi-factor multi-level orthogonal experiments, multi-sample automatic sampling technology to simultaneously measure the growth curves of multiple bacterial species, and accurately detect and control more parameters (e.g., dissolved oxygen (DO) and pH). At the same time, it is also necessary to develop more functions in the field

of microbiology to apply MMC to more practical scenarios, such as optimization of medium compositions, determination of minimum inhibitory concentration (MIC), co-cultivation of microorganisms[25], etc.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

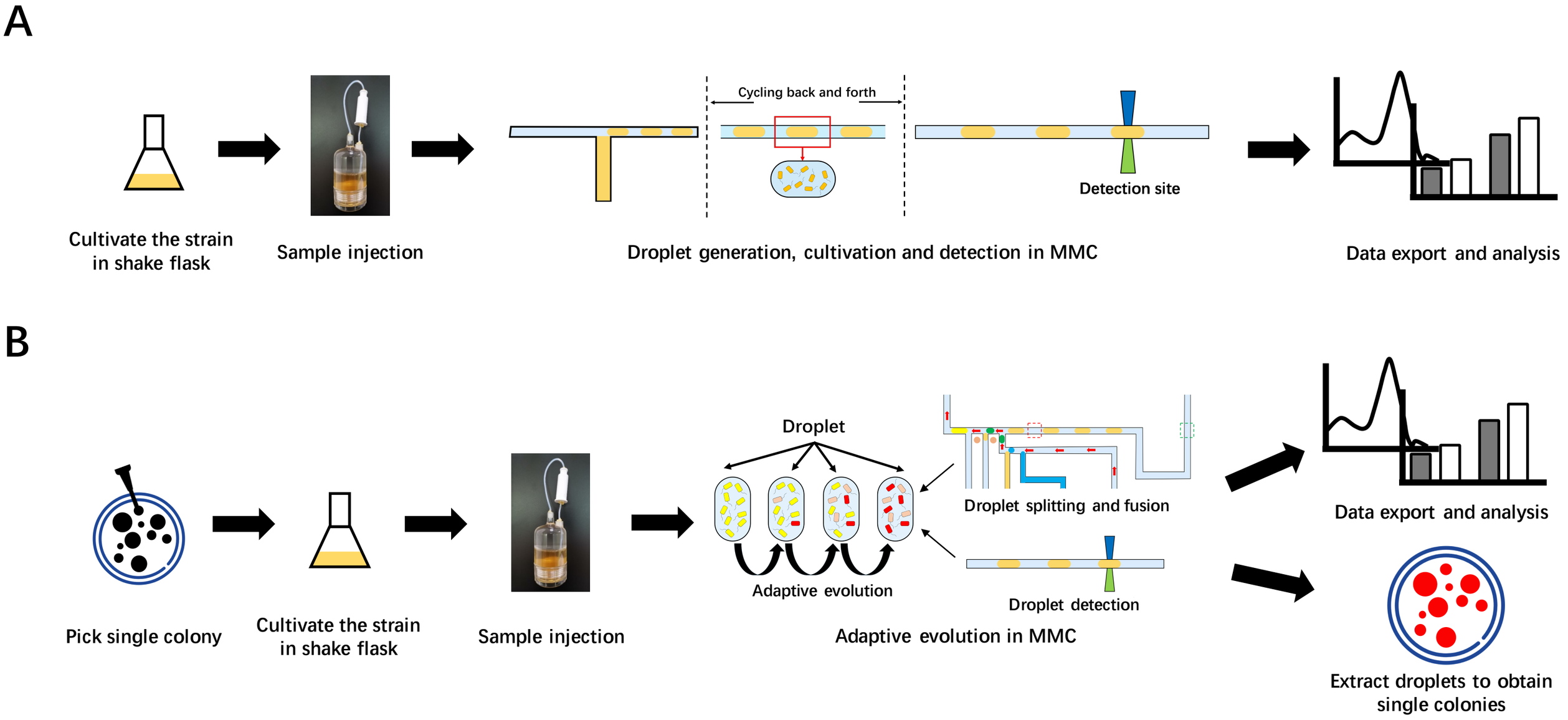

Figure 2

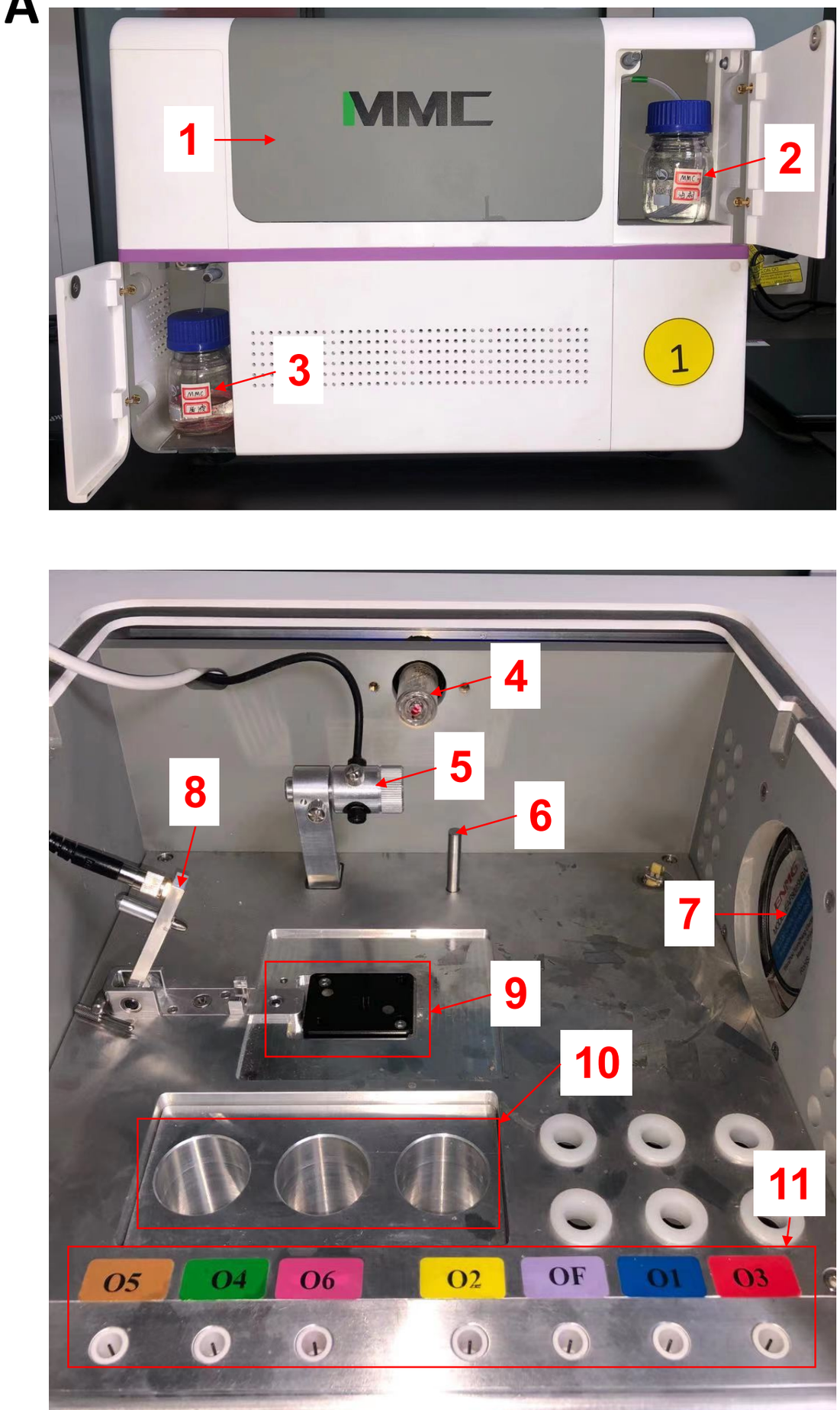

Figure 3

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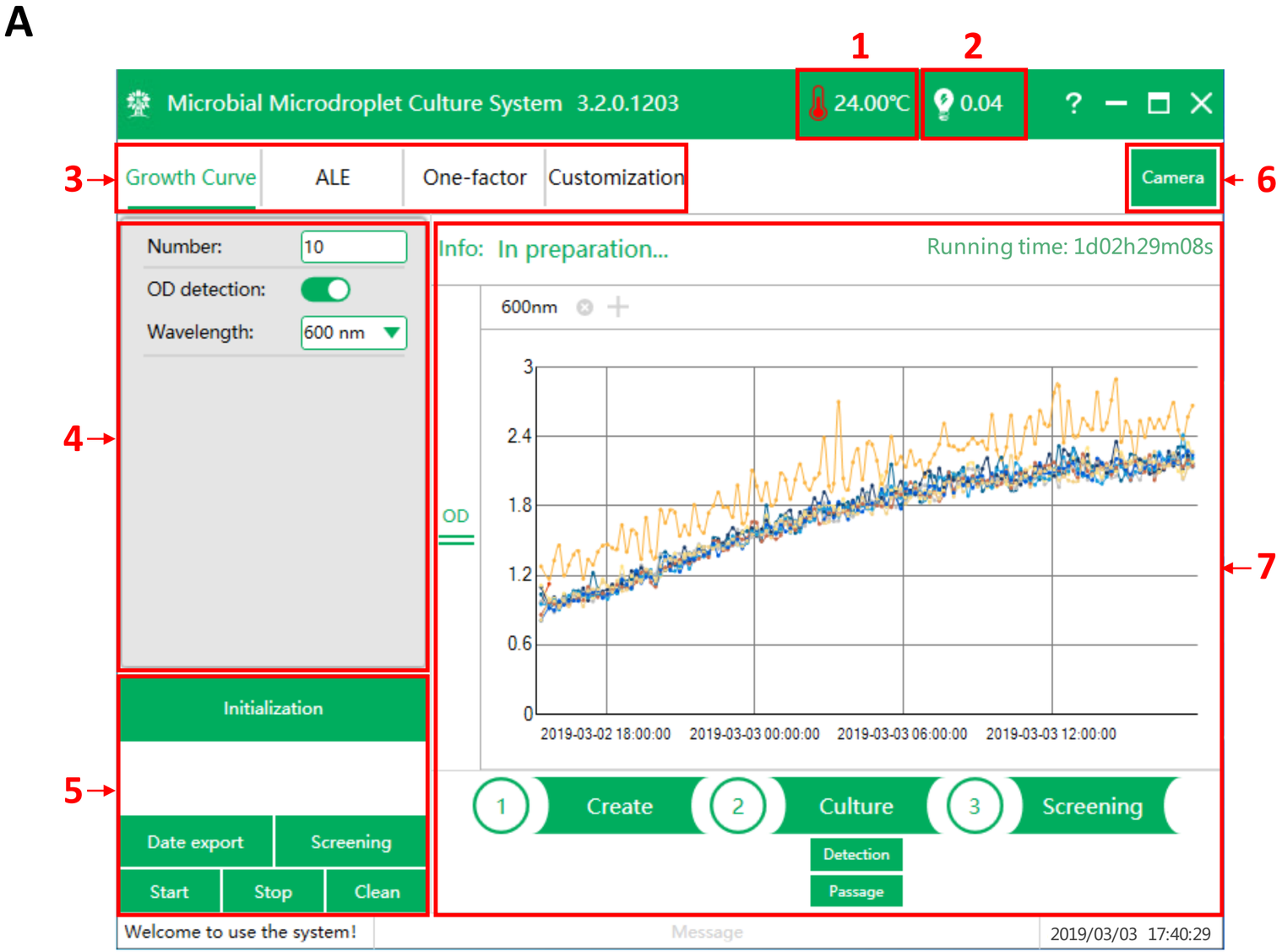

