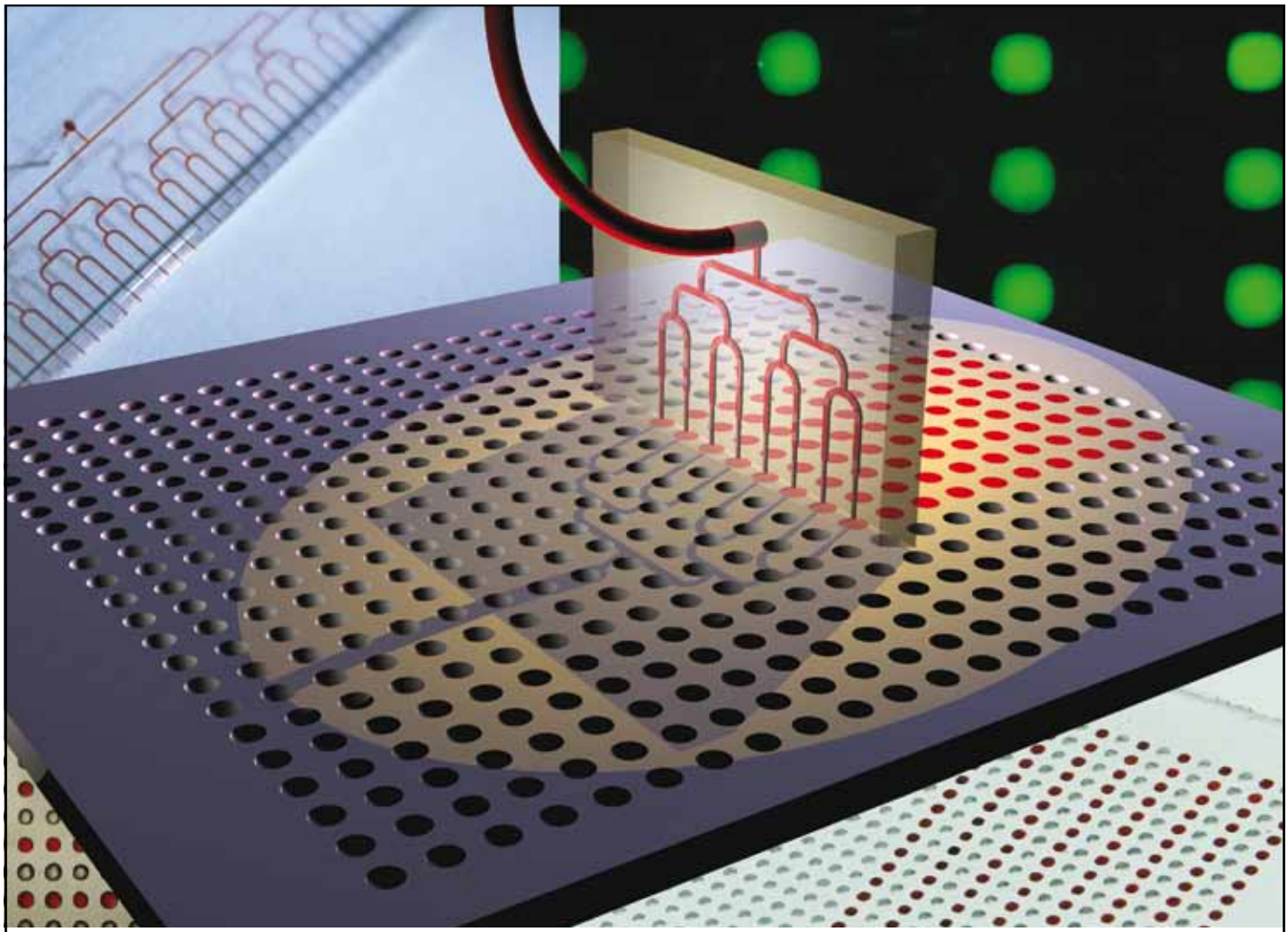

Featuring research from the groups of Prof. Yanyi Huang and Prof. Jianzhong Xi at the College of Engineering, Peking University, Beijing, China.

Title: A chip-to-chip nanoliter microfluidic dispenser

A high-throughput, contamination-free, chip-to-chip nanoliter microfluidic dispenser is demonstrated to perform the accurate dispensation of liquid samples from tens to hundreds of nanoliters, indicating the high flexibility and wide applications of this novel system.

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A chip-to-chip nanoliter microfluidic dispenser†

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A high-throughput microfluidic device is developed to handle liquid dispensation in nanoliter range. The dispenser system shows no cross-contamination between the microwells, indicating its great potential in large-scale screening experiments. An array of 115 nl PCR reactions, as well as the single channel addressable chip demonstrate the high flexibility and wide applications of this novel system.

