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Sequential Operation Droplet Array: An Automated Microfluidic Platform for Picoliter-Scale Liquid Handling, Analysis, and Screening

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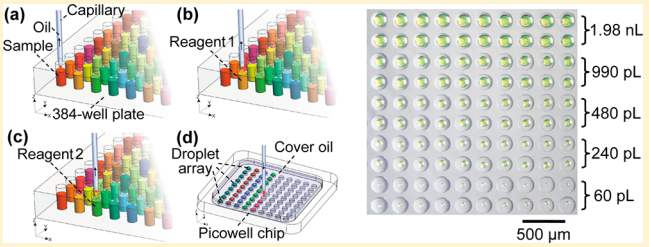
Supporting Information

ABSTRACT: This contribution describes a sequential operation droplet array (SODA) system, a fully automated droplet-based microfluidic system capable of performing picoliter-scale liquid manipulation, analysis, and screening. The SODA system was built using a tapered capillary-syringe pump module and a two-dimensional (2D) oil-covered droplet array installed on an $x-y-z$ translation stage. With the system, we developed a novel picoliter-scale droplet depositing technique for forming a 2D picoliter-droplet array. On this basis, an automated droplet manipulation method with picoliter precision was established using the programmable combination of the capillary-based liquid aspirating−depositing and the moving of the oil-covered droplet array, the so-called "aspirating−depositing−moving" (ADM) method. Differing from the previously reported droplet systems based on microchips, microcapillaries, or digital microfluidics, this method can achieve complete and flexible droplet manipulations, including droplet assembling, generation, indexing, transferring, splitting, and fusion in the picoliter range, endowing the present system with ultralow sample/reagent consumptions and substantial versatility in analysis and screening for multiple different samples. To demonstrate its feasibility and versatility, we applied the SODA system in multiple experiments required in drug screening, including the screening of inhibitors for capases-1 from a chemical library, the measurement of IC_{50} values for the identified inhibitors, and the screening of the synergistic effect of multiple inhibitors. In the experiments, the consumptions of samples and reagents are only 60−180 pL for each droplet microreactor, which are commonly 3−5 orders of magnitude lower than those of conventional multiwell plate systems, and 1−2 orders of magnitude lower than other droplet-based microfluidic systems for multiple sample screening. The ability of the SODA system in performing complicated and multistep droplet manipulations was further demonstrated in the serial dilution of nanoliter-scale inhibitor droplets with concentrations spanning 6 orders of magnitude for IC_{50} profiling, which includes droplet generation, indexing, splitting, transferring, and fusion with picoliter precision.



Automated liquid handling, analysis, and screening are attracting increasing attention in the fields of drug discovery, combinatorial chemistry, protein crystallization, synthetic biology, and clinical diagnosis. Conventional liquid handling and screening systems based on 96- or 384-well plates commonly consume relatively large volumes of samples and reagents (20−200 μL) and thus results in relatively high research costs.[1,2] The miniaturization of assay volumes will not only significantly reduce the cost of analysis and screening but will also enable many new biological studies to be carried out, where only limited amounts of samples or reagents are available.[3,4] By using 1536- and 3456-well plates, the assay volumes can be reduced to the several microliter even submicroliter range.[5,6] The state-of-the-art liquid handling robotics, such as Mosquito and Phoenix developed by TTP Labtech and ArtRobbins Instruments, can reliably handle submicroliter-scale liquid with the smallest volumes of 25 nL and 50 nL, respectively. However, great challenges exist in the development of novel platforms capable of handling liquids and performing screening experiments in the several nanoliter to picoliter range due to the significant influence of scale effects to liquid metering and handling.[7] In addition, as assay volumes decrease to nanoliter−picoliter scale, their surface area-to-volume ratios increase dramatically, leading to high liquid evaporation rates[8] and evident nonspecific adsorption of biological molecules on the phase interface.[9]

Microfluidic techniques provide an efficient way to miniaturize chemical and biological assays by manipulating small-volume liquids in the nanoliter−picoliter range in microchips with micropumps, microvalves, microwells, and other control modules.[10] Among them, droplet-based microfluidics is one of the most promising techniques for achieving assay miniaturization.[11−15] Each droplet can be regarded as a microreactor to perform reaction and assay independently.