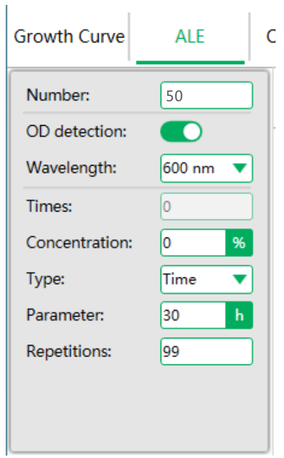

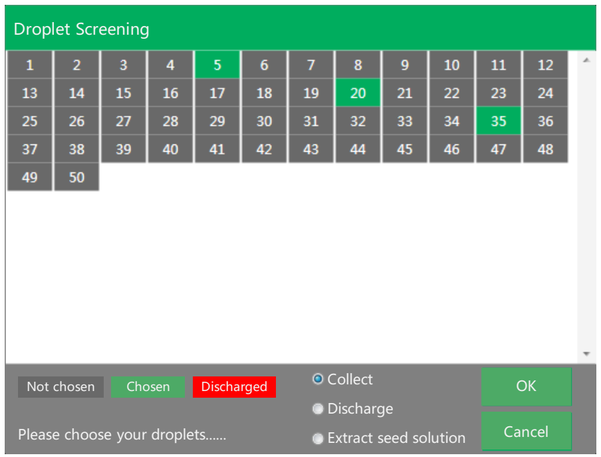

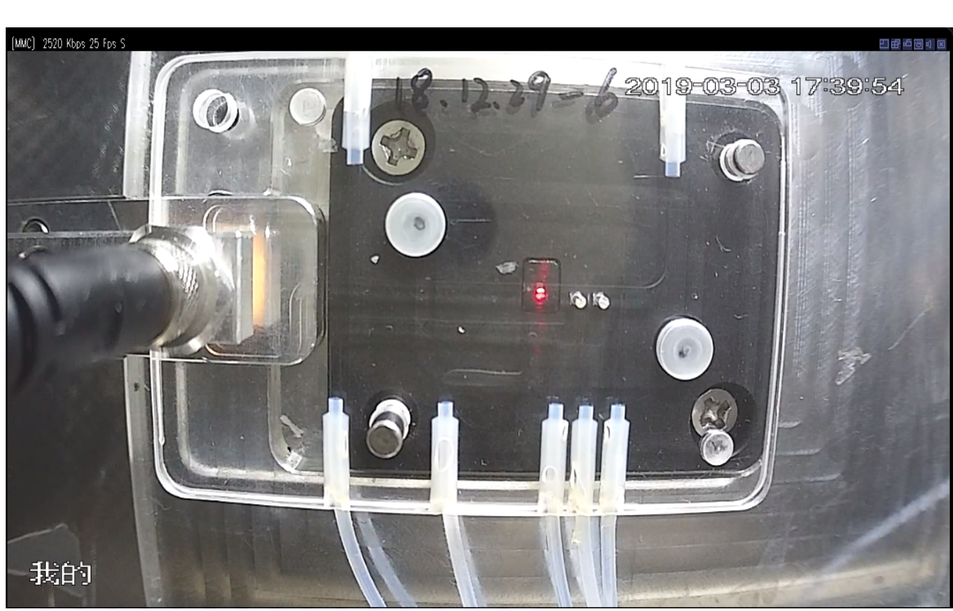

Figure 4

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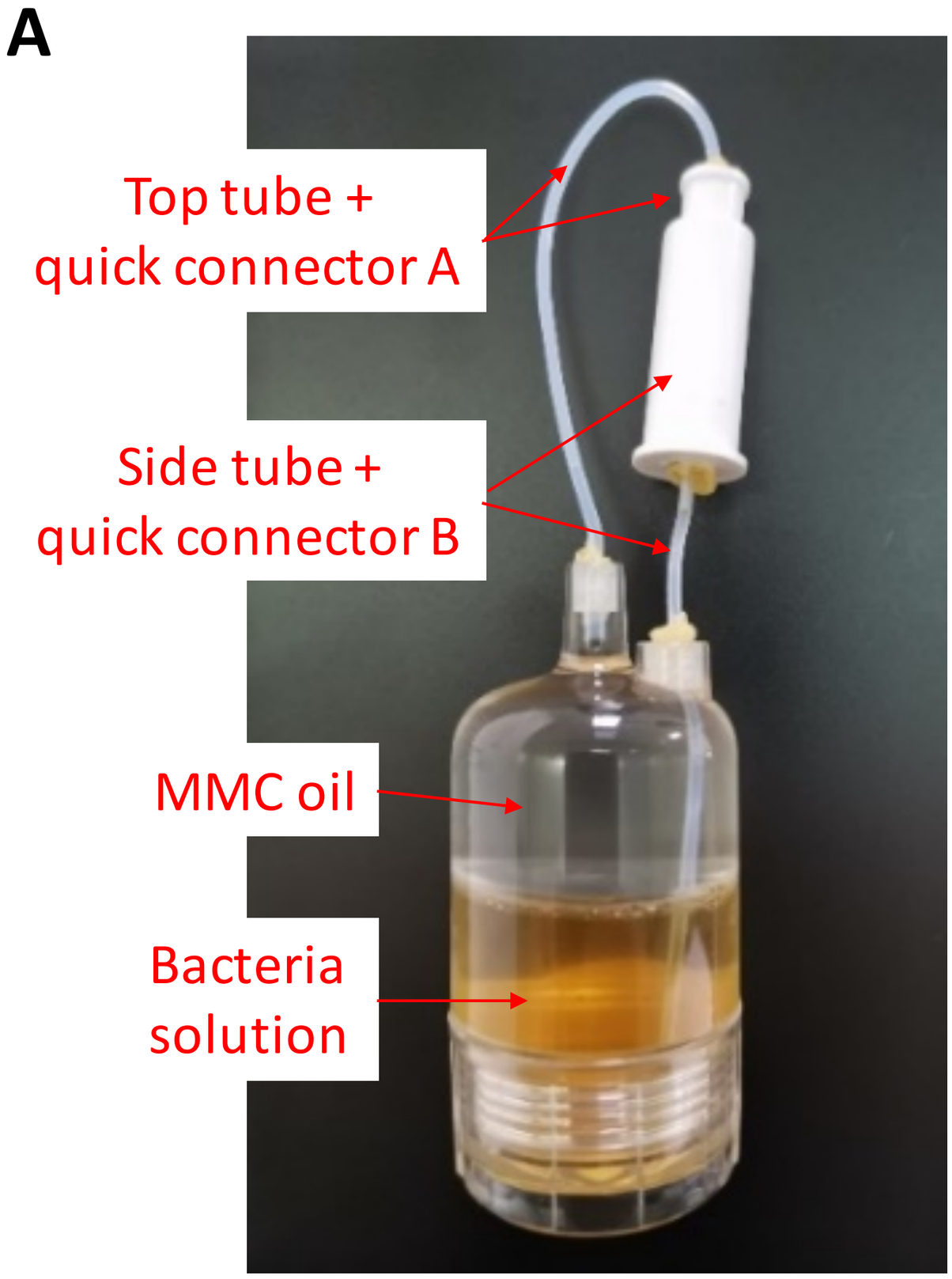