Most biochemical reactions and cell based chemical assays, especially the large-scale screening experiments and single cell studies, are performed in liquid phase with a volume range from nanoliters to microliters.[1–5] A small volume reaction not only reduces the cost for each reaction, but also contributes to the increase of the reagent concentration for an efficient reaction, and generates much less waste. These experiments are usually laborious, most of them requiring numerous times of pipetting actions to transfer the liquid samples. The accurate small-volume sample dispensation, which may be easily achieved by commercially available instruments in the range of microliters but difficult to scale down to nanoliter range, remains one of the major challenges in high-throughput reactions. On the other hand, a few alternative methods, including non-contact liquid-jet printing[6–8] and ultrasonic droplets generation,[9] have been introduced to liquid transfer. But most of these technologies deal with liquid in picoliter range. They are expensive and hard to be integrated into a highly parallel way for higher throughput fashion. Microfluidic technology shows promising potentials for accurate liquid manipulation, making itself an ideal platform for nanoliter scale reactions.[10–13] A high-throughput miniaturized reaction array with accurate sub-microliter liquid handling is becoming a powerful tool for drug discovery,[14,15] target gene detection,[16] protein crystallization,[17–21] reaction condition screening,[22–24] and cell related studies.[25–28] Several methods of effective dispensation have been reported as alternatives to conventional methods, including highly integrated chips with multi-layer soft lithographically fabricated pneumatic valves,[29,30] and time-serial droplet based microfluidic chips for parameter screening.[31–33]

Although these methods dramatically reduce the reaction volumes and improve the experimental throughput, they are usually designed to work in closed reaction space, *i.e.* the PDMS micro-compartments or liquid plugs. A comparable nanoliter liquid dispensation with open configurations, which makes the inter-device mass transfer, multi-step dispensation/reaction and sample access much easier, is also desirable. A few examples, such as the open-end microchannel electrospray devices for online mass spectrometry and for MALDI-MS sample loading,[34–36] and the microwell filling method by PDMS microchannel degassing,[37] have been demonstrated recently. Some reports[38,39] on microfluidic spotters show promising solutions to handle small samples. Herein we present a novel high-throughput microfluidic nanoliter liquid dispensation system with open accessibility and improved reproducibility and controllability. This method also reduces the expense and manipulation difficulty at the same time. Although a multichannel spotting device has been reported,[38] our system extends the application of open-end microfluidic sample dispensation devices by introducing the surface modified microwell arrays and automated moving process. This configuration provides a new approach to reduce the difficulty of forming uniform droplets at the end of branched outlets,[39] making the sample dispensation an easier operation for high throughput small-volume reactions.

The dispensation system consists of two major components: a microfluidic dispenser chip and a glass microwell array chip, as shown in Fig. 1. We fabricated microfluidic chips with parallel microchannels through a simple soft lithographic[40–42] method. The mold for thick PDMS replicas (thickness ~ 4 mm) was a silicon wafer with positive relief channel patterns made of SU-8 photoresist (Fig. 1c). We then peeled the cured PDMS replicas off the wafer, punched the inlet holes, and placed them on a freshly cured thin layer of flat PDMS (thickness ~ 200 μm) to bond these two layers to form fully sealed microchannels. After curing, we cut the PDMS chip with a razor blade (by hand, easy to perform), making these microchannels openly accessible (Fig. 1f). The cut was sharp and straight within the whole area of the chip when examined by microscope. We left the facet of the chip with a small angle (~15°) so that the channel outlets locate on the tip of the facet for easier alignment and dispensation operation.

The chip is designed to have binary divided bifurcating channels with arc turns so that fluidic resistance for all branches is equal,[26] ensuring the identical dispensation volume for each outlet. In the main body of the chip, the height of the microchannels is 100 μm and the width is 200 μm. We tapered the end part of the microchannels to generate the quadrate exits (100 × 100 μm in cross-section) for the outlets. We employed a chrome coated glass plate to generate the microwell array through photolithography and wet etching. The

