Supplementary information

A vacuum-assisted, highly parallelized microfluidic array for performing multi-step digital assays

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Supplementary methods:

Mold fabrication

Molds were fabricated by standard photolithography technique (***Fig. S1***). Masks were printed by CAD/Art Services, Inc. (Bandon, OR, USA). Two 4-inch silicon wafers (Polishing Corporation of America, Santa Clara, CA) were dehydrated at 200 °C for 1 h to increase adhesion with photoresist.

For the microarray mold, a ~100-μm-thick layer of SU8-3050 (MicroChem Corp., Newton, MA) was spun-coated onto the wafer at 1500 rpm for 35 s. After soft baking at 95 °C for 30 min, the wafer was exposed under an aligner for 25 s (Mercury lamp power: 9.3 mW/cm^2) and briefly baked at 95°C for 3 min. Next, another layer of ~100-μm-thick SU8-3050 was spun-coated onto the wafer at 1500 rpm for 35 s, soft-baked at 95 °C for 1 h, aligned and exposed for 40 s, and then post-exposure-baked at 95 °C for 10 min. Finally, the wafer was developed in SU-8 developer (MicroChem Corp., Newton, MA) for 20 min to generate the solid pattern of microarray, followed by hard baking at 200 °C for 1 h.

To prepare the mold for the suction layer, SU8-3050 was spun-coated onto a dehydrated silicon wafer at 1500 rpm for 35 s to achieve a thickness of ~100 μm. Next, the wafer was soft baked at 95 °C for 30 min, exposed for 30 s on the aligner, baked for another 5 min at 95 °C, and developed for 15 min to generate the pattern of microchannels and micropillar array. Finally, the wafer was hard baked at 200 °C for 1 h.

Chip fabrication

Before PDMS chip fabrication, molds were treated with chlorotrimethylsilane (Sigma Aldrich, St. Louis, MO) for 10 min to provide smooth demolding of PDMS. Chip fabrication and assembly were shown in ***Fig. S1***. First, 10:1 (w/w) PDMS (SYLGARD 184 Silicone Elastomer Kit, Dow Corning, Midland, MI, USA) was spun onto the microarray mold at 100 rpm for 2 min, and placed into a vacuum chamber for 5 min to remove air bubbles, followed by baking at 80 °C for 15 min. Next, the microarray layer was peeled off from the mold, cut into individual chips and plasma bonded with a clean glass coverslip (45 mm × 50 mm, Ted Pella, Inc., Redding, CA).

Two blank wafers were used to fabricate thin PDMS membranes. In this process, 12:1 (w/w) PDMS was spun onto one blank wafer at 100 rpm for 2 min to generate a sacrifice layer that can facilitate demolding of thin PDMS membrane in the following steps. The sacrifice layer was then

baked at 80 °C for 10 min and peeled off for later use. Next, 5 g 5:1 (w/w) PDMS was spun-coated onto another blank wafer at 700 rpm for 35 s to form a ~100-μm-thick thin PDMS membrane, which was baked at 80 °C for 6 min. The sacrifice layer was aligned onto the membrane and baked at 80 °C for 6 min. The ultra thin PDMS membrane can then be stripped off from the wafer because the adhesion between two PDMS layers is stronger than that between PDMS and silicon wafer. Then the stacked blank PDMS was cut into individual pieces and holes were punched with 1.0-mm puncher to access fluidic inlets of the microarray. To assemble microarray and thin PDMS membrane, both microarray and stacked blank PDMS were plasma treated, aligned carefully, and baked at 80 °C briefly to ensure tight bonding between microarray and the thin PDMS membrane.

At the same time, ~30 g 10:1 (w/w) PDMS was poured onto the mold of the suction layer. The mold was then placed in a vacuum chamber for 20 min to remove trapped air bubbles and baked at 80 °C for 40 min. The cured suction layer was removed from the mold and cut into separate modules. Sample loading inlet and vacuum access holes were punched with 1.0-mm puncher (Miltex Disposable Biopsy Punch) and homemade sharpened 20-gauge needles respectively. To assemble the multi-step loading chip, the sacrifice layer was removed, and the suction layer was aligned onto the top surface of the thin PDMS membrane under a microscope. The device was baked at 80 °C overnight before use.

