

Digital Polymerase Chain Reaction in an Array of Femtoliter Polydimethylsiloxane Microreactors

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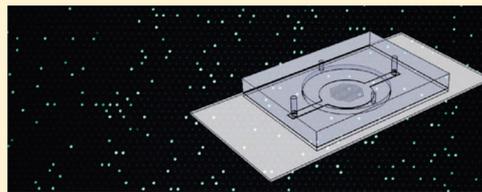
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Supporting Information

ABSTRACT: We developed a simple, compact microfluidic device to perform high dynamic-range digital polymerase chain reaction (dPCR) in an array of isolated 36-femtoliter microreactors. The density of the microreactors exceeded 20 000/mm². This device, made from polydimethylsiloxane (PDMS), allows the samples to be loaded into all microreactors simultaneously. The microreactors are completely sealed through the deformation of a PDMS membrane. The small volume of the microreactors ensures a compact device with high reaction efficiency and low reagent and sample consumption. Future potential applications of this platform include multicolor dPCR and massively parallel dPCR for next generation sequencing library preparation.



Digital polymerase chain reaction, or dPCR, employs parallel, isolated PCR reactions to amplify and/or quantify nucleic acids.^{1,2} By amplifying templates in many separate reactions, each with typically one or zero templates, and then identifying the reactors with and without amplicons, the absolute number of template molecules can be counted without errors from amplification bias or noise. The identification of positive PCR reactions is usually based on TaqMan chemistry³ due to high specificity and signal intensity of the fluorescence-based signal. Digital PCR, originally performed using multiwell plates, has been applied to assess the allelic imbalance, or loss of heterozygosity, in complex cell population such as tumors,^{4–8} to quantitatively measure the concentration of DNA from human samples,⁹ and to count cDNA templates in cancers.¹⁰ Many methods, including statistical analysis^{11,12} and multivolume digital assays,^{13,14} have been applied to extend the dynamic range of dPCR.

With the help of microfluidic methods, dPCR becomes much easier to perform, requires less sample, and reduces contamination during experiments.^{15–17} For example, the glass-based SlipChip provides a simple way to perform dPCR experiments.¹⁸ Polydimethylsiloxane (PDMS)-based microfluidic devices with many microcompartments have been developed to carry dPCR experiments for single bacteria gene analysis,¹⁹ single bacteria–virus infection detection,²⁰ single cell transcription profiling,^{21,22} chromosomal aneuploidy detection,^{23,24} copy number variation studies and validations,^{25,26} absolute quantitative detection of rare mutations in can-

cers,^{27–29} genetically modified food quantification,³⁰ and viral load monitoring.³¹ The same method can be extended to other amplification technologies, such as multiple displacement amplification (MDA)³² and loop-mediated amplification (LAMP).³³ Recently, with two-phase isolation, the density of microreactors for dPCR has been expanded to over 4400/mm² with picoliter compartments, greatly extending the dynamic range to seven logs with a large-scale chip format.³⁴ Another approach, which often employs microfluidics, involves the generation of microemulsions to isolate individual reactions.^{35–39} Although this method is highly scalable,⁴⁰ the emulsion generation process and the reactions are cumbersome and the experiments usually require complex control apparatus.

We present a novel PDMS-based microfluidic device to perform dPCR in femtoliter microreactors. This device utilizes the elasticity of PDMS to create a high-density array of fully sealed and isolated microreactors through PDMS deformation^{41,42} under hydraulic pressure.⁴³ This simple, space-saving design allows for a density of microreactors exceeding 20 000/mm² and allows rapid sample loading with a one-shot pipet injection of less than 4 μ L of sample solution. Compared to previous nano- and picoliter approaches, our femtoliter-scale method reduces the number of thermal cycles required for detection, eliminating false positive counts. This design is