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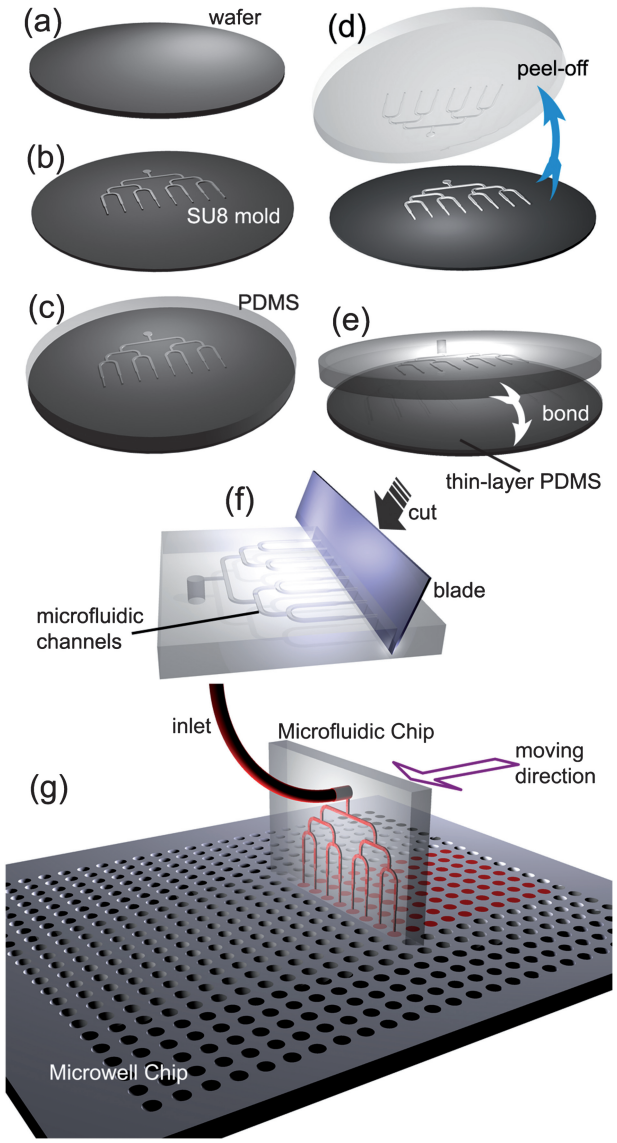

Fig. 1 Fabrication process of microfluidic dispensers. (a) A layer of SU-8 photoresist is spin-coated on a silicon wafer. (b) SU-8 mold is fabricated. (c) Uncured PDMS is poured on the mold and cured. (d) Peel the PDMS replica off the mold and punch the holes as inlets. (e) Bond the replica against a flat thin layer of PDMS on the silicon wafer. (f) Cut the chip with a razor blade to create open channels. (g) Align the microfluidic chip perpendicularly to the glass microwell chip for sample dispensation. The relative position of the chips is controlled by a 3-axis motorized moving stage.

volume of each single microwell is around 120 nl (a bowl shape with ~850 μm diameter and ~250 μm depth) and the pitch between the microwells matches the interval of the dispensing outlets of the corresponding PDMS chip (1.3 mm). Since it is crucial to guarantee the relative position of the two chips during the dispensation, a three-axis motorized moving stage is used to control the movements of the chips. We applied compressed air to drive the fluid flow and to deliver the nanoliter liquid samples into microwells. The perpendicular microfluidic dispensing chip was moved to align over the microwell chip (0.8–1.0 mm above the chip). Once it was well aligned, liquid samples were injected into the targeted microwells. A computer program ensured the dispensation process was highly precise and swift (>3 row/s).

We observed that with a low driving pressure, the flow rates in different microchannels were slightly different, because the resistance in different routes were not exactly the same due to the imperfection of microfabrications. Therefore we applied a relatively high pressure (varies with different chip design, >0.03 MPa for a 16-channel chip), saturating the flux capacity of all the channels to minimize the unevenness between channels. We could control the size of the droplets by applying pulsed pressure and adjusting the pressure and/or the pulse width (with accuracy to milliseconds with a computer controlled solenoid valve). We performed the dispensation with both ferric thiocyanate ($Fe(SCN)_3$) and fluorescein to generate the