Bacteria concentration measurement

Each time before on-chip single methicillin-resistant *S. aureus* detection, fresh cells were resuspended in GW medium and adjusted to OD_{600} of 0.1 as the initial concentration for serial dilution afterward. The colony-forming units (CFU) of the *S. aureus* strain (ATCC BAA-44) at OD_{600} of 0.1 was determined via plating. To this end, *S. aureus* suspensions were diluted in GW medium to achieve expected concentrations of 5×10^2 CFU/mL, 2.5×10^2 CFU/mL, and 1.25×10^2 CFU/mL based on approximate ~1×10^8 CFU/mL at OD_{600} of 0.1. Next, 200 μL of the three diluted bacteria sample were spread onto Trypticase soy agar plates and incubated at 37 °C overnight, and the CFU numbers of each sample were counted. We determined the density of the *S. aureus* suspension at OD_{600} of 0.1 is 9×10^7 CFU/mL.

Device optimization

We found that low viscosity silicone oil can lead to PDMS swelling, thus interrupting oil partitioning. This issue can be mitigated by increasing the viscosity of silicone oil. We found 100-cSt silicone oil can effectively prevent PDMS swelling. However, increased oil viscosity results in higher flow resistance in a microchannel, which disables the oil loading on the microarray. In the rectangular channels of the microfluidic device, the hydraulic resistance R_h can be expressed as follows,

$$R_h = \frac{12\mu L}{(1 - 0.63\frac{h}{w}) \times h^3 w}, \quad (1)$$

where μ is the viscosity of the oil. L (length), h (height), and w (width) refer to the channel dimension. Equation 1 suggests that the hydraulic resistance is negatively correlated with channel height. We found that a height of 100 μm is sufficient to allow for smooth loading of the silicone oil for complete partitioning; whereas a lower channel height (e.g. 30 μm) restricts the flow and results in incomplete partitioning. (***Fig. S3***).

Poisson distribution statistics for digital assays

The digital assay is performed by distributing single molecules or single cells across a large number of partitions. The distribution of molecules or cells by partitioning follows the Poisson distribution. The average number of copies per partition (λ), defined as the ratio of copies of target

(m) to the total number of microchambers (N=4096), can be estimated based on the proportion of negative signals using the Poisson distribution.
The possibility E that a partition contains no target can be expressed as,

$$E = P(k = 0) = \frac{e^{-\lambda}\lambda^{0}}{0!} = e^{-\lambda}, \quad (2)$$

where k is the number of targets present in a single microchamber. Then, the average number of copies per partition can be expressed as

$$\lambda = -\ lnE = \frac{m}{N}. \quad (3)$$

The number of positive microchambers (M) on the chip can be directly counted based on the fluorescence signals from each chamber. Equation 2 therefore becomes

$$E = 1 - P(k = 1,2,\ldots) = 1 - \frac{M}{N}. \quad (4)$$

Finally, the copy number of the targets can be calculated as follows,

$$m = -\ N \times ln\left(1 - \frac{M}{N}\right). \quad (5)$$

Supplementary table:

Table S1. Sequences of primers, probes, and synthetic DNA used in this paper.

Name	***Sequence***
Forward primer_ *RecA*	5'-GTCTTTGCCCTGACCGATTT-3'
Reverse primer_ *RecA*	5'-CAGTTGGGAAGGCGAATTGA-3'
Probe_ *RecA*	/56-FAM/CACGCGCCG/ZEN/GATTTGTTGATGATG/3IABkFQ/
Forward primer_ *MecA*	5'-CATTGATCGCAACGTTCAATTT-3'
Reverse primer_ *MecA*	5'-TGGTCTTTCTGCATTCCTGGA-3'
Probe_ *MecA*	/5HEX/TGGAAGTTA/ZEN/GATTGGGATCATAGCGTCAT/3IABkFQ/
Synthetic *RecA* fragment	5'-ACGTTGTCTTTGCCCTGACCGATTTGCCAGCTCACGCGCCG GATTTGTTGATGATGTCGCTCGATCAATTCGCCTTCCCAACTGATGCC-3'