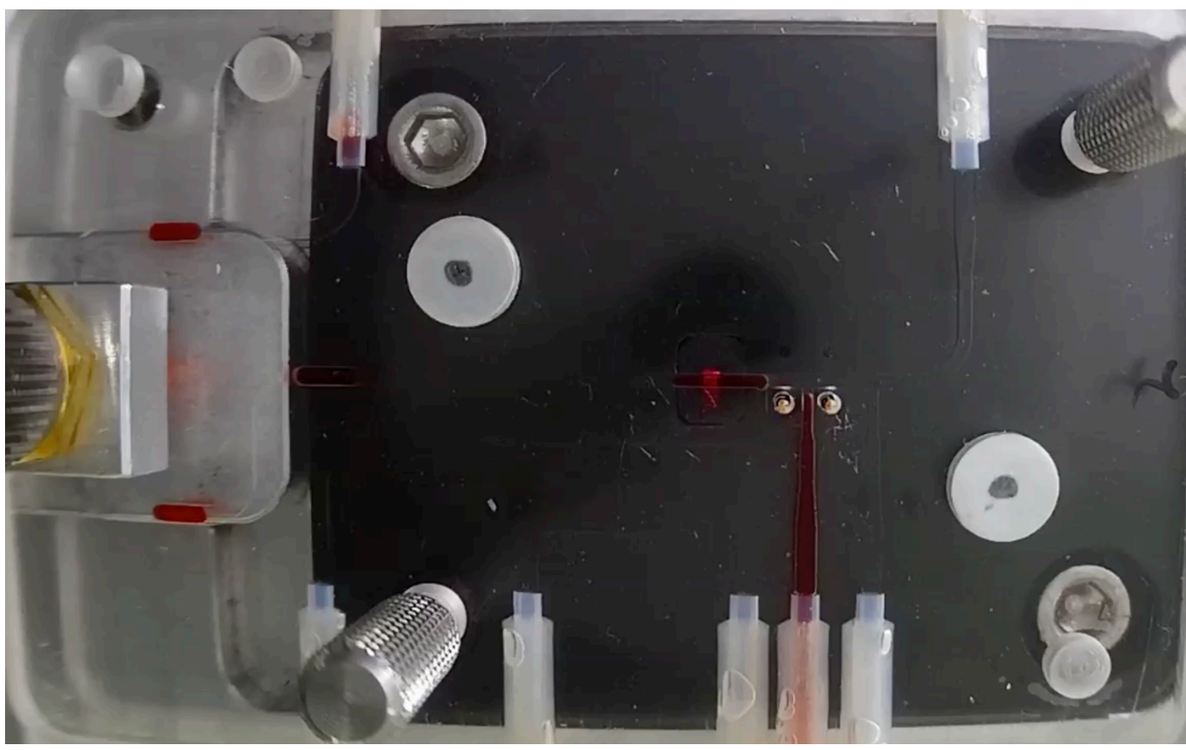

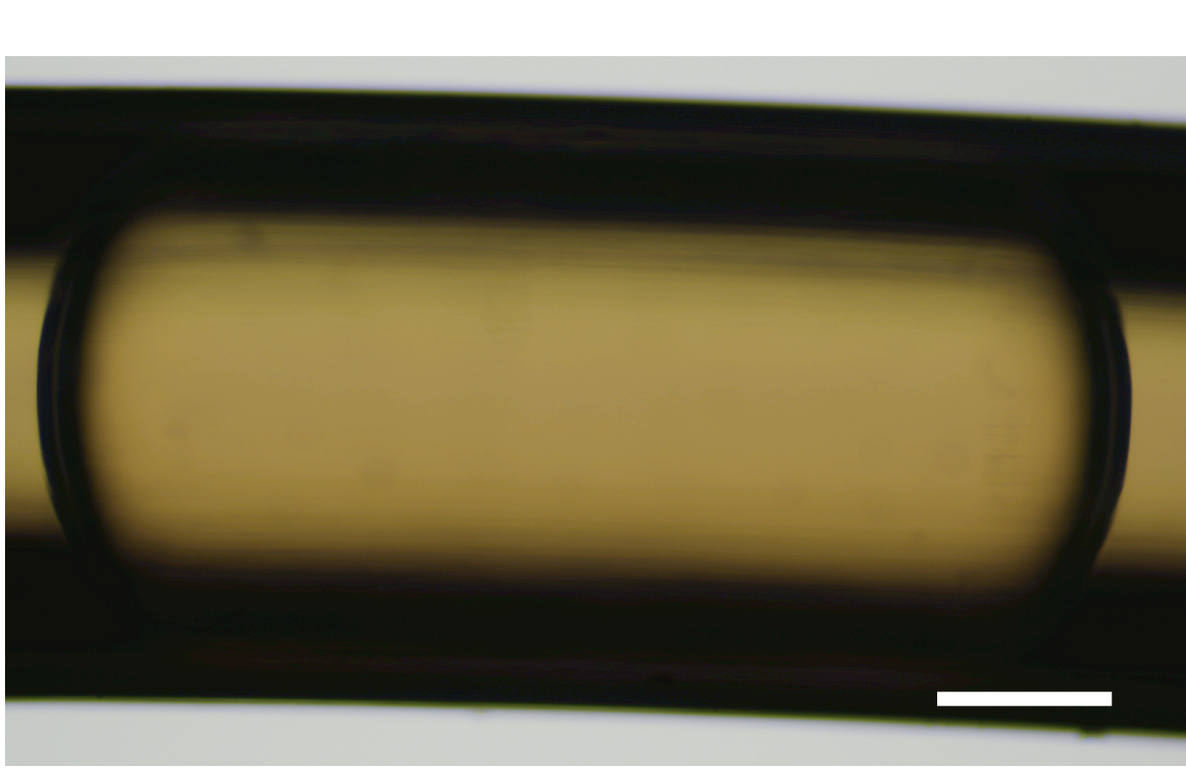

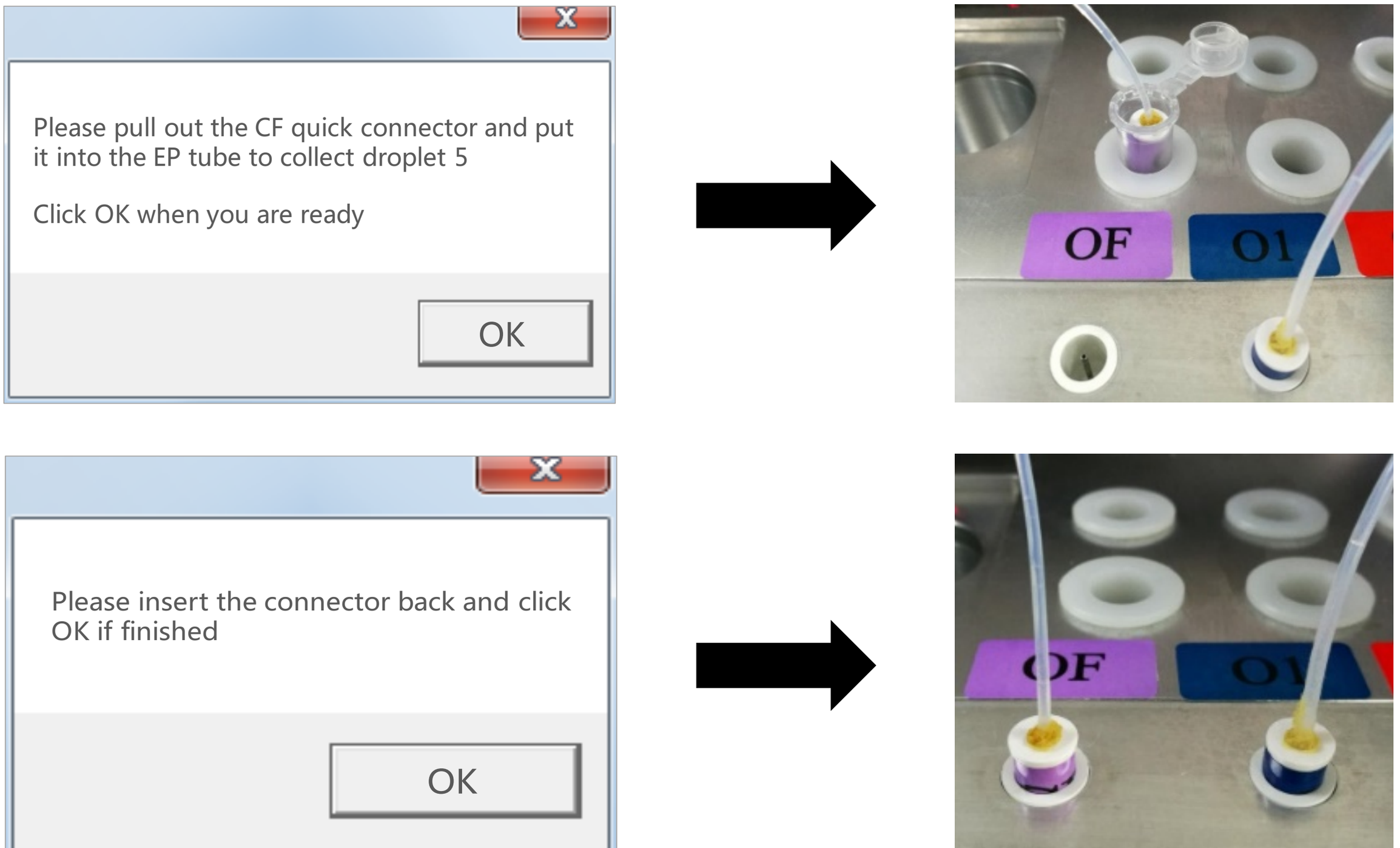

Figure 5

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A

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
1	dateTime	drop1	drop2	drop3	drop4	drop5	drop6	drop7	drop8	drop9	drop10	drop11	drop12	drop13	drop14	drop15
2	2019/8/29 11:54	0.12054735	0.0249613	0.16196179	0.03580889	0.02900588	0.00627116	0.0205246	0.19958158	0.02437642	0.04706487	0.06168714	0.13669907	0.11553461	0.09432766	0.04539266
3	2019/8/29 12:08	0.20206495	0.09458383	0.16966697	0.23353983	0.28764521	0.13566215	0.23993041	0.14152663	0.15785078	0.06183473	0.01663347	0.08914915	0.14495788	0.21987027	0.11888792
4	2019/8/29 12:22	0.29956759	0.34300347	0.30417926	0.26529247	0.46999706	0.35340247	0.32819324	0.16718481	0.20368882	0.25929193	0.21811709	0.16653715	0.11346028	0.20789435	0.26766369
5	2019/8/29 12:36	0.36279243	0.4129116	0.36673499	0.39706098	0.32821302	0.39366855	0.39736946	0.25743321	0.20813606	0.24885085	0.19599746	0.24058022	0.23468511	0.24996099	0.14598479
6	2019/8/29 12:50	0.62476155	0.45040164	0.55554528	0.43571445	0.51849248	0.45112129	0.35414929	0.57505075	0.45086426	0.55189987	0.47171054	0.33401539	0.40110733	0.53073759	0.44963065
7	2019/8/29 13:04	0.67509341	0.59731124	0.61914147	0.68081107	0.53384313	0.75257921	0.59411524	0.57381671	0.54159273	0.57402571	0.51535797	0.56587889	0.54841242	0.52080557	0.55429979
8	2019/8/29 13:18	0.90365007	0.77027656	0.90005247	0.99175496	0.81576094	0.85088613	0.82649809	0.74314302	0.65843697	0.67436295	0.67293307	0.66758653	0.78696679	0.72100219	0.5851478
9	2019/8/29 13:32	0.82782514	0.87085052	1.00738775	1.03982035	0.80359079	0.82208441	0.89124971	0.99337851	0.93659961	0.98154813	0.71245993	0.89529215	0.76141228	0.81342704	0.84648418
10	2019/8/29 13:45	1.06951486	1.01511206	1.06496235	0.99907859	0.98934215	1.13414259	1.01240999	0.92206566	1.01599457	1.04639085	1.06118908	0.97928824	1.00755961	1.02062936	0.93019162
11	2019/8/29 13:59	1.09212582	1.12720249	1.06778199	1.20659872	1.04362453	1.13403322	0.98815229	1.04747806	1.12026491	1.0631349	1.04853951	0.99464196	1.00630345	1.13883519	1.0021922
12	2019/8/29 14:13	1.18817262	1.28651851	1.24223982	1.2056997	1.255086	1.38517324	1.3829662	1.3609296	1.38523133	1.34877778	1.21450624	1.18873924	1.10806707	1.11732703	1.2414412
13	2019/8/29 14:27	1.48539789	1.40029658	1.4278995	1.39691849	1.29307934	1.46598947	1.3261944	1.29457372	1.3337583	1.28957438	1.31552485	1.39767552	1.45964795	1.21314022	1.25237982