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Picoliter and nanoliter-scale droplets can be generated, transported, manipulated, and stored long-term without sample dispersion and cross contamination. Surrounding the droplets with immiscible oil can avoid liquid evaporation, eliminate sample absorption on the solid channel surface, and provide the microreactions with biocompatible environments.[16] Various droplet-based microfluidic techniques have been successfully applied in the fields of single-cell and single-molecule analysis,[17] screening of protein crystallization conditions,[18] enzyme evolution,[17] and gene quantification with digital polymerase chain reaction (dPCR).[19]

Currently, there are five major types of microfluidic methods for droplet generation and manipulation as shown schematically in Figure 1a–e.[20−26] In continuous flow-based droplet systems,

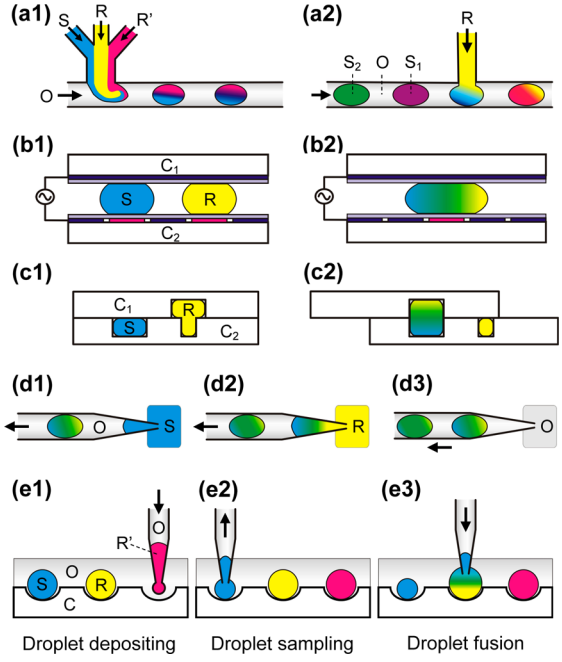

Figure 1. Schematic diagrams of five typical microfluidic methods for droplet generation and manipulation including continuous flow (a), electrowetting on a dielectric (EWOD) (b), SlipChip (c), sequential droplet assembling (d), and 2D droplet array (e). S, sample; S_1, sample 1; S_2, sample 2; R, reagent; R′, second reagent; O, oil; C_1, top chip; C_2, bottom chip; C, chip with well array.

droplets are generated by merging an aqueous stream with an immiscible stream in a T-junction or a flow-focusing microchannel.[20] The sample and reagent can be premixed before droplet formation, and their concentrations can be adjusted by changing their relative flow rates (Figure 1a1). For screening multiple samples, a droplet cartridge preloaded with droplets of different samples is connected with a microchip to merge its droplets with the reagent stream for reaction and screening (Figure 1 a2).[18] Electrowetting on a dielectric (EWOD) is another useful method for droplet manipulation (Figure 1b). Microliter–nanoliter-scale droplets can be flexibly moved, merged, and split on an open electrode array chip without the need of external pumps and valves.[15] SlipChip is a recently developed method for performing multiplex droplet manipulation[21] and has been successfully applied in nanoliter-scale protein crystallization condition screening with free interface diffusion and microbatch methods.[22] In a typical SlipChip, reagent droplets are preloaded into microwells on the chip (Figure 1c1), and the sample solution in the microchannels of the chip was then mixed with these droplets by slipping the two plates of the chip (Figure 1c2). In 2006, Chabert et al. first reported a droplet generation and reagent mixing method based on the sequential-injection mode (Figure 1d) using a capillary probe to sequentially aspirate multiple liquids into the capillary to form microliter-scale samples and reagent droplets and then to mix them.[23] In 2010, the authors' group developed a DropLab system using a sequential droplet assembling method to synchronously achieve the reagent addition operation in the droplet generation process in a capillary (Figure 1d), which has high controllability on the composition and volume of each droplet in the nanoliter–picoliter range.[24,25] From the capillary probe, the generated droplets can also be deposited on a microwell chip to form an oil-covered two-dimensional (2D) droplet array (Figure 1e1).[24] Compared with one-dimensional (1D) droplet array generated and stored in a capillary, 2D droplet array has the advantages of high droplet capacity, ease of droplet indexing, and a semiopen feature.[26] The 2D droplet array systems were applied to identify reducible carbohydrates[24] and to quantify microRNA using real-time reverse transcription polymerase chain reaction[26] with droplet volumes in the range of 25–500 nL. Despite the obvious progress of the above droplet-based microfluidic systems, droplet systems with picoliter-scale consumption and complicated droplet manipulation ability that are suitable for screening of multiple samples are still scarce and in great demand.