Supplementary figures:

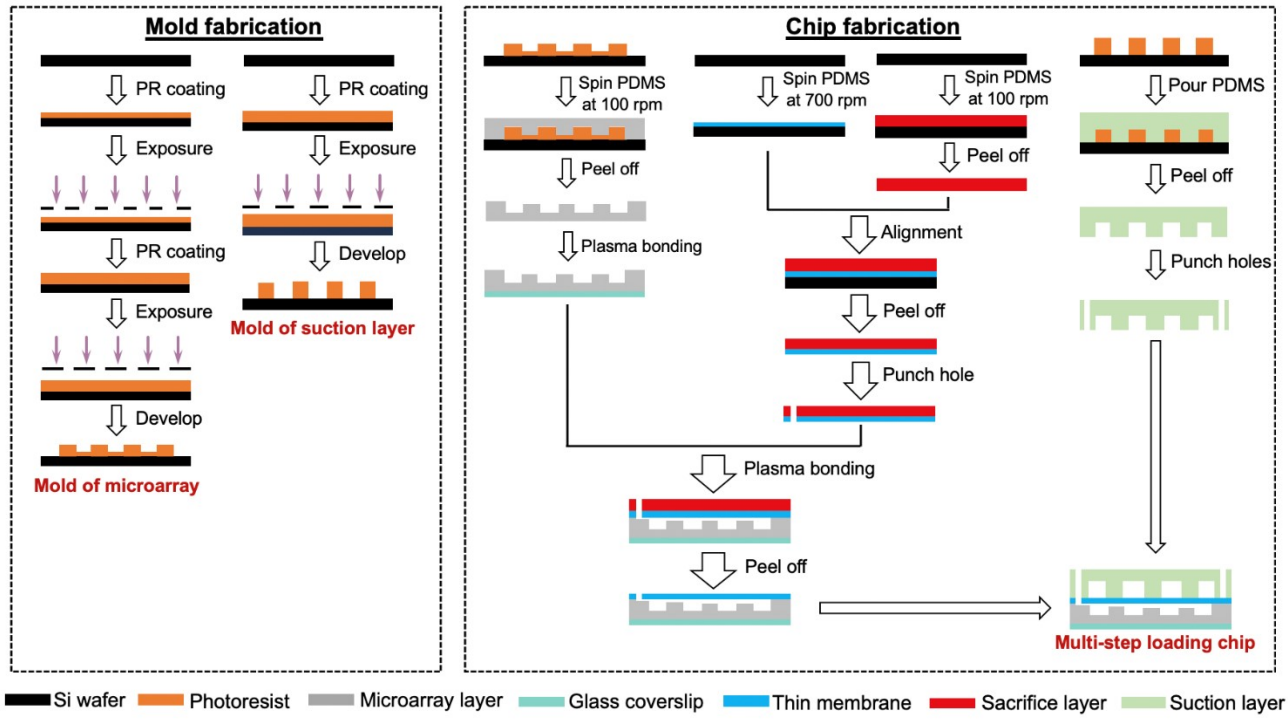
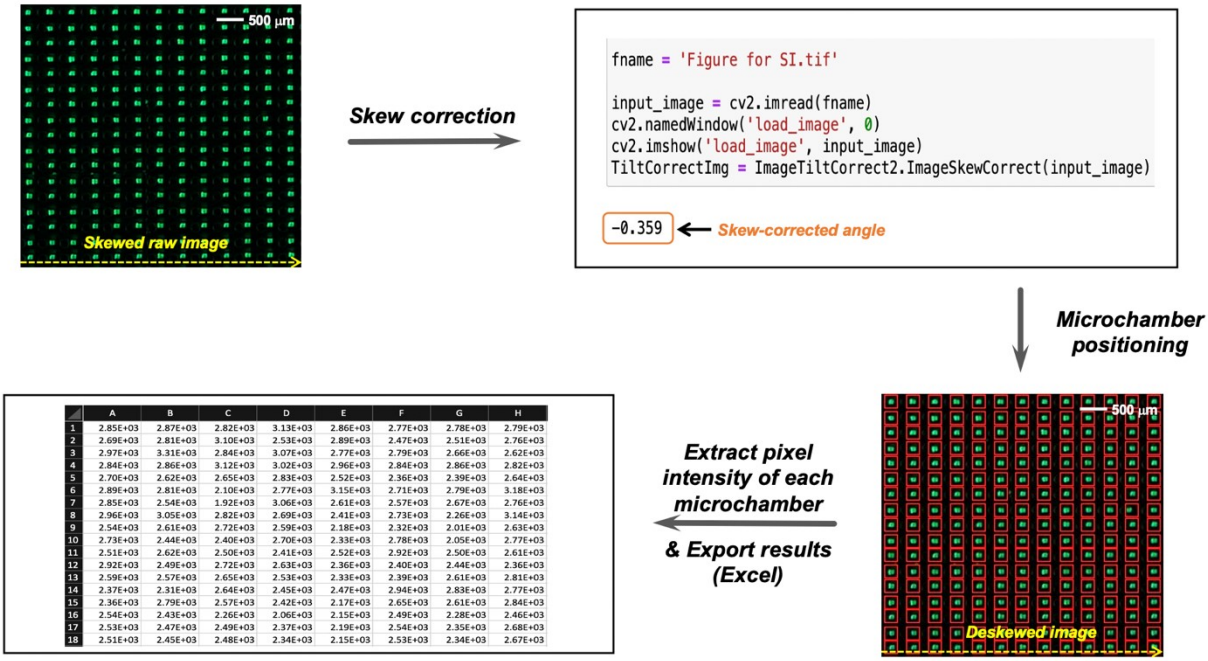