14	2019/8/29 14:41	1.51322048	1.61519458	1.53562372	1.5530004	1.52559337	1.73464941	1.61382306	1.77714417	1.62629474	1.59684785	1.60071618	1.57326224	1.44183692	1.55128467	1.45427386
15	2019/8/29 14:55	1.83946548	1.83308004	1.83989545	1.68045286	1.85059091	1.63956209	1.68280104	1.67479602	1.70704602	1.67148804	1.62131662	1.5813836	1.64207788	1.69949919	1.71345138
16	2019/8/29 15:09	2.0146842	1.99082071	2.044047	1.9139442	1.97034303	2.07751211	2.00230387	2.1954139	2.02469059	1.8625705	2.07117514	1.8704097	1.95241385	1.79432219	1.8968858
17	2019/8/29 15:23	2.22418536	2.25521724	2.30316753	2.37952392	2.34511815	2.18139349	2.13768303	2.16043608	2.28422558	2.11913282	2.40456862	2.06677733	2.01018037	2.12053372	2.11295908
18	2019/8/29 15:37	2.5997207	2.438057	2.61494753	2.62652321	2.49468302	2.43020947	2.50051553	2.47975265	2.61937152	2.57361788	2.43712553	2.43200441	2.30680899	2.27376425	2.19846198
19	2019/8/29 15:50	2.73580978	2.92844029	2.77971504	2.92519103	2.76939096	2.7624041	2.81118606	2.78075513	2.9423707	2.76074019	2.75838882	2.6695508	2.56872716	2.65820862	2.67995068
20	2019/8/29 16:04	3.22932987	3.11792124	3.19350225	3.10723422	3.09548074	3.1771778	3.18803272	3.28138551	3.04377149	3.07317558	3.06118559	3.06226179	2.87521029	2.86218713	2.85484481
21	2019/8/29 16:18	3.42290973	3.32354678	3.39857049	3.40847744	3.44196759	3.53535677	3.65353007	3.44023316	3.37677391	3.35496264	3.2319503	3.24268012	3.24496024	3.13915203	3.25614971
22	2019/8/29 16:32	3.64538518	3.69204635	3.79165099	3.93576449	3.71845388	3.74298706	3.5933724	3.58113388	3.82219889	3.7729532	3.63546633	3.48846124	3.56891427	3.47737063	3.38890096
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25	2019/8/29 17:14	4.73926447	4.69898467	4.68111887	4.72979441	4.81427361	4.65400136	4.94061916	4.66092547	4.67329879	4.73271387	4.51075224	4.55250374	4.33412813	4.42475514	4.33850441
26	2019/8/29 17:28	5.06803175	5.16337452	5.19906362	5.22260176	5.29363945	5.10967809	5.30549389	5.1270106	5.33011299	5.16561203	4.92338484	4.92447008	4.99104409	4.72257772	4.53044218
27	2019/8/29 17:42	5.30759034	5.33152768	5.28740702	5.78125189	5.33942536	5.29176924	5.3163395	5.37315227	5.5223849	5.25915936	5.15326261	5.00665738	5.13774693	5.0439709	5.00908958
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30	2019/8/29 18:24	5.49328752	5.71988429	5.47970032	5.55955893	5.70004987	5.52273986	5.41320103	5.46065763	5.64746798	5.45478459	5.45110372	5.3004732	5.51341144	5.31571089	5.46812966
31	2019/8/29 18:37	5.68393896	5.78297982	5.95192617	5.81929514	5.72292312	5.78267535	5.99507154	5.60187898	5.67182073	5.6460437	5.67822806	5.72917039	5.71094619	5.83082473	5.57622966