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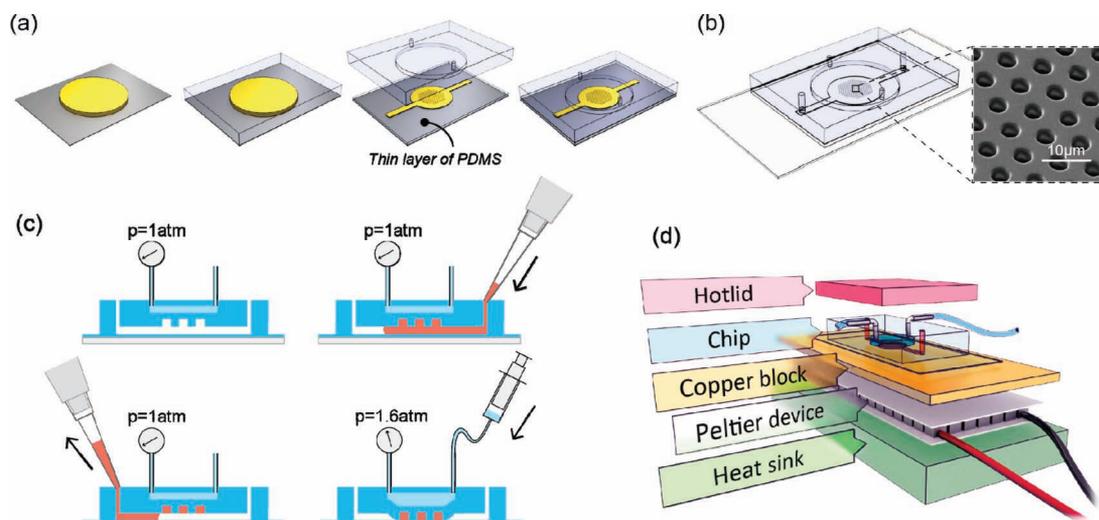


Figure 1. The fabrication, structure, and operation of the dPCR chip. (a) The fabrication procedure for the PDMS dPCR chip. Two molds, both generated through photolithography, are used to cast the two layers of chambers, respectively. The two PDMS slabs are bonded into a monolithic device. (b) The structure of the dPCR chip. The femtoliter microwells are fabricated in the bottom surface of the PDMS membrane between two chambers. The SEM image shows the dimension of the microwells. (c) The dPCR operation. The top chamber was filled with water, for compensating the loss of water during PCR thermal cycles. The sample was injected into the lower chamber to fill all the microwells. Excess sample was removed, and then, the top chamber was pressurized to seal and isolate all microreactors. (d) The thermocycler for dPCR. A Peltier device array, bonded with a copper block, was used to control the reaction temperature. The chip was placed directly onto the copper block.

intrinsically scalable, offering the possibility of performing dPCR with less sample consumption and a dynamic range of 10^5 with a 4 mm^2 array.

We employed multilayer soft lithography to fabricate the microfluidic dPCR chip with a high-density array of microwells (Figure 1a). The mold for the array is made from photoresist (SU-8, MicroChem) patterned with two-step photolithography on a silicon wafer. The chip has two layers of liquid chambers separated by a thin (1 mm), deformable PDMS membrane. The upper and lower chambers are 110 and $70 \mu\text{m}$ tall, respectively. The microwells are in the bottom surface of this membrane. Each well is $3.3 \pm 0.1 \mu\text{m}$ in diameter and $4.2 \pm 0.1 \mu\text{m}$ deep, with a volume of $36 \pm 2 \text{ fL}$ (Figures 1b and S3, Supporting Information). We fabricated a hexagonally close packed array with a period of $7.5 \mu\text{m}$ containing 82 000 wells within a $2 \times 2 \text{ mm}$ array area.

As shown in Figure 1c, we loaded the sample solution into the lower chamber with a pipet, and then, excess solution was removed by evacuating the liquid from the outlet of the chamber before actuating the membrane. Without this evacuation step, excess liquid in the lower chamber can cause imperfect sealing upon membrane deformation and eventually lead to the unreliable quantification. With positive pressure applied to the upper chamber, the PDMS membrane deforms to seal all the microwells, creating isolated microreactors. To prevent evaporation through the water-permeable PDMS membrane, we loaded the upper chamber with water ($\sim 5 \mu\text{L}$). We found that our method to prevent evaporation was insufficient if the thickness of the PDMS membrane exceeded 1 mm. The bottom of the lower chamber is a coverslip coated with a $10 \mu\text{m}$ PDMS layer. The hydrophobic nature of this PDMS layer enhances the sealing of the microreactors. The performance of sealing is evaluated and confirmed through a photobleaching test before each experiment. Fluorescein solution is sealed into the reactors, and a portion of the field of view is illuminated with the laser for a lengthy exposure time. A few minutes after exposure, we take wide-field fluorescence

images (Figure S4, Supporting Information). If the microreactors are well-sealed, a photobleached area can be clearly observed; otherwise, the bleaching pattern vanishes because dyes diffuse between the microreactors.