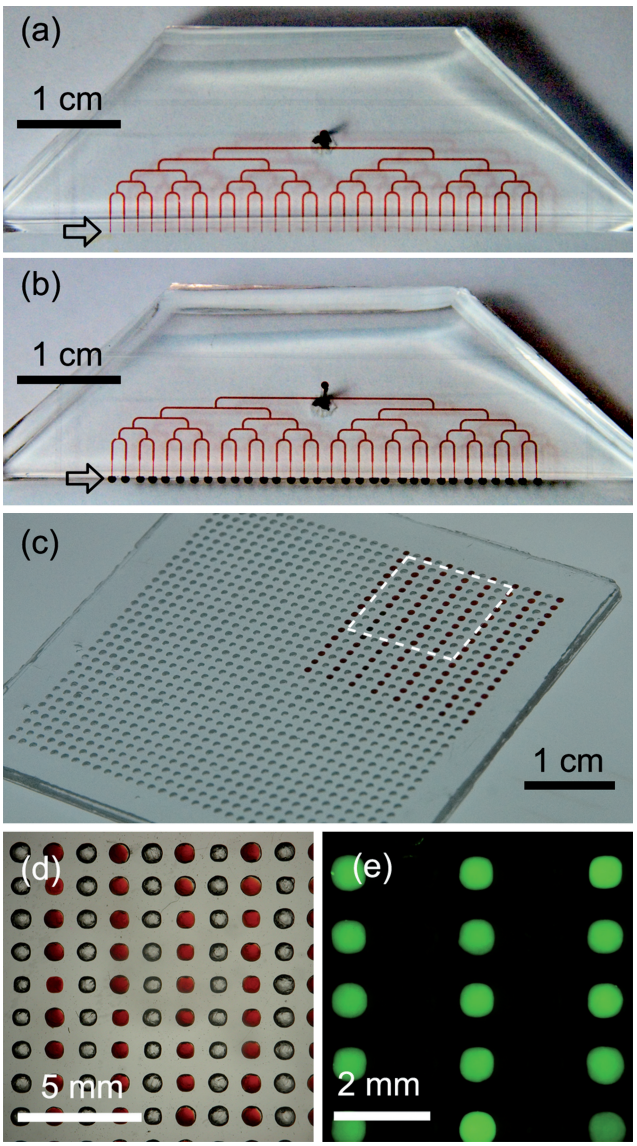

Fig. 2 A 32-channel microfluidic dispenser chip (a) before and (b) during the dispensation. (c) Fill the alternative columns of a microwell array using a microfluidic chip with the double-channel-width gap between the adjacent channels. (d) Micrograph of the dash square area in (c). (e) Fluorescent micrograph of a microwell chip with fluorescein solution dispensation.

microarrays of liquid samples (Fig. 2c–e) from 56 to 170 nanoliters with current design of microwells. The lower limit of volume is controlled by channel cross-section, the gap between the two chips and the surface tension. We can change the former two to achieve different dispensation range while the surface tension is controlled by the materials themselves. To determine the dispensation volume, we measured the length of sample plug consumed in the Tygon tubing and calculated the volume assuming a uniform inner diameter. The range of the dispensation is related to microwell dimension (see ESI).† The coefficient of variation (CV) of the dispensation at 115 nl is less than 6% by colorimetry, fluorimetry and image processing and higher when the dispensation volume reached the lower or higher limits of the microwell containers (CV ~ 14% for 56 nl and ~10% for 80 and 170 nl) due to the physical shape of the wells.

The plateau of the microwell chip was selectively modified with perfluorosilane using microcontact printing[43] to create a hydrophobic surface with the intact intrinsic hydrophilic surface inside the microwells. Control experiment showed that such water reception discrimination plays a key role to guarantee the successful dispensation. The perfluorosilane treatment is easy to perform and does not need a clean room. The graft is so robust that it can maintain the property after we submerge the plate in 1 M hydrochloric acid for cleaning. In order to further understand the dispensation process, we monitored it with a high speed CCD camera to capture the transient actions. Frame-by-frame analysis of the slow motion movies (also see ESI)† shows a complete dispensation includes four steps (Fig. 3a–d): (1) move the dispenser outlets towards the microwells, (2) generate droplets by a pressure pulse, (3) the droplets grow and touch the bottom of the microwells and wet the hydrophilic surface inside the wells, (4) droplets stay in the microwells and the channels are ready to move to the next destination. Such process suggests that there is no connection between the adjacent wells, preventing the reagent contamination between the wells. For experimental validation, we placed $FeCl_3$ and KSCN into alternative rows (see ESI, movie 1),† dried the wells, and refilled all the microwells with pure water (see ESI, movie 2).† If any detectable aliquot from a microwell had entered a neighbouring well, a dark red product $Fe(SCN)_3$ would be observed. The result (Fig. 3f) indicates no detectable (<100 ppm, see ESI)† cross-contamination between the wells. Usually it takes more than 10 minutes to completely dry the wells and our fully automated system can finish the three-round dispensation mentioned above in less than 2 minutes. We did not control the humidity on purpose and we found the evaporation was negligible under our experimental conditions. However, if an ultra-high throughput experiment is performed, humidity control should be considered.

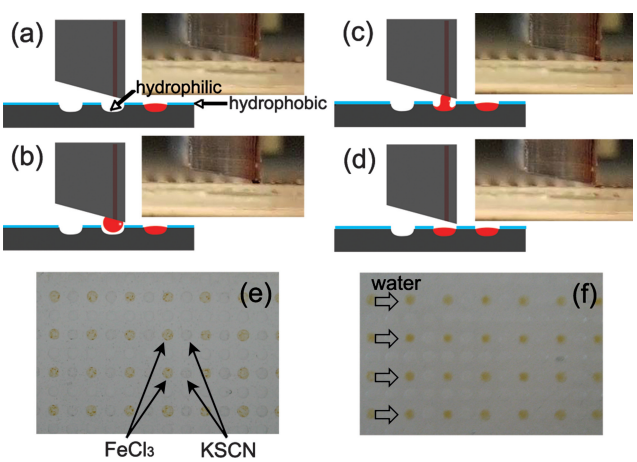

Fig. 3 Illustrations and pictures of the dispensation process. (a)–(d) Dispensation process. (e) $FeCl_3$ (yellow) and KSCN (clear) solutions are dispensed into alternative microwells. (f) After drying the microwells, refill with water.