32	2019/8/29 18:51	5.74864065	5.627643	5.75215665	5.84300756	5.83366217	5.90535494	5.99415904	5.6765566	5.8917415	5.9739825	5.61459764	6.00151817	5.65786825	5.85348345	5.64338536
33	2019/8/29 19:05	5.68861184	5.95303763	5.6922065	6.30586618	5.89944049	5.91534797	5.7511517	5.9475687	6.05181693	5.92994942	5.86006623	5.7632355	5.76656178	6.16190776	5.67205758
34	2019/8/29 19:19	5.6575104	5.86392496	5.76109899	6.51097476	5.97439801	5.95831538	6.07304948	6.07973993	6.36098858	6.01869085	5.88940778	5.91773896	5.74684755	5.81077789	5.69119239
35	2019/8/29 19:33	5.79863453	5.8229765	6.11938346	5.9684407	5.92006781	6.06702995	5.95069379	5.90500532	5.80351729	5.90191384	5.73190139	5.858657	5.80931948	5.81268059	5.65942924
36	2019/8/29 19:47	5.87523435	6.0253125	5.95104668	5.87696318	6.09578492	6.02365455	5.93596942	5.98200913	5.98675163	6.13645872	6.22672678	6.12123397	5.83072021	5.90457393	5.75134086
37	2019/8/29 20:01	5.95728884	5.9914914	6.00389906	5.93305209	5.99941337	5.98909553	5.92798626	5.95251848	6.18597901	5.90992047	5.75694282	5.91755469	5.81755505	5.95552554	6.36535609
38	2019/8/29 20:15	5.80563042	6.16352544	6.03973054	6.08067532	5.94138848	6.04842669	5.89652765	5.89909898	6.05136249	6.05125762	5.85856941	5.93880336	5.90342069	6.00858676	6.18461425
39	2019/8/29 20:29	5.74499627	5.93658637	5.9235036	6.17691675	6.01845698	5.90058373	6.02095751	6.00690235	5.99775389	6.14594369	5.81900383	5.98716323	5.89668472	6.00024787	5.90807934
40	2019/8/29 20:42	5.76665793	6.05265573	5.94366011	5.83532872	6.17233892	5.85917197	5.87291702	5.99093302	5.80371697	6.01671427	5.97448533	6.9258849	6.04814099	5.71325404	5.93392896
41	2019/8/29 20:56	6.68757663	5.861129	5.84785862	6.05316303	6.10999675	6.16150926	6.04573449	6.12044136	6.10367339	5.89296649	6.12382035	6.07696037	5.95692513	6.66300292	6.06688756
42	2019/8/29 21:10	5.94550391	6.08987174	6.10469657	5.84048353	6.01127188	6.07612317	6.00254041	6.06962454	6.08892641	5.83885519	5.93735987	5.75554643	5.87659655	5.86607207	6.35541486
43	2019/8/29 21:24	6.09827325	6.41720476	6.32008209	5.96649664	5.98894035	6.17985689	6.16378746	6.25050731	6.498879	5.98514401	6.03061439	5.90025549	5.89589288	5.84947395	5.79170567
44	2019/8/29 21:38	6.51726987	6.19616605	5.86710962	6.03153706	6.1264526	5.99856322	6.07498574	6.000012	6.15859231	6.18291549	6.14213732	5.91959308	5.92943416	6.0250974	5.84353418
45	2019/8/29 21:52	6.05943311	6.14509756	6.11805625	5.94842714	6.06655394	6.06948791	6.26708107	6.0028	6.3379963	6.14065475	6.00903791	5.95834172	6.0797654	5.75290307	5.74525419