We built a customized thermocycling system (Figure 1d) for our dPCR chip. This system uses four Peltier devices (TE-31-1.4-1.15R, TE Technology, Inc.) connected in series underneath the chip to control the temperature of microreactors. We also apply another Peltier device to keep the top of the device at 95°C . This hot lid increases the temperature ramping rate and also helps prevent evaporation. The pressure (170 kPa) of the upper water chamber was constantly monitored using an electronic pressure sensor to ensure the microreactors were well-sealed during the experiment. After loading the sample and confirming the seal, we placed the chip on our dPCR thermocycler. Samples were typically run for 30 two-step cycles (5 s at 90°C and 20 s at 55°C) with an initial denaturing step (60 s at 90°C , 60 s at 55°C , 60 s at 90°C , and 60 s at 55°C) for the reaction. The entire experiment is completed within $\sim 35 \text{ min}$.

After dPCR thermocycles, we transferred the chip to an inverted fluorescence microscope (Ti-E, Nikon) with a $20\times$ N.A. 0.75 objective for observation. We used TaqMan probes to detect the individual PCR reactions in each microreactor. Amplified target template within a microreactor generates fluorescent dye that is confined within the microreactor. Microreactors that are positive for template amplification can be detected within 30 amplification cycles.

Fluorescence images were captured with a scientific CMOS camera (Neo-DC-152Q-FI, Andor), and each field-of-view contained 11 855 microreactors ($825 \text{ mm} \times 700 \text{ mm}$, ~ 70 pixels/microreactor). Each array was fully quantified with 9 images. The fluorescence was excited by a 473 nm DPSS CW laser, and the power density at the focal plane was $13 \text{ mW}/\text{mm}^2$. Figure 2a shows a typical dPCR image with ~ 3100 microreactors. After 30 dPCR cycles, the microreactors with zero copies of template exhibit a small amount of residual

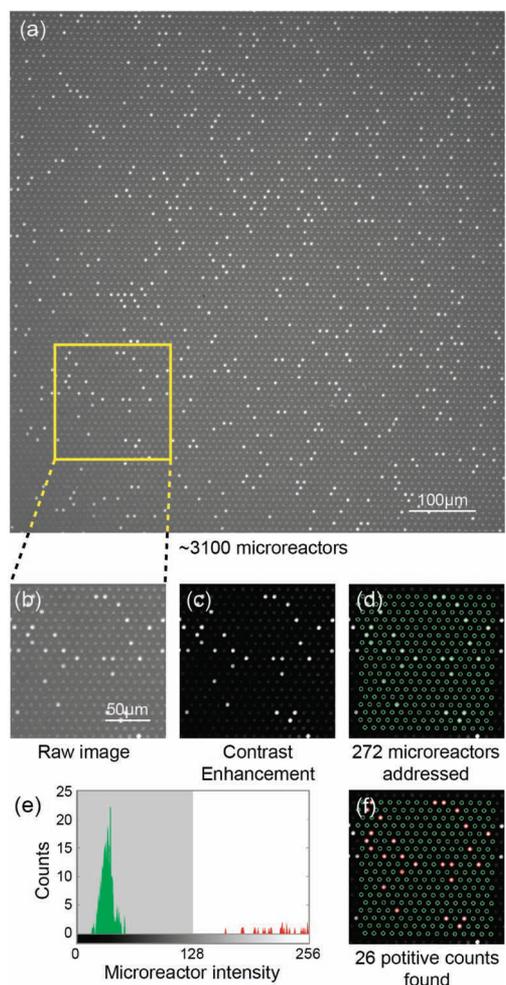


Figure 2. The dPCR quantification pipeline. (a) A region of a raw experimental image containing ~ 3100 microreactors. (b) A region of interest with 272 microreactors. (c) Bright PCR microreactors can be distinguished from dark microreactors after contrast enhancement (see text). (d) Locations of the microreactors are determined. (e) A histogram of the integrated intensity from each microreactor. (f) 26 microreactors are found to be positive.

fluorescence, due to the autofluorescence of PDMS and the background of the TaqMan probe, which is significantly weaker than the fluorescence generated by TaqMan probes in the positive microreactors. We used a top-hat filter to subtract much of this background and enhance the contrast (Figure 2c). The hexagonal alignment of the microreactors allows for simple segmentation (Figure 2d), and the integrated intensities from bright and dark reactors are well-separated (Figure 2e), allowing digital identification of reactors that were positive and negative for template amplification (Figure 2f). This quantification pipeline provides the absolute number of amplicon-containing microreactors, as well as the ratio between the positive counts and the total number of microreactors.