Herein, on the basis of the DropLab system,[24] we built a sequential operation droplet array (SODA) system, an automated and flexible microfluidic platform for picoliter-scale droplet manipulation, analysis, and screening of multiple different samples. The SODA system mainly consisted of a capillary-syringe pump module coupled to a movable oil-covered 2D droplet array. With the SODA system, a novel picoliter-scale droplet depositing technique was developed for forming a 2D picoliter-droplet array by overcoming the influence of scale effects induced by the decrease of droplet volumes from nanoliter- to picoliter-scale. On this basis, we established an automated droplet manipulation method with picoliter precision using the programmable combination of the capillary-based liquid aspirating–depositing operation and the moving of the oil-covered 2D droplet array. This method can achieve complete and flexible droplet manipulations, including droplet assembling, generation, indexing, transferring, splitting, and fusion in the picoliter range (Figure 1 e2 and e3). To demonstrate its potential and versatility in drug screening, we applied the SODA system in multiple types of enzyme inhibition assays, including screening of the inhibitors for caspase-1 from a chemical library, wide-range of serial dilution and measurement of IC_{50} values of the identified inhibitors, and screening of synergistic effect of multiple inhibitors. A significant improvement in sample/reagent consumptions in multisample analysis and screening was obtained compared with conventional multiwell plate systems and other droplet-based microfluidic systems.

EXPERIMENTAL SECTION

Building of the SODA System. The SODA system is composed of four parts: a thin-wall capillary with a tapered tip for liquid aspirating, droplet assembling, and droplet depositing; a microchip with 2D picowell array for droplet storage and reaction (Figure S1 in the Supporting Information [SI]); a high-precision syringe pump (PHD 2000, Harvard Apparatus,

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Holliston, MA) with a 1-μL syringe (7000 series, Hamilton, Reno, U.S.A.) for liquid metering; and an automated $x-y-z$ translation stage (Zolix, Beijing, China) on which the droplet array chip and a 384-well plate (Corning Life Sciences, San Nicolas, NL) for sample and reagent storage were installed. A program written with Labview (Labview 8.0, National Instruments, TX) was used to control the syringe pump and the $x-y-z$ translation stage simultaneously. Unless mentioned otherwise, the syringe pump was operated at a flow rate of 80 nL/min and the $x-y-z$ translation stage moved with an initial velocity of 6 mm/s, an acceleration of 10 mm/s^2, and a uniform velocity of 10 mm/s. A stereomicroscope (SMZ 850T, Touptek, Hangzhou, China) equipped with a CCD camera (UMD 300, Glory Technology, Hangzhou, China) was used to monitor and record the droplet-generation process.

The tapered tip of thin-wall capillary (100 μm i.d., 150 μm o.d., 12 cm length, Reafine Chromatography Co., Yongnian, China) was fabricated by heating its middle region (2 mm length) with a butane lighter and then pulling it to form two tapered capillaries. Using the manual fabrication method, the tip diameters commonly varied in the range of 10−30 μm (i.d.) and 20−40 μm (o.d.). Only the capillaries with tip size of ∼20 μm i.d. and 30 μm o.d were chosen for use. The inner and outer wall surface of the capillary were silanized to be hydrophobic with 1% OTCS in isooctane (v/v).[27] The capillary was connected to the syringe via a 10-mm-long Tygon tubing (130 μm i.d., 1.7 mm o.d., Saint-Gobain Performance Plastics, France). The microchip with picowell array was fabricated on glass substrate using standard photolithographic and wet etching technique as described previously.[28] The size of each well is ∼60 μm in depth, and 170 μm in diameter. The center-to-center distance between adjacent wells is 250 μm, and the density of wells is 1600 droplets/cm^2. The surface of the picowell chip was modified to be hydrophobic as described above. Finally, a 2-mm thick glass frame was glued on the microchip surrounding the picowell array for containing the cover oil. A homemade fluorescence detector was used to measure the fluorescence intensity of the droplet array as described previously.[29]