We further performed Polymerase Chain Reaction (PCR) to explore the dispenser's feasibility in biochemistry applications. We chose pDsRed1 plasmid as template and designed the primers and a Taqman probe according to a previous report.[44] We loaded 115 nl mixed reagents into each microwell, and examined the products after 35 thermal cycles. Experimental group showed significant increase of the fluorescence in the wells (Fig. 4a, 4b). The amplicon extracted from the single wells and the 20 wells were detected in the experimental groups, while no detectable band was observed in the control groups (Fig. 4c).

To extend the application of our dispenser, we integrated an additional control layer containing monolithic elastomer valves and the microfluidic multiplexer (Fig. 5a).[19,29,30] We changed the height of the fluid channels to 10 μm for the complete seal of the microvalves. We also modified our dispensation method by adding a main valve for all the channels and by applying constant pressure to the sample

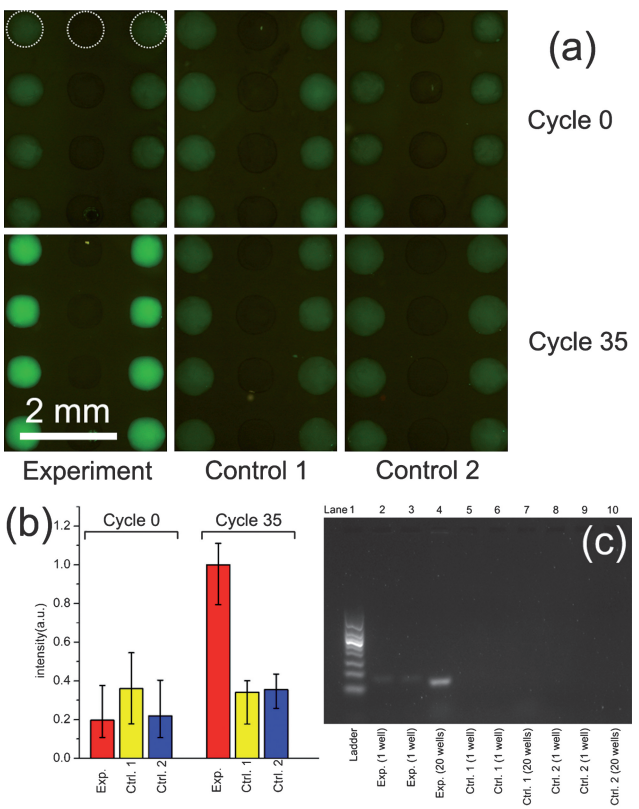

Fig. 4 Taqman PCR result of sequence in DsRed1 gene. The pre-mixed PCR reagents are dispensed into the microwells and amplified for 35 cycles. Control 1 does not contain template (vector) and control 2 does not contain primer. (a) Fluorescent micrographs of the microwell chip. The dashed circles indicate the locations of microwells. (b) Fluorescent intensities of different groups. (c) Agarose gel electrophoresis of the amplicons.

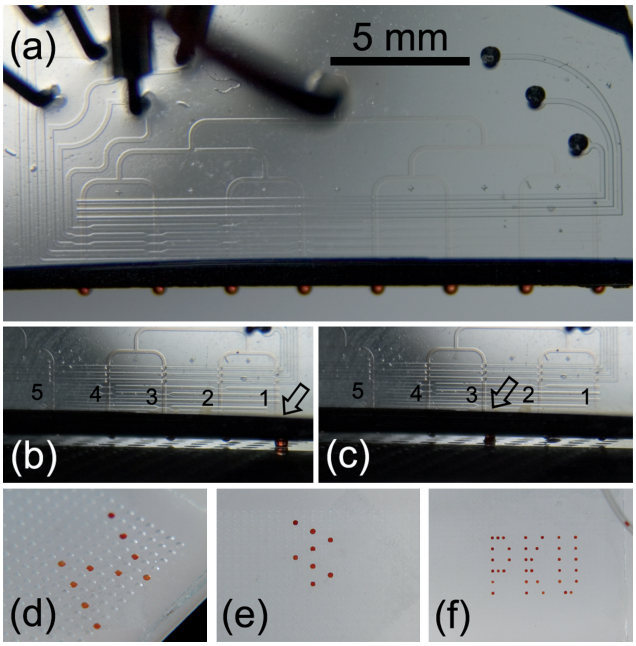

Fig. 5 Single-channel addressable microfluidic dispenser. (a) Micrograph of an 8-channel microfluidic dispenser with control channels. (b) and (c) Channel 1 and 3 are selectively opened. (d)–(f) Patterns printed by dispenser: the character "N", zigzag and "PKU", the abbreviation of Peking University.

inlet. During the dispensation, we selected the channel through the multiplexer and opened the main valve by releasing the pressure in the control channel. The pictures show the switch among different channels clearly (Fig. 5b and 5c show the droplets generated from the first and third outlets, respectively). Since each single channel was addressable, patterns could be designed at will (Fig. 5d–f), which makes the dispensation highly flexible, thus an ideal choice to handle complicated high-throughput reactions.