Fig. S1. Fabrication processes of the molds and the multi-step loading chips.



	A	B	C	D	E	F	G	H
1	2.85E+03	2.87E+03	2.82E+03	3.13E+03	2.86E+03	2.77E+03	2.78E+03	2.79E+03
2	2.69E+03	2.81E+03	3.10E+03	2.53E+03	2.89E+03	2.47E+03	2.51E+03	2.76E+03
3	2.97E+03	3.31E+03	2.84E+03	3.07E+03	2.77E+03	2.79E+03	2.66E+03	2.62E+03
4	2.84E+03	2.86E+03	3.12E+03	3.02E+03	2.96E+03	2.84E+03	2.86E+03	2.82E+03
5	2.70E+03	2.62E+03	2.65E+03	2.83E+03	2.52E+03	2.36E+03	2.39E+03	2.64E+03
6	2.89E+03	2.81E+03	2.10E+03	2.77E+03	3.15E+03	2.71E+03	2.79E+03	3.18E+03
7	2.85E+03	2.54E+03	1.92E+03	3.06E+03	2.61E+03	2.57E+03	2.67E+03	2.76E+03
8	2.96E+03	3.05E+03	2.82E+03	2.69E+03	2.41E+03	2.73E+03	2.26E+03	3.14E+03
9	2.54E+03	2.61E+03	2.72E+03	2.59E+03	2.18E+03	2.32E+03	2.01E+03	2.63E+03
10	2.73E+03	2.44E+03	2.40E+03	2.70E+03	2.33E+03	2.78E+03	2.05E+03	2.77E+03
11	2.51E+03	2.62E+03	2.50E+03	2.41E+03	2.52E+03	2.92E+03	2.50E+03	2.61E+03
12	2.92E+03	2.49E+03	2.72E+03	2.63E+03	2.36E+03	2.40E+03	2.44E+03	2.36E+03
13	2.59E+03	2.57E+03	2.65E+03	2.53E+03	2.33E+03	2.39E+03	2.61E+03	2.81E+03
14	2.37E+03	2.31E+03	2.64E+03	2.45E+03	2.47E+03	2.94E+03	2.83E+03	2.77E+03
15	2.36E+03	2.79E+03	2.57E+03	2.42E+03	2.17E+03	2.65E+03	2.61E+03	2.84E+03
16	2.54E+03	2.43E+03	2.26E+03	2.06E+03	2.15E+03	2.49E+03	2.28E+03	2.46E+03
17	2.53E+03	2.47E+03	2.49E+03	2.37E+03	2.19E+03	2.54E+03	2.35E+03	2.68E+03
18	2.51E+03	2.45E+03	2.48E+03	2.34E+03	2.15E+03	2.53E+03	2.34E+03	2.67E+03