Procedure. Before an experiment, the 384-well plate was loaded with the mineral oil, sample, reagent, and washing buffer (water); the droplet array chip was covered with a layer of oil (2 mm thickness); the syringe and the capillary were filled with degassed water, and the capillary tip was filled with 10 nL of mineral oil as carrier. The SODA system was operated in an "aspirating−depositing−moving" mode (Figure 2a and c). Each droplet microreactor is assembled by moving the $x-y-z$ stage to allow the capillary tip to sequentially insert into the solutions preloaded in different wells of the 384-well plate and aspirate definite volumes of sample, reagent 1, and reagent 2 solutions into the capillary using the syringe pump to form a droplet (Figure 2 a1−a3). The droplet is then deposited on the picowell-array chip by moving the $x-y-z$ stage to make the capillary tip aligned to the target picowell and pushing the droplet out from the capillary tip with the syringe pump (Figure 2a4 and c2). The droplet settles into the picowell by gravity. Finally, the droplet array was incubated for reaction and detected with the fluorescence detector. All the procedures are controlled with a computer program. Two movie clips (Movies S1 and S2 [si_002.avi and si_003.avi] showing the droplet assembling and depositing processes are provided in the SI.

Enzyme Inhibition Assay. In the screening experiment for enzyme inhibitors from the chemical library, the final concentrations of enzyme, substrate, and inhibitor in each droplet reactor were 6 mU/μL, 20 μM, and 100 μM, respectively. The concentrations were adapted from the protocol of the screening kit for caspase-1 from BioVision (Mountain View, CA) with slight modification. During the droplet microreactor generation process, the microchip was kept at a temperature of 4 °C to minimize the enzyme reaction before incubation. A washing step was added between the assembling operations for different inhibitor droplets in order to minimize cross contamination. After the droplet generation process was completed, the droplet array was incubated at 35 °C for 1 h and detected with the fluorescence detector. To measure the IC_{50} value for an identified inhibitor, the inhibitor solution was diluted sequentially with water and loaded into the 384-well to perform the enzyme inhibition assay. For comparison, the same enzyme assay was also performed in a 384-well plate (50 μL, product #3544, Corning Lifesciences, Tewksbury, MA) according to the protocol of the screening kit for caspase-1.

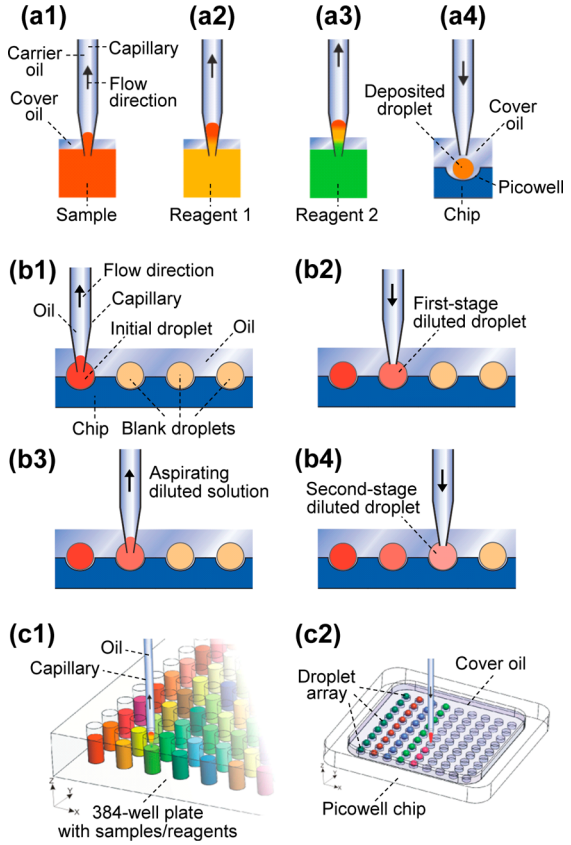

Figure 2. Schematic diagrams showing the working principle for droplet array generation (a1−a4) and serial dilution in droplets (b1−b4), and setup (c1−c2) of the SODA system.

