Supporting information

Programmable Static Droplet Array for the Analysis of Cell-Cell Communication in a Confined Microenvironment

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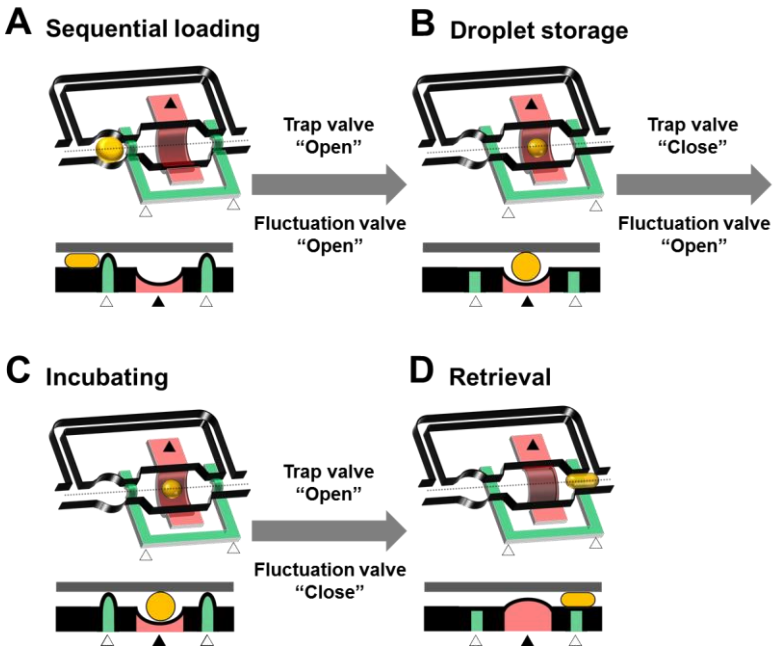

Figure S-1. A schematic diagram showing the operation of valves according to unit operation. White and black triangles represent trap and fluctuation valves, respectively. The dashed line in the 3D image indicates the direction of the cross section image. (A) The operation of valve during sequential loading. When the trap valve closes the channel, droplet cannot pass through. The fluctuation valve is suctioned by negative pressure, which forms the bottom surface of concave shape. (B) A step of droplet storage. When the trap valve opens, the droplet goes through. The pressure balance between the Laplace pressure of the droplet and the hydraulic pressure drop results in the moving of droplet from microwell to reaction chamber. Because the fluctuation valve is deflated downward in the reaction chamber, the height of microfluidic channel in reaction chamber becomes higher than that of the surrounding microfluidic channel. The height difference induces stable immobilization of the droplet in the reaction chamber. (C) The incubation. Trap valve is re-expanded as much as possible to fully close the channel. (D) A step of droplet retrieval. Trap valve is opened to retrieve the droplet from the reaction chamber. Once applying positive pressure to the fluctuation valve, the fluctuation valve expands. As a result, the droplet is moved along the microfluidic channel.

Cell loading procedure

The number of mixed *MAT***a** and *MAT*$\boldsymbol{\alpha}$ cell in droplets were manipulated by repeating of double sequential loading and droplet storing procedures. The detailed procedure is as follows.

1. Double sequential loading and merging of droplets

Using the fully integrated SDA, we can individually address droplets since they are sequentially immobilized into hydrodynamic trap. Since this addressable droplet immobilization technique is conducted in a passive manner, our SDA does not require any additional control to immobilize droplets. We demonstrate the individual addressability of the droplet array and the easiness of SDA operation (Figure S-2).

After a first droplet is trapped in a microwell, all the flow is directed toward the bypass channel if the first droplet plugs the microwell. Otherwise, a second droplet should be also entered into the microwell. By an alternative explaining way, trapping mode continues until the microwell is fully occupied. As shown in Figure S-2, in the case of double sequential loading of 27 pL droplet, one microwell can accommodate two droplets which finally merged into a large droplet. The size of the merged droplet during the double sequential loading is 54 pL. When the droplet immobilized into microwell is changed into a plug to block the capillary path located at the exit of the microwell, subsequent droplets are no longer trapped into the microwell but moves along the bypass channel toward next vacant microwell.

In this study, droplets containing *MAT***a** cells expressing green fluorescent protein to distinguish each mating type of cell are arrayed ahead. Then, we subsequently generate droplet containing culture media from droplet generation part, and it is loaded as a droplet array. This entire procedure is accomplished within 1 minutes. For example, we obtained a droplet encapsulating four *MAT***a** cells through the result of the first double sequential loading and droplet storing (first step in Figure S-2).

2. Storage

Next, we perform a droplet storing procedure to dramatically increase of the number of droplet stored in the specific position. This method allows us to create single droplet composed of eight droplets on a single hydrodynamic trap with zero loss in the SDA. The ‘‘storing’’ method, illustrated in Figure S-2, uses the exact simultaneous opening trap valve. The storage process is to transfer droplet immobilized in a microwell to reaction chamber. The principle of storage is based on the correlation between hydraulic pressure-drop and Laplace pressure of droplet.