To achieve robust and quantitative dPCR results, proper surface treatment of the microreactors is required. Hydrophobic macromolecules and fluorophores can adsorb to bare PDMS surfaces. Because of the large surface area to volume ratio of the femtoliter microreactors, surface adsorption is an especially acute possibility and could severely hinder the efficiency of PCR. We passivated the microreactors by soaking

the lower chamber with the reaction buffer without template for 30 min and air drying the chamber before loading the sample.

To test our dPCR chip, we synthesized an 88 nt template sequence, from the mouse homeobox A1 gene (*Hoxa1*), and prepared standard solutions with the final concentration ranging from 1.25 to 20 pM. Each sample was tested 3 times, and each experiment was performed on a newly prepared chip. Figure 3a shows two sets of the experimental results. As the template concentration increases, we observed more positive microreactors under the microscope. The bright microreactors are randomly distributed, but the ratios between the positive

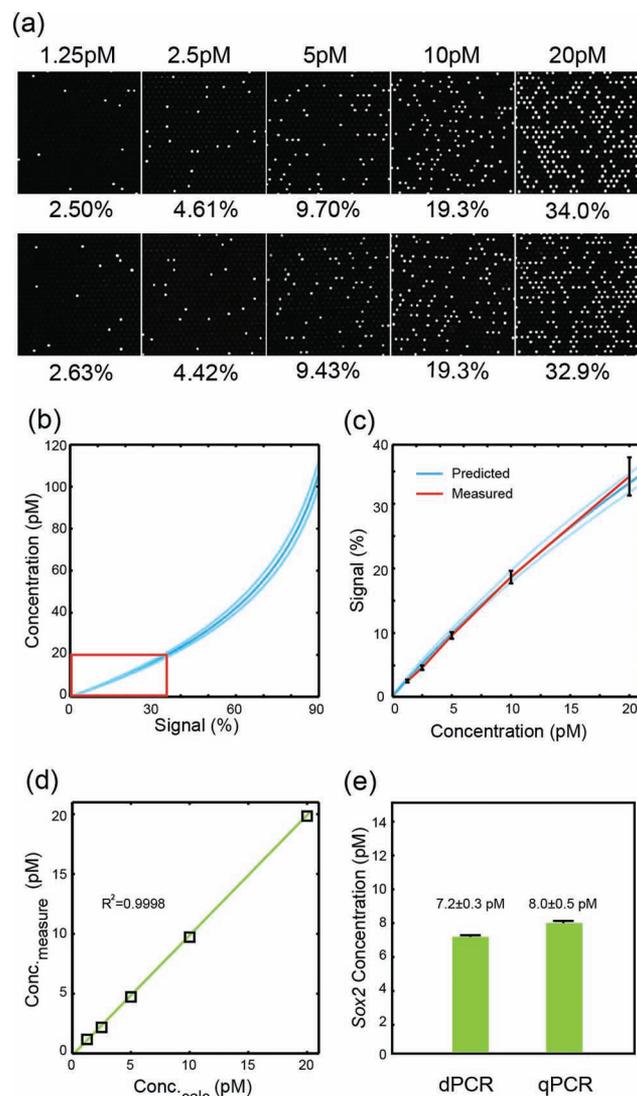


Figure 3. dPCR experimental results (a) Two independent sets of experiments of dPCR with 5 different concentrations of templates. The percentage of the counts of each experiment is shown. (b) The concentration of the templates can be calculated from the fraction of positive microreactors. The thick blue curve is the theoretical result calculated from Poisson statistics. The two thin curves indicated the uncertainties of the calculation due to uncertainties in the volume measurement of the microreactors. (c) The measured fraction of positive microreactors match well with the predicted values according to Poisson statistics. (d) The correlation between the measured concentration and the prepared concentration of cDNA templates. (e) The quantitative real-time PCR and dPCR quantifications of concentrations of *Sox2* gene cDNAs.

counts and the total number of microreactors are consistent in different experimental runs, indicating the robustness of the dPCR chip.