Automated Serial Dilution. For producing inhibitor droplets with concentrations spanning 6 orders of magnitude, seven droplets including one 5.28-nL inhibitor droplet (100 μM) and six 4.32-nL blank droplets (water) were first deposited on the chip (Movie S3 [si_004.avi] in SI). The first-stage dilution was conducted by moving the $x-y-z$ stage to allow the capillary tip to insert into the inhibitor droplet and aspirate 480 pL of inhibitor solution from the droplet to the capillary tip (droplet sampling, i.e. splitting) (Figure 2b1). Then, the capillary tip was inserted into the adjacent 4.32-nL blank droplet to inject the 480-pL inhibitor solution into the

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droplet (droplet fusion); thus, a 10-fold dilution was achieved (Figure 2b2). The second-stage dilution was conducted by aspirating 480 pL of inhibitor solution from the droplet with 10-fold diluted inhibitor concentration (Figure 2b3) and injecting it into the next blank droplet to achieve 100-fold dilution (Figure 2b4). The next four stage dilutions were performed similarly. To measure the IC_{50} value of the inhibitor using the serial diluted droplets, each droplet reactor was assembled by aspirating the enzyme and substrate solution from the 384-well plate and aspirating the inhibitor solution from the inhibitor droplets with serially diluted concentrations.

RESULTS AND DISCUSSION

Design of the SODA System. The main objective of this work is to develop a droplet-based microfluidic system for automated analysis and screening of multiple samples with picoliter-scale resolution. The SODA system was built mainly on the basis of the DropLab system,[24] which consisted of a tapered capillary-syringe pump module coupled to an oil-covered 2D droplet array and a 384-well plate for sample and reagent storage fixed on an $x-y-z$ translation stage (Figure 1e1). However, a droplet manipulation method different from the DropLab system[24,25] and other droplet systems[15,18,20−22] (Figure 1a−d) was adopted to achieve multiple picoliter-droplet manipulations, using sequential operations combining the liquid aspirating-depositing operation through the tapered capillary and the moving of the oil-covered droplet array and 384-well plate, which can be referred to as "aspirating−depositing−moving" (ADM) method.

Although the similar operation method has been used in conventional liquid-handling robot system[5] or sequential injection analysis (SIA) systems,[30,31] the sample and reagent volumes handled in these systems are usually in the microliter to hundreds nanoliter range. SIA is a well-established automated solution treatment technique in the microliter range.[30,31] A typical SIA system consists of a syringe pump and a multiposition selective valve for sequentially switching different samples and reagents, which can automatically perform various complicated solution treatment operations. This selective valve function for liquid switching was accomplished in the SODA system by sequentially moving the oil-covered droplets array and sample/reagent wells to the tapered capillary according to computer program.

In the SODA system under the ADM working mode, the oil-covered 2D droplet array was used to generate a quadratic increase in droplet capacity compared with droplet systems with a 1D droplet array.[20−25] The droplet indexing for each droplet could also be achieved conveniently by simply using its position information in the array. Furthermore, compared with other droplet systems where droplets are stored in closed microchannels and capillaries,[13,20−25] the semi-open feature of the oil-covered droplet array allows the tapered capillary to easily contact each droplet in the array through the "virtual wall" of covering oil.[26] This makes the SODA system capable of performing complicated droplet manipulations, such as sampling from a definite droplet (i.e., droplet splitting, Figure 1e2) or adding reagent to a droplet (i.e., droplet fusion, Figure 1e3) which has not been demonstrated in the DropLab system.[24] Differing from systems using femtoliter to picoliter wells to meter and generate discrete liquid,[32−35] the picowells here were designed for maintaining the positions of in-oil aqueous droplets to facilitate the spatial indexing and subsequent manipulation of droplets. The syringe pump-based liquid driving method was chosen because it has reliable abilities in accurately controlling the volume, flow rate, and flowing direction of the liquid in the capillary independent of liquid property,[24] compared with the solid pin transferring and inkjet printing methods commonly used for microarray chips.[7] The 384-well plate has large storage capacity for multiple samples and reagents and can achieve seamless coupling with routine chemical libraries based on multiwell-plate systems. The $x-y-z$ translation stage can achieve automated moving of the droplet array with micrometer-scale moving and droplet positioning precision.