B

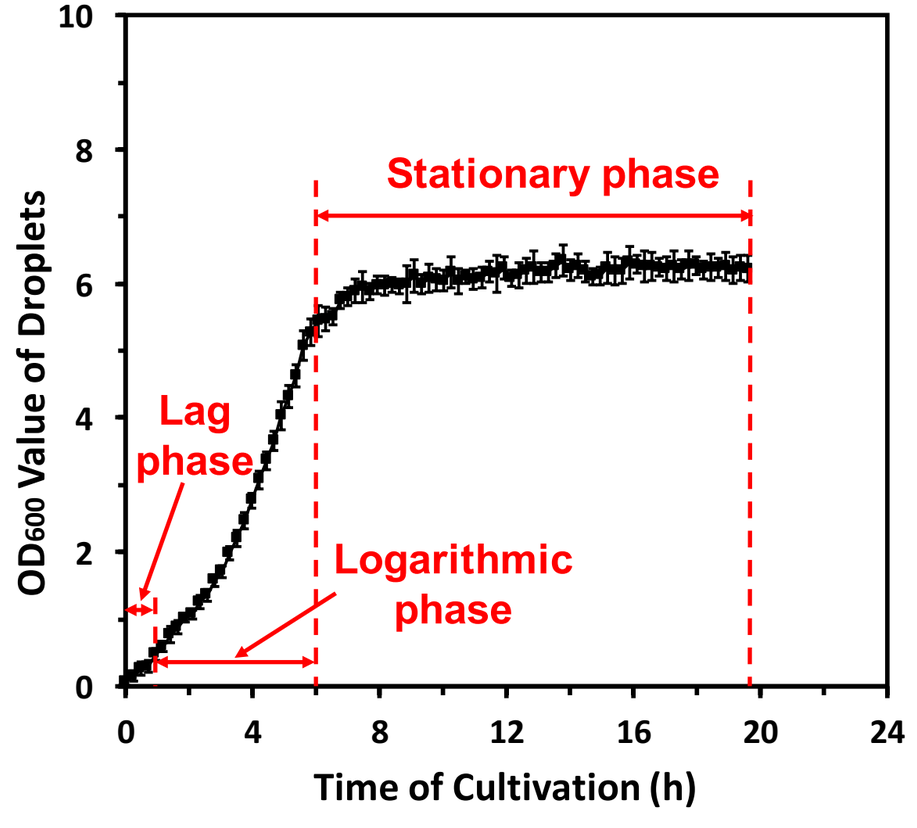

Figure 6

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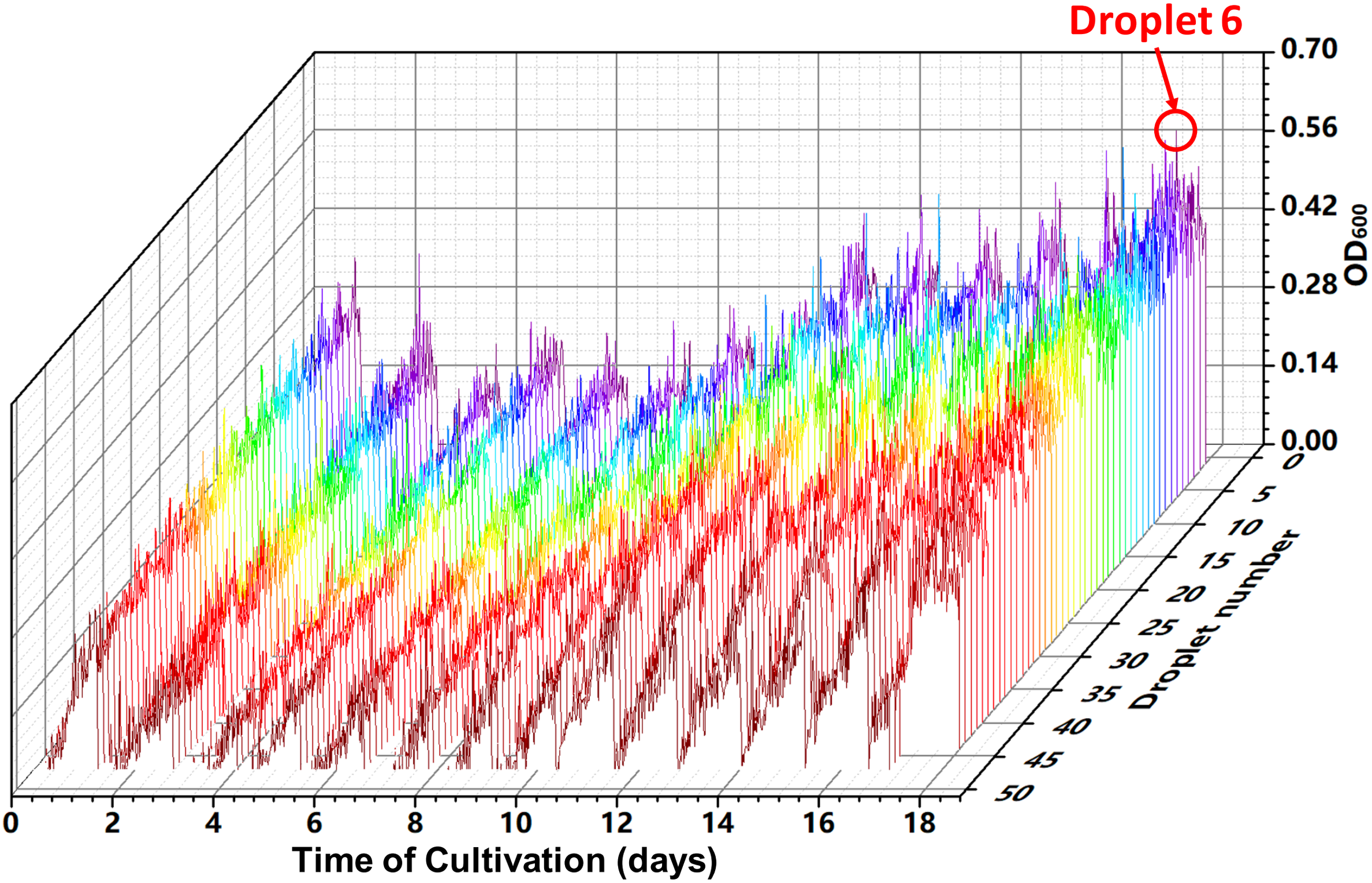
A



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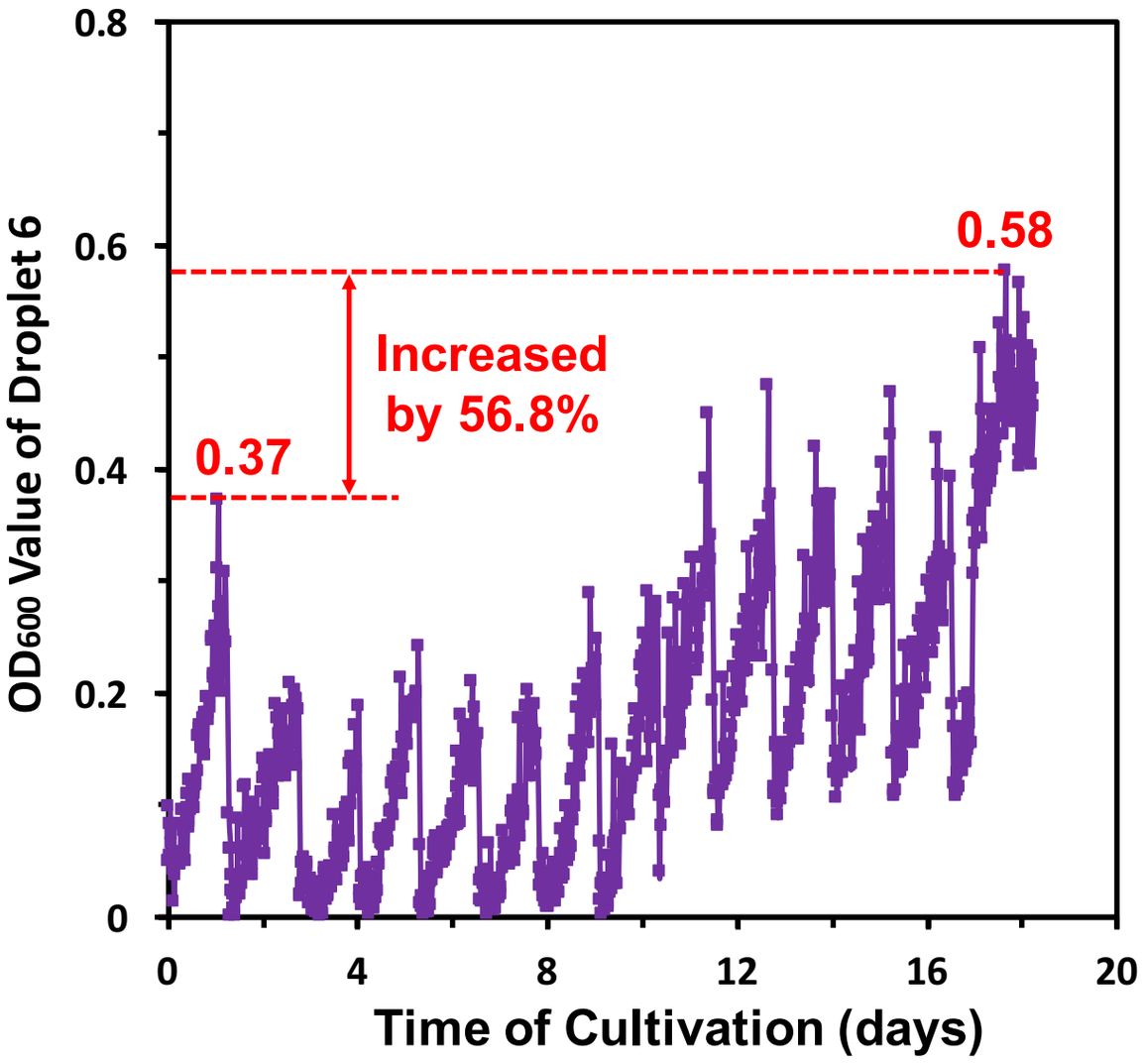