The relationship between the concentration and the expected fraction of positive reactors from Poisson statistics is shown in Figure 3b. Given the measured fraction of positive reactors, we can calculate the absolute number of molecules within all the reaction volumes and, therefore, the concentration of template in the solution. Our experimental results are consistent with the theoretical curve, as shown in Figure 3c. We tested the accuracy of our dPCR system by calculating the correlation between the measured concentration of template from digital counting and the prepared concentration from serial dilutions. The correlation between the two sets of values is 0.9998.

We then prepared a cDNA solution through reverse transcription of total RNA extracted from 1.4×10^5 cells (mouse embryonic stem cells). We applied our dPCR chips to measure the concentration of mouse SRY-box containing gene 2 (*Sox2*), the gene of the key transcription factor that is essential to the self-renewal capacity of embryonic stem cells, from the sample, and compared the result with conventional q-PCR. As indicated in Figure 3e, the concentration was measured to be 7.2 ± 0.3 pM from dPCR and 8.0 ± 0.5 pM from q-PCR.

The high-density microfluidic dPCR platform described here has a number of advantages over other dPCR platforms. The increased density of the array allows us to reach a dynamic range of almost 5 logs with a small sample volume on a tiny chip. Compared with most previous reports, in which 40 or more temperature cycles were typically applied to picoliter or larger reactors, our femtoliter microreactors greatly increase the effective concentration of template and consequently reduce the number of cycles needed. A typical practice in dPCR experiments is to prepare the extremely dilute samples to guarantee that the template copy number in each isolated reactor is reasonably low, enabling the digital counting. Shrinking the size of the microreactors creates the possibility of the use of samples without further dilution, eliminating possible contamination and error during the extra experimental steps. With the greatly reduced volume of each microreactor, we can directly use the samples with picomolar concentrations to perform the experiments. Additionally, compared with many other approaches such as emulsion and larger chips with microchambers, our method requires less time and effort to create a large number of isolated reactors. Many reactions can be integrated into a single device, and the image can be acquired by a scanning-based detection system, which has been widely used for microarrays and DNA sequencers.

The digital counting result can also be exploited to provide the absolute counts of expressed mRNA number of certain genes. In our case, the average number of expressed *Sox2* mRNAs was 3.2×10^2 copies/cell. The ability to count the absolute number of mRNA molecules also demonstrates the possibility of single cell measurements, which will become one of the most important applications of dPCR. The PDMS microreactor array can be seamlessly integrated with other functional components in a single chip for screening, handling, and processing individual cells. Microfluidic integration will greatly increase reproducibility while reducing contamination. In addition, because quantification is based on a microscopic readout, this platform can be easily extended to multicolor dPCR for simultaneous measurements of multiple genes of interest.

Finally, this high-density platform may have applications to high-throughput sequencing sample preparation. High-throughput sequencing libraries are often preamplified using common sequencing primers ligated to a heterogeneous population of DNA fragments to generate a diverse set of DNA fragments to sequence. However, differences in amplification efficiency can cause the relative abundances of different DNA fragments to change.^{44,45} By carrying out this amplification step in microreactors and running the dPCR reaction to completion, we anticipate that this nonlinear amplification bias will be mitigated, as every equally sized reaction chamber will generate an approximately equal number of DNA fragments. This DNA can be recovered by unsealing the reactors and flowing out the products. Such digital amplification will eliminate the biases inherent in bulk multiplex PCR and allow for a more quantitative readout of nucleic acid copy number using sequencing methods.

In summary, we have reported a novel microfluidic device with an array of femtoliter microreactors that can be simply sealed and isolated for digital PCR experiments. The 36 fL microreactors are arrayed at high density, allowing us to reach more than 20 000 reactors/mm². We characterized the performance of the dPCR on-chip and employed this device to test the cDNA sample that was reverse-transcribed from embryonic stem cells. We have demonstrated that this method is robust and reproducible for achieving high dynamic-range dPCR reactions with high efficiency, limited sample volume, and without extensive dilutions. This method is also easily scalable to millions of microreactors within a square centimeter using existing technology and may be extended to multicolor dPCR and to highly multiplexed dPCR reactions.

■ ASSOCIATED CONTENT

📄 Supporting Information

Additional information as noted in text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

P.A.S. and W.J.G. are listed as inventors on a patent application based on this work filed by Harvard University.

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Supporting Information

Digital polymerase chain reaction in an array of femtoliter PDMS microreactors

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1. Experimental

1.1 Mold fabrication

The molds of the PDMS dPCR chip are made of SU-8 photoresist on Si wafers through conventional photolithography.