We integrated these simple modules to form the SODA system for performing automated and flexible picoliter-droplet manipulation of multiple samples under the ADM mode. However, before using the ADM mode to handle picoliter droplets of different samples, an essential prerequisite is to achieve reliable picoliter-scale liquid aspirating and depositing.

Aspirating and Depositing of Picoliter-Scale Droplets by SODA. Differing from the conventional systems manipulating liquid in the microliter to nanoliter range, significant challenges are faced by the SODA system to manipulate picoliter-scale droplets with the ADM mode. Multiple special measures were developed to overcome the problems caused by the order-of-magnitude decrease in droplet volume.

For achieving picoliter-scale liquid metering, a high precision syringe pump with a 1-μL syringe was used, with which a setting resolution of 30 pL was obtained. A thin-wall capillary with a sharp tip end was adopted to reduce the tip end area and thus minimize the remaining of the liquid (oil and aqueous solutions) at the tip end, which would evidently improve the precision of liquid metering under the ADM mode (see Figure S2 in SI). In addition, in our previous study with DropLab,[25] we observed the fluctuation of ambient temperature would affect the liquid metering precision significantly by inducing the thermal expansion and contraction of the liquid filled in syringe and capillary. Thus, for minimizing this effect, the syringe and capillary in the present system were filled with degassed water (with lower thermal expansion coefficient than the oil) as carrier and protected with an adiabatic shield, except the capillary tip filled with 10 nL carrier oil (Figure S3 in SI).

Under the ADM mode, the working state of the syringe pump was frequently converted between the "aspirating" and "dispensing" states. The changes in the pump driving direction would produce mechanical backlash of the syringe pump, which may have no evident effect on microliter liquid metering but has significant effect on the accuracy of picoliter-scale liquid metering. In the preliminary experiment for droplet generation, no droplets could be generated if we set droplet volumes lower than 240 pL. To solve this problem, two additional buffering operations were performed before aspirating aqueous liquid and after dispensing the generated droplet out from the capillary, respectively. When the syringe pump was changed to "aspirating" state from "dispensing" state, 1 nL of oil was first aspirated into the capillary before aspirating target liquid. When performing the droplet depositing operation, the generated droplet was pushed out from the capillary tip and deposited on the picowell, and the 1-nL additional buffer oil was also delivered out from the capillary subsequently following the droplet (see si_002.avi and si_003.avi in the SI). The corresponding operation programs are provided in Table S2 in SI and Table S3 in SI. With such steps, a smallest droplet volume of 60 pL was obtained, and the accuracy and precision in droplet generation were also improved significantly (see

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section "Performance of the SODA System" and Figure S4 in SI).

The oil and surfactant play essential and critical roles in the picoliter-scale droplet manipulation, and biological reaction processes in the SODA system. The oil has the function of enclosing the droplets for avoiding liquid evaporation and sample dispersion as in other droplet-based systems. We tested the effect of oil on reducing evaporation by monitoring the fluorescence intensity of three 480-pL droplets for over three hours using sodium fluorescein as a model sample (see Figure S5 inSI). The fluorescence intensity has no observable change in 3 h, indicating that droplet evaporation could be ignored due to the protection of oil. In addition to this, the oil also has multiple important functions in the droplet aspirating and depositing processes in the present system. The carrier oil segmented the aspirated liquid with the carrier water, and the preaspiration of the buffer oil eliminated the mechanical backlash of the syringe pump. These two functions ensured accurate liquid metering in the picoliter range in the capillary. In the droplet depositing process, the capillary tip was immersed in the cover oil, and the extra buffer oil was pushed out from the capillary tip following the generated droplet. Both the operations facilitate the smooth removing of the generated droplet from the capillary tip, thus allowing the droplet to be deposited on the chip picowell by gravity without the need of contact with the chip surface. Without such measurements, the picoliter-scale droplet would easily adhere to the capillary tip by surface tension without being deposited on the chip.