In summary, we have developed a microfluidic dispenser for high-throughput nanoliter liquid sample dispensation. It provides an inexpensive and compact solution for liquid sample distribution in the range of tens to hundreds of nanoliters. We have achieved a CV < 6% for 115 nl dispensation and 3 row/s dispensation speed. We have also demonstrated 115 nl PCR using dispenser with microwell arrays. We anticipate that with the development of single-cell and single-molecule-based assays, this microfluidic dispensing system has great potential in large-scale chemistry and biochemistry experiments. We are also working on more sophisticated applications and on optimizing the detailed processing parameters.

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A chip-to-Chip nanoliter microfluidic dispenser

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Supporting Information

1. Microfluidic Dispenser Fabrication

We fabricated the microfluidic dispenser chips from poly(dimethylsiloxane) (PDMS) with multilayer soft lithorgraphy technology.

Single Layer Chip

First, we spin-coated a layer of SU-8 (Microchem, Newton, MA) 2050 photoresist (1700 rpm, 60 s, 100 μm) on a 3″ silicon wafer, to a thickness of approximately 100 μm, and baked it on a hotplate at 65ºC for 5 min and 95ºC for 8 min. The SU-8 was exposed to 300 mJ/cm^2 of collimated UV light (365 nm center wavelength) through a high-resolution transparency mask containing the design of the flow channels. The mask was printed at 20,000 dpi from an AutoCAD (AutoDesk, San Rafael, CA) design file. Then the SU-8 was baked at 65ºC for 3 min and 95ºC for

10 min, and then developed. The wafer was finally baked at 150ºC for 3 hours to fully crosslink the SU-8.

We treated mold with trimethylcholorosilane (Sinopharm, Beijing, China) vapor for 5 min at room temperature and poured on it uncured PDMS (RTV-615, GE Advanced Materials, Wilton, Connecticut, 5:1 ratio) to a thickness of 4 mm. After curing the PDMS by baking at 80ºC for 1 hour, the thick flow layer was peeled off the mold and punched for inlets on predefined locations, using a 20 gauge round hole cutter. These inlets were connected through Tygon tubing (0.020″ ID, 0.060″ OD, S-54-HL, Saint-Gobain Performance Plastics, Akron, OH, USA) with 23-gauge stainless steel tubes. Then the thick layer was bonded to a thin, cured, spin-coated layer of PDMS (20:1 ratio, 500 rpm 60s, ~200 μm, 80ºC for 1 hour) on a 3″ silicon wafer. We baked the device at 80ºC overnight and peeled the bonded PDMS layers off the flat silicon wafer. Before application, the chip was cut by a razor blade to form outlets. It is important to place the blanket layer face-up when cutting the chip to produce smooth edge.

Addressable Chip

The chip consists of two layers of PDMS, one with the channels where liquid flow occurs (flow layer), and the other with dead-ended channels that control the valves (control layer).

Both the molds for flow and control layer were made from AZ-P4620 positive photoresist (AZ Electronic Materials, Somerville, NJ, USA). We treated a 3″ silicon wafer with hexamethyldisilazane (Alfa Aesar, Ward Hill, MA, USA) vapor for 5 min at room temperature. Then we spin-coated the AZ photoresist onto the wafer (1000 rpm, 60 s) to a final thickness of 10 μm and baked at 65ºC for 3 min and 95ºC for 5 min. The photoresist was then exposed to 220 mJ/cm^2 UV light (365 nm) through a high-resolution transparency mask containing the design, and developed in AZ 400K developer (1:1 diluted by water). Finally, after soaked in water for 30 min to remove remnant developer, the photoresist was re-flowed and hardbaked on a hot plate ramped from 80ºC to 150ºC at 10ºC/hour, to obtain rounded patterns.