We used SU-8 2050 to fabricate the top layer mold, with thickness of 100 μm (1700 rpm for 60 s on a Si wafer). We prebaked the wafer at 65 °C for 1 h and then at 95 °C for 1h. After exposure (5 min), we postbaked the wafer at 65 °C for 1 h and then at 95 °C for 1h. The wafer was then developed and then hard-baked at 150 °C for 3 h. The mold of the lower layer, in which the microreactors are located, was made by double layer photolithography with two exposures (Fig. S1). The flow chamber was patterned using 100 μm SU-8 photoresist. A critical step is to make sure that after photoresist spun the wafer is left on the bench over night to release the stress before pre-baking. After exposure, development, and baking, as described above, we then coated another layer of thin SU-8 (5 μm) to pattern the microreactors (SU8 2010, 8700 rpm for 1 min). We prebaked the wafer at 65 °C for 1 h, and the exposure time is 2.5 s. We then postbaked the wafer at 65 °C for 5 min and at 95 °C for 5 min before development. We finally hard-baked the mold at 150 °C for at least 3 h.

1.2 The fabrication of PDMS chip

Both the molds were treated with trimethyl chlorosilane, which allows the PDMS to be peeled off of the mold easily. The procedure is shown in Fig. S2. For the top layer, we mixed the PDMS (Sylgard 184, Dow Corning) base and curing agent at an 8:1 ratio and poured 30 g PDMS over the mold, degassed the mixture, and put it into an oven at 80 °C for 15 min. We then peeled off the PDMS slab from the mold, cut it into rectangular shape and punched two holes for water injection. For the lower layer, the ratio of PDMS was 15:1. We poured 5 g of the mixture over the mold and put it into the oven at 80 °C for 15 min. We manually aligned the top layer onto the lower layer and bonded the two layers together by baking at 80 °C for 30 min. After the top layer bonded with the lower layer, we peeled off the PDMS slab and punched the holes for sample loading. We spin-coated the 8:1 PDMS mixture onto a cover slip at 4000 rpm for 60 s, released the stress for 10 min, and then cured at 80 °C for 20 min, to form a thin layer of PDMS as the substrate of the reaction chamber. The top layers were bonded to this substrate layer by baking at 80 °C over night. The dimension of microreactors is measured by a stimulated Raman scattering (SRS) microscope by monitoring the C-H stretching at 2845 cm⁻¹, and a transmission confocal microscope.

1.3 cDNA synthesis

We purified the total RNA from mouse ES Cells with RNeasy Mini Kit (Qiagen) using the protocol recommended by the manufacturer. The concentration of the total RNA was determined by Nanodrop (Thermo). The first-strand cDNA was synthesized using SuperScript III RT (Invitrogen) by the protocol provided by the manufacturer. The synthesized cDNA was then purified by Enzyme Cleanup Kit (Qiagen).

1.4 Oligo synthesis

We chemical synthesized a few oligonucleotides for testing the performance of the dPCR chip.

- (1) *Sox2* gene (Invitrogen):

```
CCCCTTTTATTTTCCGTAGTTGTATTTTAAAAGATTCGGCTCTGTTATTGG  
AATCAGGCTGCCGAGAATC
```

- (2) *Hoxa1* gene (IDT):

```
TTAAGAAGGCCTGCCAAGCACTTTGCCTGTGCCAGGTCTTCCCAAACCTCT  
AACCAATTCTTCCCTGCTTCTCTGGCCACCCTGCATTTA
```

- (3) *Sox2* primers:

```
CCCCTTTTATTTTCCGTAGTTGTATT  
GATTCTCGGCAGCCTGATTC
```

- (4) *Sox2* TaqMan-MGB-probe:

```
AAGATTCGGCTCTGTTATT
```

- (5) *Hoxa1* primers:

```
GGCCTGCCAAGCACTTTG  
AGCAGGAAGAATTGGTTAGAGTTTG
```

- (6) *Hoxa1* TaqMan-MGB-probe:

```
CTGTGCCAGGTCTT
```

1.5 Reagent for dPCR and pre-runs

Component	PCR solution (20uL)	Pre-run solution (20 uL)
MgCl ₂ (50mM, Invitrogen)	2.4 μ L	2.4 μ L
10X PCR buffer (Invitrogen)	2 μ L	2 μ L
BSA (10mg/mL, NEB)	0.4 μ L	-
dNTP (10mM each, NEB)	0.8 μ L	-
Platinum Taq DNA polymerase (Invitrogen)	1.5 U	0.8 U
Forward primer (18 μ M)	1 μ L	2 μ L
Reverse primer (18 μ M)	1 μ L	2 μ L
Taqman MGB probe (10 μ M, Invitrogen)	1 μ L	-
Template	1 μ L	-