The addition of surfactant to the oil plays an indispensable role in ensuring the sufficient reliability and stability for the above-mentioned operations. The surfactant decreases the oil–water surface tension and thus facilitates the successful aspiration of picoliter-scale aqueous solution into the tapered capillary tip. It also increases the wettability of the mineral oil on the capillary surface and helps to form an oil layer on the capillary surface, preventing possible absorption and cross contamination of adjacent droplets. With the protection of the oil layer, the cross contamination between sequentially generated droplets is less than 1/100,000 (using sodium fluorescein as model sample) in the present system. More importantly, the surfactant molecules assemble at the oil/water interface, which could prevent the possible protein absorption and denaturation on the droplet/oil interface[9] and thus provide a biocompatible microenvironment for enzyme reaction in picoliter-size droplets. In our preliminary experiments, we observed that the enzyme activities in 1.98-nL droplets enclosed by the mineral oil without surfactant were almost completely suppressed (Figure S6 in SI). In contrast, by using the mineral oil with surfactant (Span 80, 0.5%, v/v), the enzyme activities were recovered.

Performance of the SODA System. We first evaluated the performance of the present system in the generation of droplet array using fluorescent and colored dyes as model samples. As shown in Figure 3a, 100 droplets with the same volume of 480 pL and identical composition (10 mM sodium fluorescein) were deposited on the chip in a 30-min experiment, corresponding to a generation speed of 18 s per droplet. The relative standard deviation (RSD) of the droplet sizes is 2.8% (n = 100). An array of 480-pL droplets containing different dye solutions was produced to prove the flexibility of the system in handling different samples (Figure 3b). The RSD of the droplet sizes is 4.9% (n = 100). A relative large droplet array containing 384, 480-pL droplets of two different dyes is

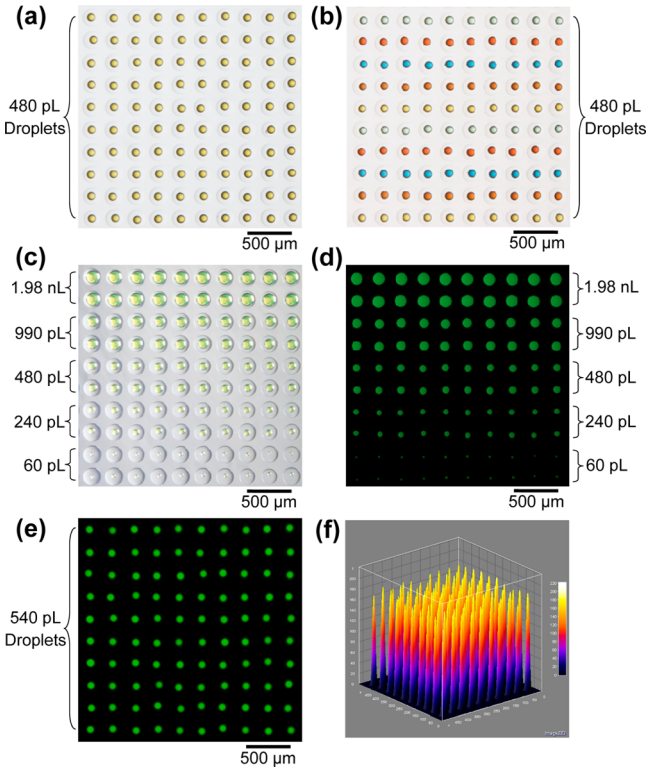

Figure 3. (a) CCD images of 480-pL droplets of 10 mM sodium fluorescein solution on the chip. (b) Image of an array of 480-pL droplets with different food dyes for showing the flexibility of the system in handling different samples. (c) Bright field image and (d) the corresponding fluorescent image showing an array of droplets with volumes of 1.98 nL, 990 pL, 480 pL, 240 pL, and 60 pL (from the top to the bottom). (e) Fluorescent image and (f) the corresponding surface plot showing an array of droplets generated by assembling 180 pL of 1 mM sodium fluorescein, 180 pL of blank, and 180 pL of 0.1 mM sodium fluorescein solutions into each droplet. The scale bar is 500 μm.

shown in Figure S7 in SI. To demonstrate the ability in generating variable volumes of droplets, an array of droplets with volumes of 1.98 nL, 990 pL, 480 pL, 240 pL, and 60 pL was generated on the chip (Figure 3c,d) and the corresponding RSDs of droplet sizes are 1.1%, 1.7%, 2.4%, 4.6% and 29.5%, respectively. To test the repeatability in generating droplet microreactors containing sample and multiple reagents, 100 droplets were formed by aspirating 180-pL 1 mM sodium fluorescein, 180-pL blank solution, and 180-pL 0.1 mM sodium fluorescein to assemble each droplet. The time required to generate each composite droplet was ~28 s, and thus, the total 100 composite droplets were generated in 47 min. The fluorescence image and the corresponding 3D surface plot are shown in Figure 3e,f, respectively. The RSD of the fluorescence intensities of the assembled droplets is 2.8% (n = 100).