After finishing the first step of double sequential loading and storing, we perform a second step of double sequential loading and storing with two droplets containing *MAT*$\boldsymbol{\alpha}$ cells (non-fluorescent cells). Also, we repeat 3 and 4 step of double sequential loading and storing with droplet containing culture media. Finally, we obtain a droplet encapsulating 4 *MAT***a** and 11 *MAT*$\boldsymbol{\alpha}$ cells through 4 repetitive steps of double sequential loading and storing (Figure S-2). In case of undesired sex ratio and cell density in a droplet, we can differently utilize the droplet containing *MAT***a**, *MAT*$\boldsymbol{\alpha}$, or culture media at each 3 and 4 step in accordance with the

status of droplet. Namely, we can execute controlling procedure through 3 and 4 step as a feed-back procedure comparing desire value of sex ratio and cell density in a droplet within 2 minute. Through these repetitive processes, we obtain a constant droplet volume (about 220 pL) containing the desired sex ratio and of cell density in a droplet at each experiment. The result proves the advance of the SDA platform in quantitative precision.

In summary, once droplets in the microwells are formed, the droplets transfer into the reaction chamber. Then it becomes possible to reconstitute vacant microwells again. We can repeat the process for four times to obtain a single droplet consisting of 8 combinatorial elements. Therefore, we can control and manipulate the appropriate sex ratio and cell density per droplet through the combination of double sequential loading and storing in this study. Furthermore, these droplets represent physically and biologically isolated microenvironment with different cell entities.

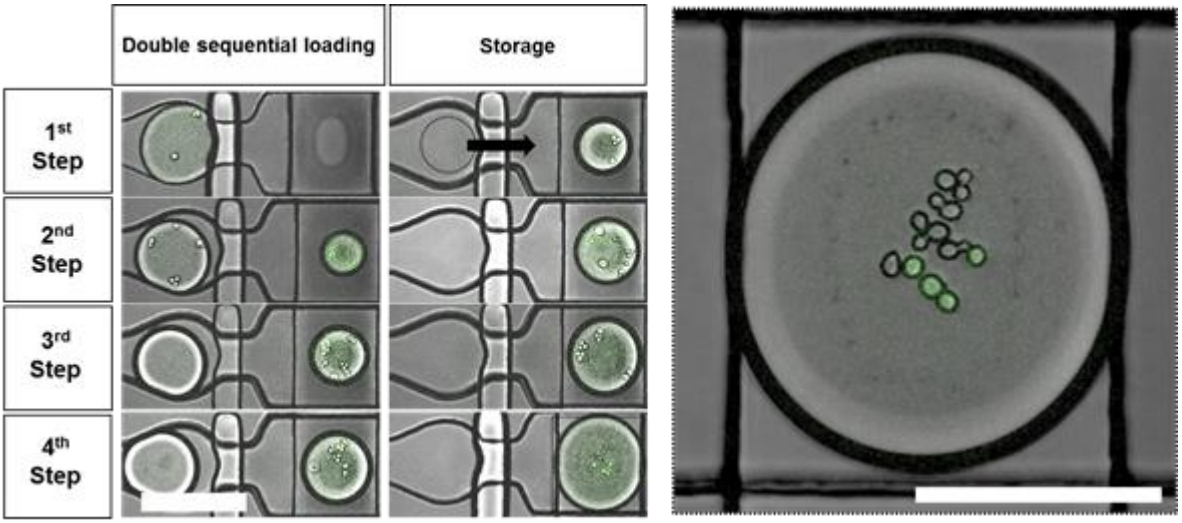

Figure S-2. Enlarged image of Figure 4A. *MAT***a** cells genetically modified to express green fluorescent protein is easily distinguished from other mating type of *MAT*$\boldsymbol{\alpha}$ cells (non-fluorescent cells). Right enlarged image shows the final droplet obtained from repeated 4 steps of double sequential loading and storage.