C

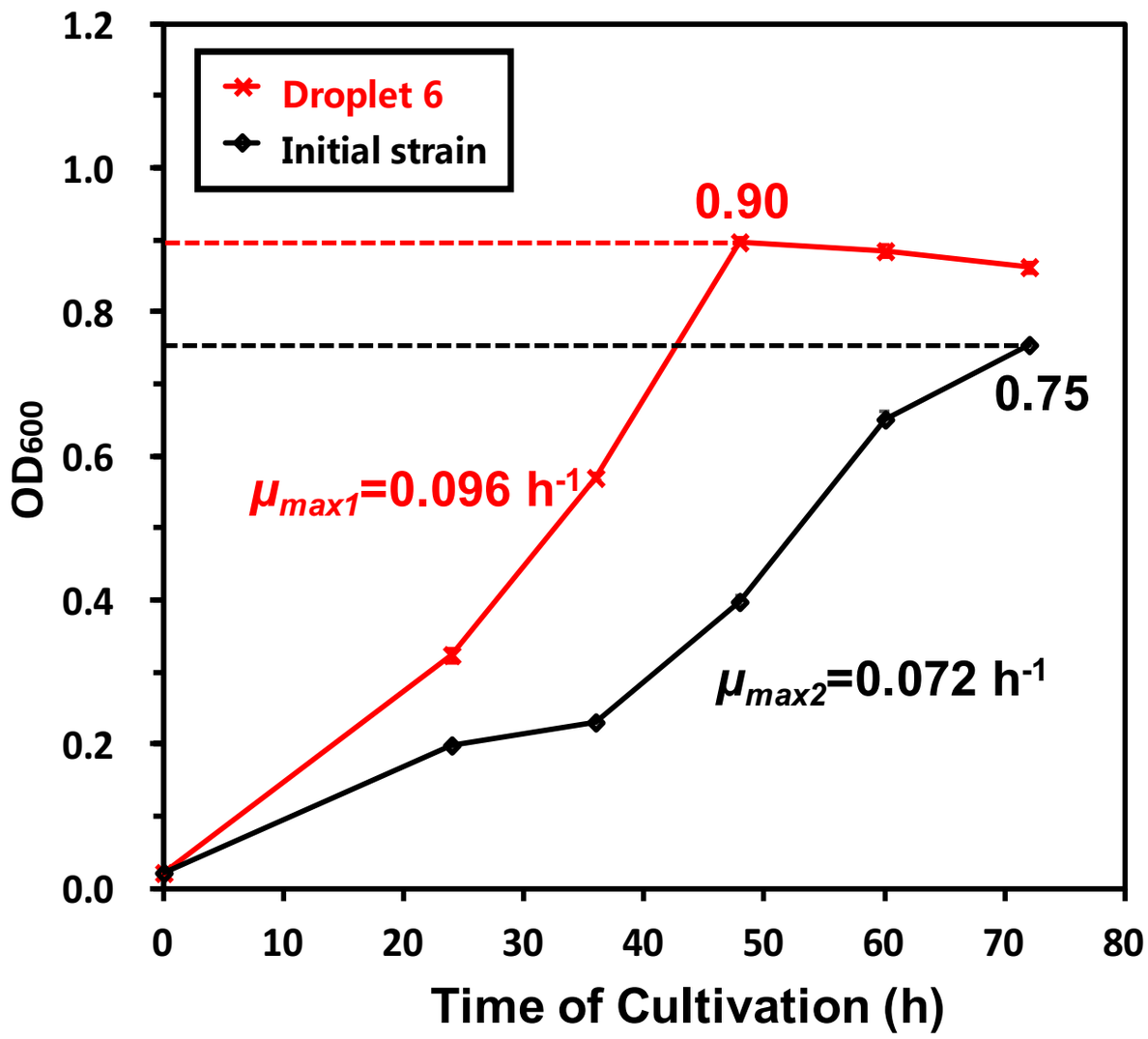

Table 1

Components	**Concentration**
$Na_2HPO_4 \cdot 12H_2O$	6.78 g/L
KH_2PO_4	3 g/L
NaCl	0.5 g/L
NH_4Cl	1 g/L
vitamin B1 (sterilized by filtration)	0.34 g/L
$MgSO_4 \cdot 7H_2O$	0.049 g/L
$CaCl_2 \cdot 2H_2O$	1.5 mg/L
Microelements:	
$FeCl_3 \cdot 6H_2O$	0.5 mg/L
$ZnSO_4 \cdot 7H_2O$	0.09 mg/L
CuSO4·5H2O	0.088 mg/L
$MnCl_2$	0.045 mg/L
CoCl2·$6H_2O$	0.09 mg/L
gluconate	1.09 g/L
methanol	500 mmol/L
isopropyl-β-d-thiogalactopyranoside	0.1 mmol/L
streptomycin sulfate	20 μg/mL
kanamycin sulfate	50 μg/mL
Add extra 15 g/L agarose to prepare solid medium.	

Table 1

Tunable
Parameter
Temperature of cultivation
Number of droplets
Concentration of inoculum
Concentration of chemical factor
The time of sub-cultivation
The number of sub-cultivations
Wavelength of OD detection
Wavelength of fluorescence detection
Accuracy
Parameter
Volume of droplets
Concentration of inoculum

parameters
Range
25–40 °C ± 0.5 °C
0–200
13.3–86.7 %
8 different concentrations, up to the maximum concentration of stored chemical factor
Up to the user
Up to the user
350–800 nm
Excitation: 470, 528 nm Emission: 350–800 nm
y parameters
C.V
1.88%
<5.0%

Amit Krishnan, Ph.D.

Review Editor, *JoVE*

October 28, 2021

Dear Dr. Krishnan,

With this cover letter, we will submit the revised manuscript (Manuscript ID JoVE62800R2) entitled, "Automated and High-throughput Microbial Cultivation and Adaptive Evolution using Microbial Microdroplet Culture System (MMC)" for publication in *JoVE*. We would like to thank reviewers for the careful and constructive reviews. Based on the comments from the reviewers, we have made substantial changes of the manuscript and given more detailed explanations to clarify some important issues raised by the reviewers. A point-to-point response to all the comments from the reviewers is also uploaded.

We really appreciate the suggestions from the reviewers and the editorial board to improve the quality of this manuscript and our work. We hope after careful revision this new version is more suitable for publication in *JoVE*.

Sincerely yours,

Chong Zhang

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The Response to comments

To the editorial comments:

1. Please note that the manuscript has been formatted to fit the journal standard. Please review. Consider revising the title to " Automated Microbial Cultivation and Adaptive Evolution using Microbial Microdroplet Culture System (MMC)".