1.6 Digital PCR Experiment

We used a pipet to inject the sample solution (typically $\sim 5 \mu\text{L}$) into the reaction chamber of the chip, incubated at room temperature for 5 min and then sucked out the excessive fluid. We applied 170 kPa to the top layer, filled with water, to push down the PDMS membrane and to seal and isolate the microreactors. We placed the chip onto the home-developed thermo-cycler, using a drop of mineral oil to facilitate the heat conductance between the copper block and the glass cover slip. We then covered the chip with a hot lid, setting at 90 °C. The temperature setting for the thermal cycling was set to be: starting temperature: 90 °C 60s, 55 °C 60s, 90 °C 60s, 55 °C 60s; cycling temperature: 90 °C 5s, and 55 °C 20s, for 30 cycles. After the reaction, we moved the chip on the stage of an inverted fluorescence microscope (Nikon Ti-E, with CNI 473 nm solid laser and Semrock GFP-A-Basic-000 filter cube). The fluorescence images were captured by an sCMOS camera (Andor) with exposure time of 1s.

To test the sealing of a dPCR chip, we loaded the chip with 500 nM fluorescein solution in 1X PCR buffer, and observed the stability of photobleached pattern under the sealing condition (Fig. S4). We typically did pre-runs to passivate the PDMS surface. After soaking with pre-run buffer for 30 min at room temperature, the dPCR chips are ready for experiment. For each gene expression counting dPCR, we used 5 μ L cDNA template solution, which equals to cDNA extracted from $\sim 1 \times 10^6$ cells.

1.7 Data processing

We flattened the background of each raw image using the top-hat method. We then manually determined a series of center coordinates of the microreactors in the image and calculated the basic unit vectors to obtain the location of all microreactors. The intensity of each microreactor was the average intensity of all pixels inside the microreactor. Through the histogram of the image we found two peaks. We set a threshold in the middle of these peaks, and the counts with intensity above the threshold were the positive counts.

2. Supplementary Figures

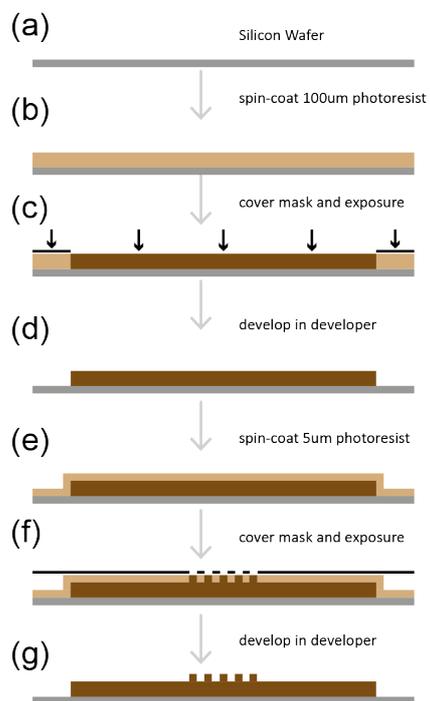


Fig S1 The fabrication of the mold of sample chamber.