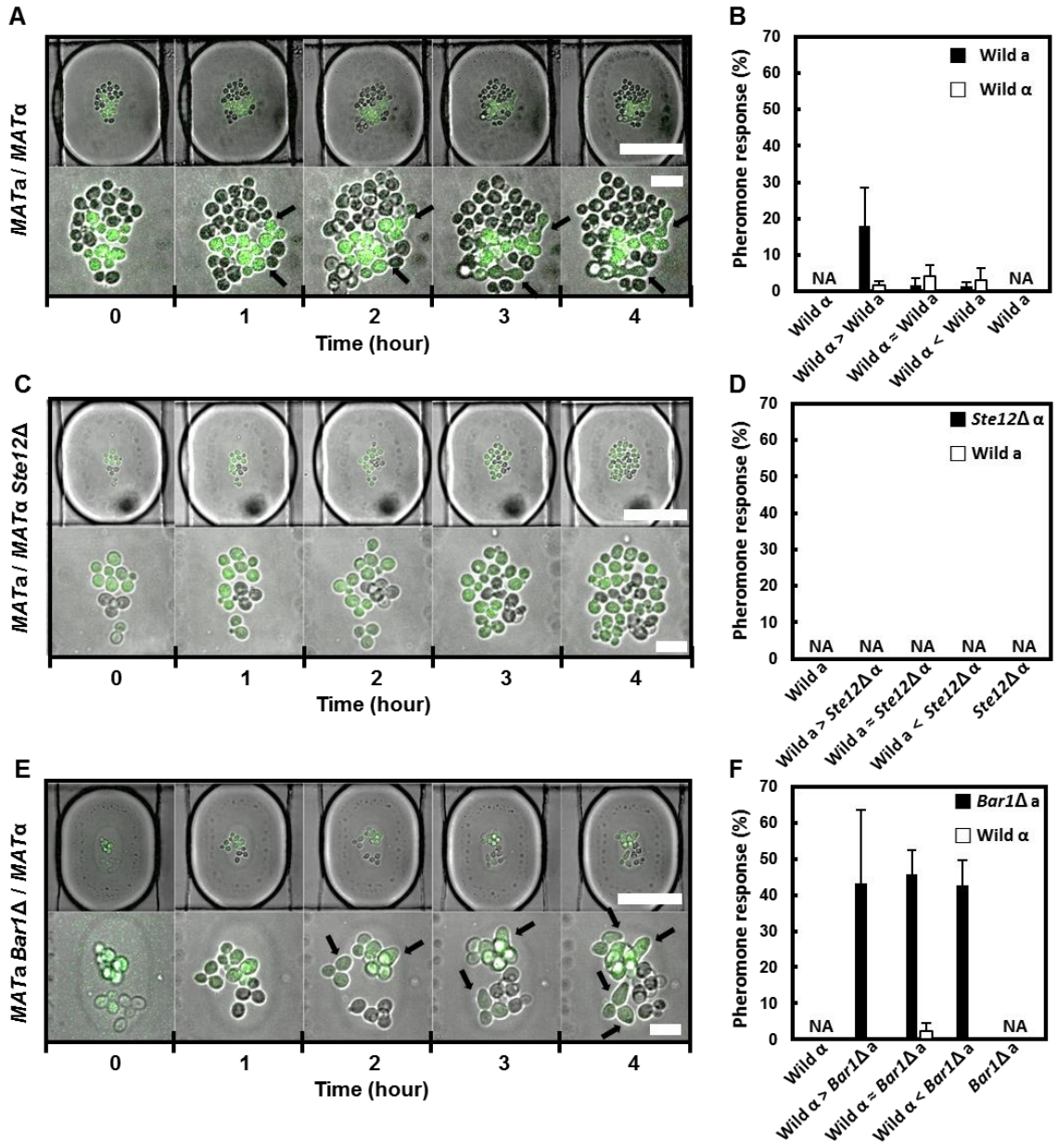

Figure S-3. Droplet-based pheromone response assay. Time-lapse images of the mating process and histogram of the pheromone response in correlation with different ratio of *MAT***a** and *MAT***α** cells. The pheromone response is characterized by morphological changes such as the formation of mating projections (shmoo) or fused *MAT***a**/**α** zygotes. NA stands for "Not Available". **(A)** and **(B)** WT and WT cells; **(C)** and **(D)** WT and *ste12*Δ cells; **(E)** and **(F)** WT and *ste12*Δ cells, respectively. The batch experiment was performed 3 times and quantified by calculating the average value. In addition, average values from 10 droplets in a single SDA device are shown for specific points.

Table S-1. Yeast strains used in this study

Strain No	Relevant genotype	Mating type	Reference
yBH100	*MAT*a *pFIG1-qV*	a	This study
ySSL93	*MAT*a *RPL9A-GFP*	a	OpenBiosystems
ySSL96	*MAT*a *bar1*Δ *HSP104-GFP*	a	This study
yMU53	*MAT*a *HOG1-mCherry::HTA2-CFP::pSTL1-qV*	a	This study
ySSL100	*MAT*α *SSA4-mCherry*	α	This study
ySSL101	*MAT*α *ste12*Δ*::HSP104-GFP*	α	This study

Throughput of the programmable static droplet array

A reasonable estimate for the throughput of the static droplet array platform requires only 5 min per single combinatorial assay with 8 different compositions. We are able to perform 11529 assays in a single day in a single SDA device. We think that it is quite competitive performance. Similarly, using robot for the screen, 73,000 assays in a day could be performed. By considering all supplies, amortization, developing trend, parallelization, and automation of the SDA, we believe that microfluidic approaches can overcome the robotic system soon.

Table S-2. Specification of programmable static droplet array

Unit operations or features	Time or frequency
Double sequential loading	50 s
Droplet storage	10 s
Repeating steps	4 steps
Reaction droplets/device	40
Combinations/reaction droplet	8
Droplet retrieval	60 s
Assay time (40 reaction droplets with 8 compositions)	5 min
Throughput (Reaction droplets with 8 compositions/day)	11520

Movie S-1. Demonstration of microfluidic static droplet array.

Movie S-1 shows the demonstration of the microfluidic static droplet array device in four representative processes such as double sequential loading, droplet storage, droplet retrieval, cell culture and monitoring.