Response: Thank you for your suggestions. After consideration, we revised the title. We also revised the description of "high-throughput" in the manuscript.

The revision is as follows：

Line 1: Automated ~~and High-throughput~~ Microbial Cultivation and Adaptive Evolution using Microbial Microdroplet Culture System (MMC)

2. Please consider including the following Figures (in the rebuttal) as Supplementary Figures in the manuscript. Please explain these results in the Representative Results section. Also, include a title and short description for the figures in the Figure and Table Legends section.

(i) Accuracy and reproducibility of droplet operations using dyed solutions,

(ii) Consistency of E. coli MG1655 cultivation, and

(iii) Evaporation of the droplet

(iv) Calculation of the OD value for a single droplet

(v) Verifying crosstalk between the droplets during the long-term cultivation.

Response: We are sorry that we think we cannot directly add these figures to the manuscript. On the one hand, the results of these figures are not obtained by the protocol, and they are not the main application of MMC too. On the other hand, these results come from some of our recent work, and we are considering combining them with our other work to publish in a new journal. Here, we will take another form (raw data or tables) to provide these results as **Supplementary Materials**. We have also given some explanation for these results in the Representative Results section

(i) Here involve two experiments: one is about "volume of droplets", and the other is about "concentration of inoculum". The C.V of both are shown in **Table 1**. The data about "volume of droplets" are detailed in **Table S1**. The data about "concentration of inoculum" comes from ref. 13, which has been marked in **Table 1**.

(ii) The accuracy and reproducibility of droplet operations have been explained in (i). The (ii) just further explains the cultivation consistency brought by the accuracy and

reproducibility. We think the information in (i) is sufficient, so we will not put this Figure in the manuscript or Supplementary Materials.

(iii) Please refer to **Table S2** for details.

(iv) Please refer to **Figure S1** for details.

(v) Please refer to **Figure S2** for details.

3. Please ensure that all the limitations mentioned in the rebuttal are included in the manuscript.

Response: We have mentioned all the limitations involved in the rebuttal in the discussion.

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Lead author	Xingjin Jian, Xiaojie Guo, Jia Wang, Zheng Lin Tan, Xin–hui Xing, Liyan Wang, Chong Zhang
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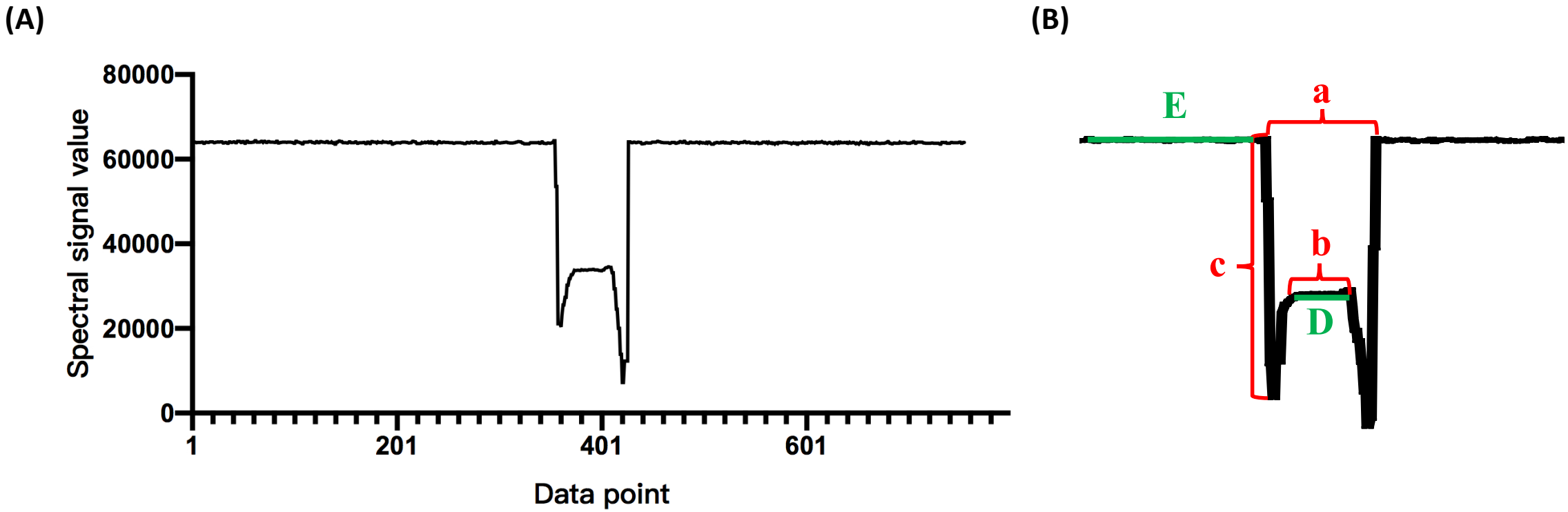

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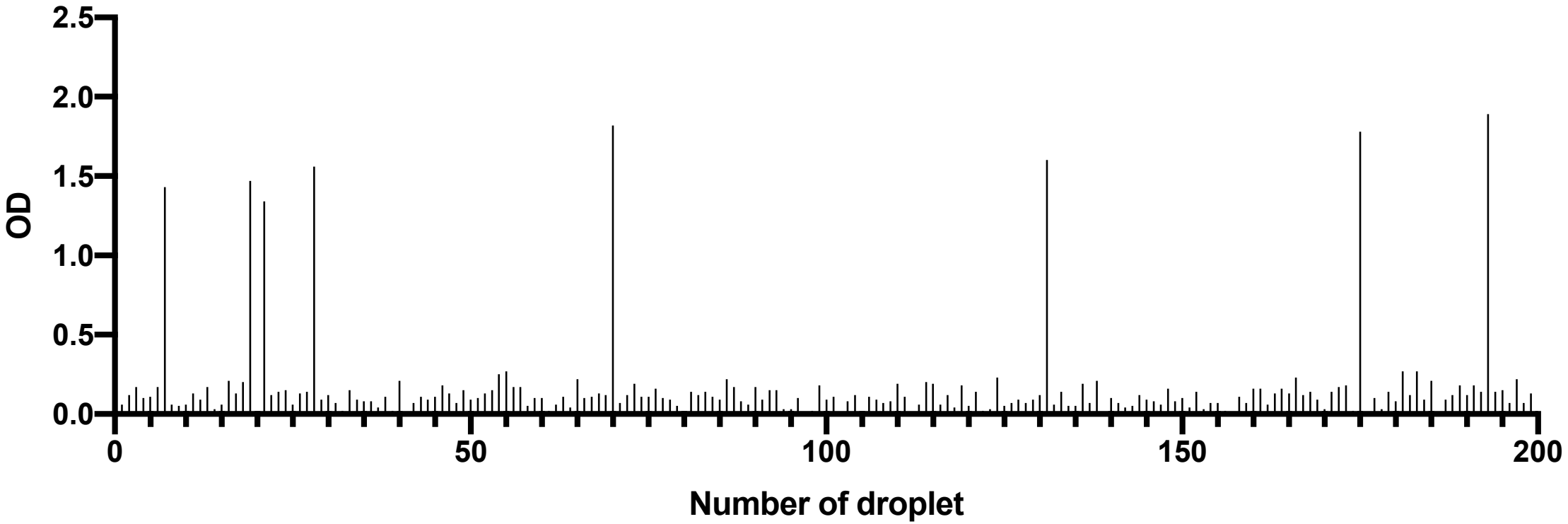

Supplemenary Table 1

Number of data points	Number of droplets	C.V
68	8	
69	33	
70	74	
71	122	1.88%
72	86	
73	60	
74	14	

Supplemenary Table 2

Time/days	Length of droplet/mm	Percentage of reduction
0	2.19	0.00%
6	2.18	0.50%
12	2.15	1.80%
18	2.06	5.90%
24	1.97	10.00%
30	1.92	12.30%

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