Fig. S2. Fluorescence intensity extraction of each microchamber. Raw chip image with slight tilt angle was first skew corrected to align the microchambers. Based on the regular structure of the microarray, each microchamber was precisely located and labeled by a rectangular frame on the deskewed image. The fluorescence intensity of each microchamber was then extracted by accumulating all the pixels within the frame, and the results can be exported into an Excel file for analysis. Scale bar: 500 μm.

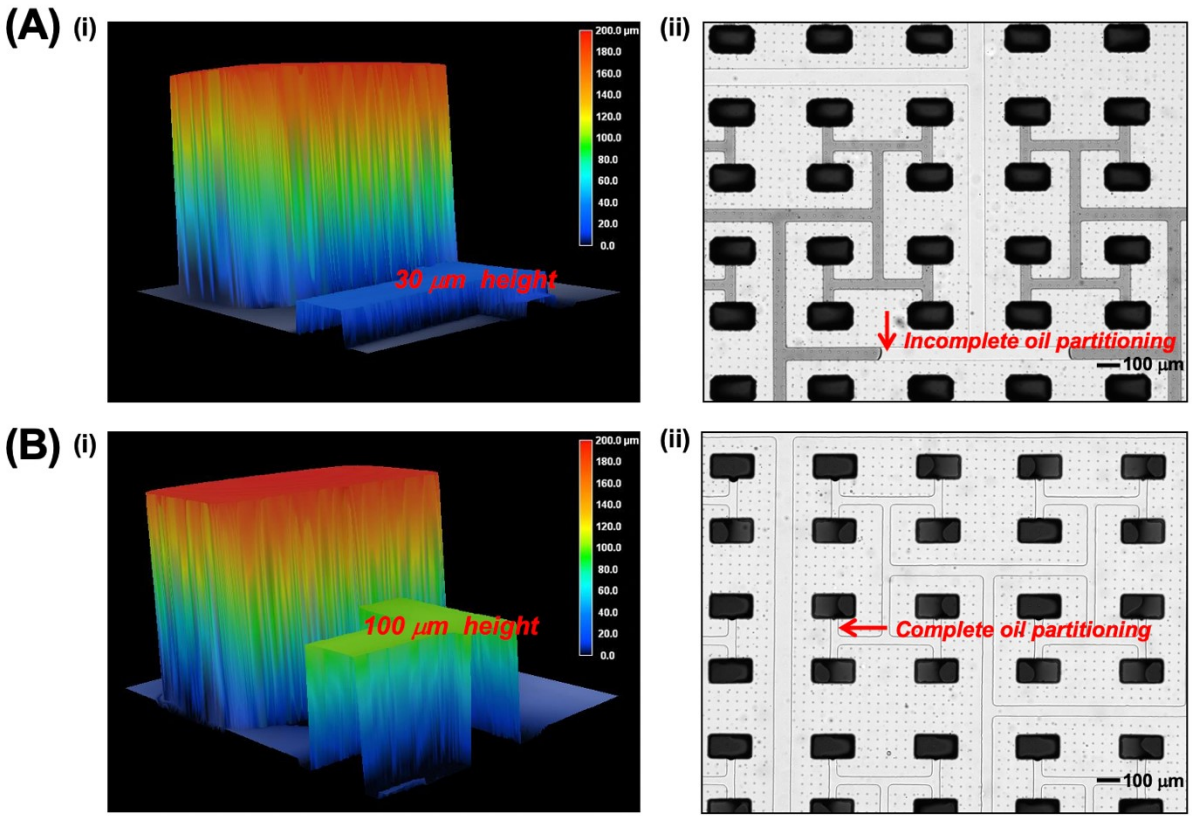


Fig. S3. Optimization of microchannel height to facilitate smooth loading of silicone oil. (A) The surface profile of microarray structure with channel height as 30 μm was acquired using Keyence laser scanning microscope (i). Oil cannot approach the dead-end microchambers in this design due to the high flow resistance (ii). (B) When the channel height was increased to 100 μm (i), oil partitioning can be performed efficiently (ii). Scale bar: 100 μm.

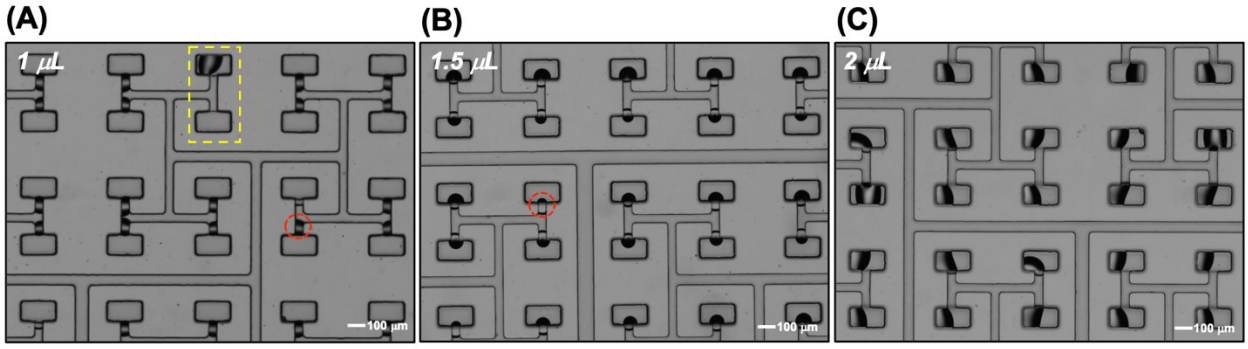


Fig. S4. Sample partitioning effects with different loading volumes. (A) When the loading volume was set to 1 μL (water), it didnot effectively split at some junctions and thus flowed into a single microchamber, leaving the other mcirochamber empty (marked with a yellow rectangle). Some were stagnant within the microchannels (marked with a red circle). (B) When sample volume was set to 1.5 μL, it can well split, but failed to load into microchambers. (C) When sample volume was increased to 2 μL, the sample can be efficiently “digitalized” into all the microchambers. Scale bar: 100 μm.

Supplementary videos:

Video S1. 3-step sample loading and oil partitioning on our device. Before first loading, the device was connected to an external vacuum via tubing for 1 min to create negative pressure and continuously subjected to vacuum in the sequential multi-step loading steps. A 3-step loading and oil partitioning can be completed in 10 min. Specifically, oil was directly added into a pipette tip that was preloaded with the reagent for third-step loading, where a clear oil-water interface can be generated without introducing any air gaps during oil partitioning.

Video S2. Scalable multi-step sample digitalization on our device. Two microliters of red food dye and blue food dye were alternately loaded into the device six times with desirable uniformity.

Video S3. The operation procedure of our device. Precise volume of sample (green food dye as mock sample) that was pre-loaded using a 10-μL pipette tip was autonomously loaded into the device via a vacuum. After the sample loading is complete, the vacuum was disconnected, and the suction layer on top of the device was removed. Then ~2 mL thermosetting oil was poured onto a clean glass slide, and the chip was carefully aligned with the glass slide to avoid trapping air bubbles. Finally, this newly assembled chip was directly put onto a flat PCR machine for amplification.