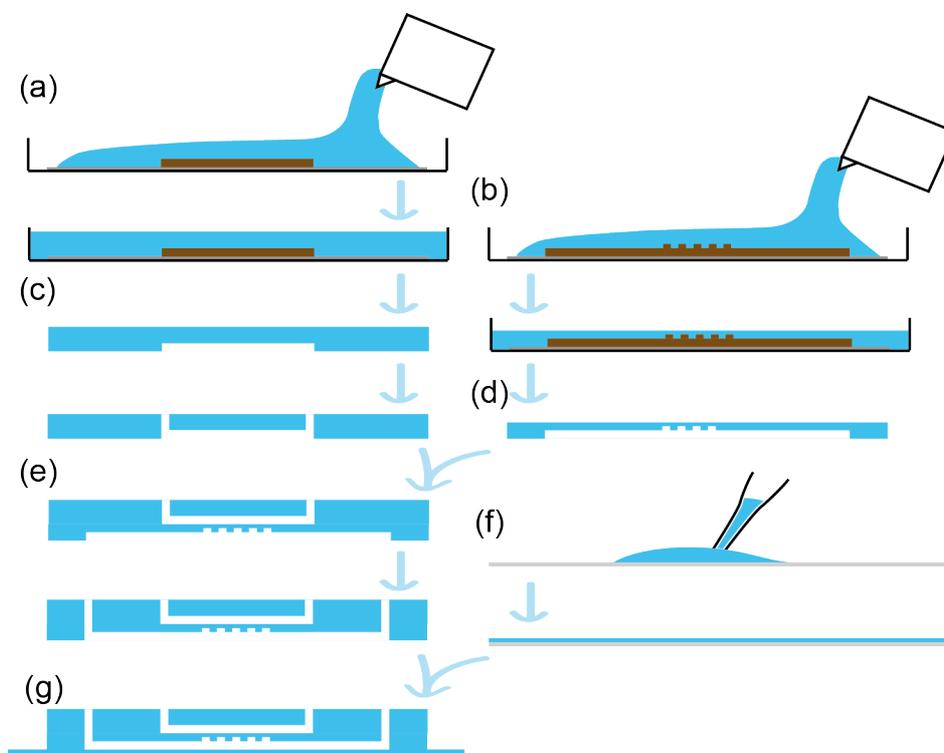


Fig S2 The fabrication procedure of a dPCR chip.

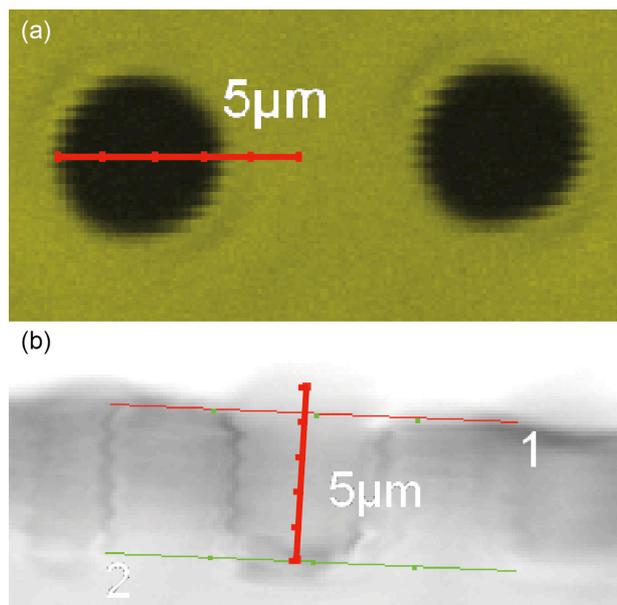


Fig S3 The measurement of the microreactors through SRS microscopy (a) and transmission confocal microscopy (b). The diameter is measured to be $3.3\ \mu\text{m}$ and the depth is $4.2\ \mu\text{m}$. The uncertainty of the measurement is around $0.1\ \mu\text{m}$ after multiple measurements.

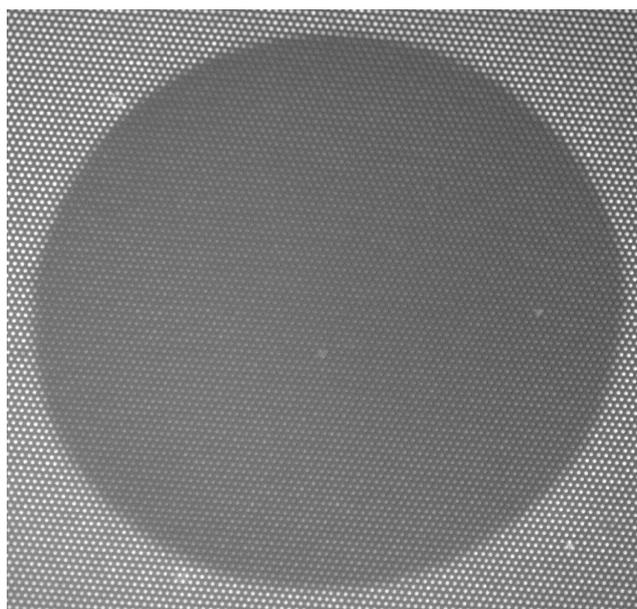


Fig S4 The image of a photobleached area, containing hundreds of microreactors when sealing is well performed. After photobleaching, we wait for 5~10 min to capture the fluorescence images. The clear edge of the bleached pattern indicates the good sealing.