Before the PDMS chip fabrication, both molds were treated with trimethylcholorosilane vapor for 5 min at room temperature. We poured uncured 5:1 PDMS onto the control mold to a thickness of 4 mm to form the control layer. The flow layer was made by spin-coating uncured 20:1 PDMS onto the flow mold (2000 rpm, 60 s) . It then stayed horizontally for 20 min to form a uniform 35 to 40 μm thick layer. After curing the PDMS on the molds by baking at 80ºC for 1 hour for both layers, the thick control layer was peeled off its mold, punched for inlets and aligned over the fluid layer. Irreversible bonding of the two layers was achieved by baking them at 80ºC for 1 hour. After bonding, the layers were peeled off the flow mold, punched for fluid inlets and bonded to a thin, cured, spin-coated layer of PDMS (20:1 ratio, 500 rpm 60s, ~200 μm, 80ºC for 1 hour), by baking at 80ºC overnight. We finally peeled the device off the flat silicon wafer. Before application, the chip was cut by a razor blade to form outlets.

2. Glass Microwell Array Plate Fabrication

The microwell array was etched from a glass plate with a photo-lithographically patterned chrome (Cr) film. We found that the original positive photoresist (Shipley S1805) on the Cr film was fragile and may produce unwanted pinholes in the glass after wet etching. We removed the original photoresist with acetone and spin-coated SU-8 2010 (2000 rpm, 60 s, ~15 μm) onto the Cr film as a replacement and prebaked it on a hotplate at 65ºC for 3 min and 95ºC for 5 min. The SU-8 was exposed to 150 mJ/cm^2 365 nm UV, postbaked at 65ºC for 3 min and 95ºC for 5 min and then developed. The plate was finally baked at 150ºC for 3 hours to fully crosslink the SU-8.

The Cr film was patterned by a home made etchant (5 g $(NH_4)_2Ce(NO_3)_6$ and 4 ml concentrated nitric acid in 50 ml water). This layer of Cr was later used as etch-mask for glass (*Caution: the used etchant contains Cr^{6+} and is extremely toxic. It must be treated with reducer such as $FeSO_4$ before disposure*).

For protection, the backside of the glass plate was then coated with a layer of cured SU-8 before etching. Due to the isotropic etching process using a mixture of

hydrofluoric acid and hydrochloric acid ($HCl:HF:H_2O$ = 1:1:2), the diameters of the microwells in the glass plate increase as they go deeper. This effect had been taken into account in the mask design to compensate the diameter increase. After 2-hour etching, the microwells were in the shape of bowl with ~ 850 μm diameter and ~ 250 μm depth. The volume for each microwell is ~ 120 nl. The remaining SU-8 and Cr were removed by hot Piranha solution (a 3:1 mixture of concentrated H_2SO_4 and 30% H_2O_2 solution) and Cr etchant, respectively. (*Caution: Pirahna solution is very acidic and corrosive, and explosive when mixed with organic solvents. Mixing the solution is exothermic and it should not be stored in a closed container.*)

We used perfluorosilane (1H,1H,2H,2H-perfluorodecyltriethoxysilane, 97%, Alfa Aesar, Ward Hill, MA, USA) to modify the glass surface. 0.8 ml silane solution (5 μl silane in 2.5 ml toluene) was spin-coated (1000 rpm, 15 s) on a silicon wafer and then transferred onto a plat PDMS stamp for 5 min. We then stamped the PDMS onto the microwell plate and let it sit for 20 min for reaction. The contact angle of modified glass was 95°. This silane treatment is critical for a successful dispensation. After the contact transfer of silane, when we poured the aqueous solution onto the treated plate, it went straight into microwells. An untreated or uncompleted treated microwell plate would cause cross contamination between adjacent microwells.

3. Dispensation

We used a home-made CNC control box to drive a 3-axis moving stages, with a commercial control software (Mach3, Newfangled Solutions, Carmel, ME). We employed a digital I/O device (USB-6501, National Instrument, Austin, TX, USA) with a home-made driving circuit to control a solenoid valve (S15MM-30-12-3E, Pneumadyne, Plymouth, MN, USA) to switch compressed air on and off. A LabVIEW (National Instrument, Austin, TX, USA) program was also used to control the single channel addressable dispenser with integrated valves. The moving speed of the stages was 20 mm/s for X and Y axis and 10 mm/s for Z axis. Before the dispensation, we aligned the microwell glass chip and the PDMS microfluidic dispenser chip by adjusting the moving stages and two rotating stages. During the

dispensing process, we moved the dispenser chip directly above the wells and applied a pressure pulse (0.05 MPa, 18 ms) to drive the liquid through an 8-channel PDMS dispenser chip, and waited for 30 ms for the droplets to grow. Such parameter usually fills the microwells with about 115 nl liquid (variable with connection resistance and other conditions; calibration is needed if specific volume is desired). An even higher pressure (0.06-0.07 MPa) was applied to fill the microwells completely. For the single channel addressable dispenser, we applied a constant pressure in the fluid layer (0.06 MPa) and actuated the microvalves with a higher pressure (0.15 MPa). We controlled the addressable dispensing by releasing the pressure of the microvalves of certain channels through a LabVIEW program. Every single channel dispensation took 100 ms to complete the valve-switching and droplet generation. The slow-motion movies were taken with a high-speed CCD camera (Marlin F-033B, Allied Vision Technologies, Germany) at 200 frame/s and a digital camera (EX-F1, Casio, Japan) at 1200 frame/s and reedited to adjust the frame rate for viewing.

To investigate the dispensation range, we filled 128 microwells with both ferric thiocyanate ($Fe(SCN)_3$) and fluorescein using a 4-channel dispenser with different dispensation parameters. We measured the length of sample plug consumed in the Tygon tubing and calculated the volume assuming a uniform inner diameter. When we kept the pulse width constant and reduced the pressure to 0.01 MPa, we could filled the microwells with as little as 56 nl liquid. Even though our microfluidic dispenser could generate much smaller droplets when driven by shorter pulse, the microwells were too deep for the droplets to contact the hydrophilic inner surface and subsequently was trapped within the microwells. On the other hand, when we increased the pressure to 0.045 MPa, the dispensation could be as much as 170 nl., Our microfluidic dispenser could generate much larger droplets, but that would lead to overflow. Within the range between 56 – 170 nl using currently fabricated microwells, the best uniformity was achieved when the dispensation volume was ~ 115 nl with a coefficient of variation (CV) less than 6%. This condition was also used in the PCR experiments. The CV was higher when the dispensation volume reached the lower or higher limits of the microwell containers (CV ~ 14% for 56 nl and ~ 10%

for 80 nl and 170 nl) due to the physical shape of the wells. The dispensation volume beyond the current range (56 – 170 nl) could be achieved by changing the microwell chips with different well dimensions.

The dispensation variation was determined by filling the microwells with fluorescein, taking fluorescent microscopic images of each microwell with an Eclipse 80i microscope (Nikon, Japan) and integrating the fluorescence intensity through a home-written image process program. To prevent discrimination, we moved each microwell to the same location before taking a picture, so that the illumination intensity was equal. In the contamination examination, we rewrote the code of moving stage and the dispenser moved twice the pitches every time it filled the microwells (see the slow-motion movie #1). So they were alternatively filled with $FeCl_3$ and KSCN. Then we used the normal code to fill every microwell with water (see the slow-motion movie #2). No manual operation was needed during the 3-step reaction, ensuring the precision of the operation. We mixed $FeCl_3$ and KSCN solution in different ratio and observed the color as standard solution. We found that even 100 ppm of $FeCl_3$ in KSCN could produce the red product in the solution which was clearly observable, indicating that we were able to detect contamination above 100 ppm with this reaction system.

4. Polymerase Chain Reactions

We designed primers (71 bp amplicon) and a TaqMan probe (29 mer) for DsRed1 gene. All the oligonucleotides were synthesized by Invitrogen with sequences listed as following:

Forward primer	5’-GCAGCTGCCCGGCTACT-3’
Reverse primer	5’-CGATGGTGTAGTCCTCGTTGTG-3’
TaqMan probe	[FAM]-5’-CTACGTGGACTCCAAGCTGGACATCACCT-3’-[DAB]

pDsRed1 plasmids were amplified by DH5α cells and extracted using commercial kit (TIANprep Mini Plasmid Kit, Tiangen, Beijing, China). The 20 μl reaction system contained 100ng DNA, Taq PCR MasterMix (Tiangen, Beijing, China), 250 nM of each forward and reverse primer, 250 nM TaqMan probe, and

ddH_2O. Sample without template DNA was used as control 1, while sample without primers as control 2. In control 1 and control 2, the missing reagents were substituted by ddH_2O.

PCR experiments were carried out by a thermocycler (Dongsheng, Beijing, China) with a flat heating block. The thermal cycling protocol consisted of initial activation of the Taq polymerase (Tiangen, Beijing, China) at 95℃ for 5 min, followed by 35 cycles at 95 ℃ for 25 second and 60℃ for 75 second. The PCR results were examined by both 2% agarose gel electrophoresis and the fluorescence image analysis. The fluorescence micrographs were taken under a fluorescence microscope (Eclipse 80i, Nikon, Japan) by a CCD camera (DS-5Mv, Nikon, Japan). The intensity of a single well was extracted through image processing using Matlab (Mathworks, Natick, MA).

5. Slow-motion Movies

(1) Dispensing process of filling every alternative row of microwells. The movie was re-edited to play at 1/8 of the real speed. The movie was recorded with a Marlin F-033B CCD camera (Allied Vision Technologies, Germany).

(2) Dispensing process of filling every row of microwells. The movie was re-edited to play at 1/8 of the real speed. The movie was recorded with a Marlin F-033B CCD camera (Allied Vision Technologies, Germany).

(3) Dispensing process for parallel filling. The movie was re-edited to play at 1/10 of the real speed. The movie was recorded with an EX-F1 digital camera (Casio, Japan).