We also tested the cross contamination between sequentially generated droplets by alternatively generating 5 nL of 10 mM fluorescein droplets and blank droplets on the chip. To quantitatively evaluate the contamination of the blank droplets, the fluorescence intensity of the blank droplets was measured and the fluorescein concentrations in the droplets were calibrated using a series of standard fluorescein droplets with concentrations in the range of 10 nM to 1 mM. Four blank droplets were measured, and the average fluorescein concentration was calculated as 79 ± 17 nM. Thus, the cross

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contamination between sequentially generated droplet is less than 1/100,000 in the present system. The cross contamination in parent solutions in the 384-well plate induced by successive inserting of the capillary tip from one well to another during the droplet assembling process was also tested by sequentially inserting the capillary tip into two wells containing 1 mM sodium fluorescein and buffer. After repeating the operation 100 times, the solution concentration in the buffer well was 2.6 nM, corresponding to a cross contamination of ~3/100,000 for each operation. When a washing step was added, detectable signal (3.0 nM) was observed after repeating the operation for 900 times. Such ultralow cross contaminations can be attributed to the protection effect of the surfactant-contained oil to the capillary surface during the droplet assembling and depositing process. Due to the high wettability of the surfactant-contained oil on the capillary surface, the droplet was separated from the hydrophobic capillary surfaces with a layer of oil.[25] In addition, the oil covered the sample/reagent wells and the droplet array also has a washing function to the capillary tip. Thus, possible absorption and cross contamination could be effectively depressed. In addition, if required, the cross contamination can be further reduced by adding a washing step between the operations for different samples or reagents, as in commercial automated screening instruments, such as microarray spotter and protein crystallization screening station.

Screening of Inhibitor for Caspase-1. To demonstrate the potential of the SODA system in drug screening, we applied it in the screening of inhibitor for caspase-1. Caspase enzymes have been shown to have key roles in the cellular apoptosis induced by cancer, inflammation, and autoimmune disorders. These enzymes have become important drug discovery targets because their inhibitors could be potential treatments of apoptosis-related diseases such as Huntington's disease, acute pancreatitis, and rheumatoid arthritis.[36] We first evaluated the repeatability and reliability of the SODA system in enzyme inhibition assays by measuring the Z'-factor.[37] The Z'-factor is defined as $Z' = 1.0 - (3.0 \times (SD_{neg} + SD_{pos})/(Avg_{neg} - Avg_{pos}))$, where SD_{neg} and SD_{pos} are the standard deviation of the response of the negative and positive controls, respectively, and Avg_{neg} and Avg_{pos} are the average response of positive and negative controls, respectively. Z' over 0.5 is generally considered necessary for a high throughput screening experiment. In this experiment, a total of 84 enzyme reactions were conducted in droplets including 42 droplets without inhibitor as negative controls and 42 droplets with a known inhibitor (Ac-(NMe)Tyr-Val-Ala-Asp-CHO, 100 μM) as positive controls (Figure S8 in SI). The Z'-factor was calculated as 0.85, demonstrating the high reliability of the system in an enzyme inhibition assay.

Then, we performed the inhibitor screening with a set of 32 small-molecule compounds (Table S1 in SI) by incubating them with the enzyme and substrate in a series of droplets. To ensure the reliability of the screening results, each compound was tested in quintuplicate, and thus a total of 160 droplet microreactors was assembled and detected. Figure 4a shows a typical result of the 32 droplet reactors. Three compounds (Nos. 28, 30, and 32) from the 5 known caspase inhibitors (Table S1 in SI) were successfully identified as inhibitors of caspase-1 in the five parallel experiments (Figure S9 in SI and Figure 4b). The other two known caspase inhibitors (No. 29, and No. 31) have no observable inhibitory effect on caspase-1. The reason is not well understood. To confirm these results, we also performed a comparison experiment using a conventional 384-well plate system, similar results were obtained.

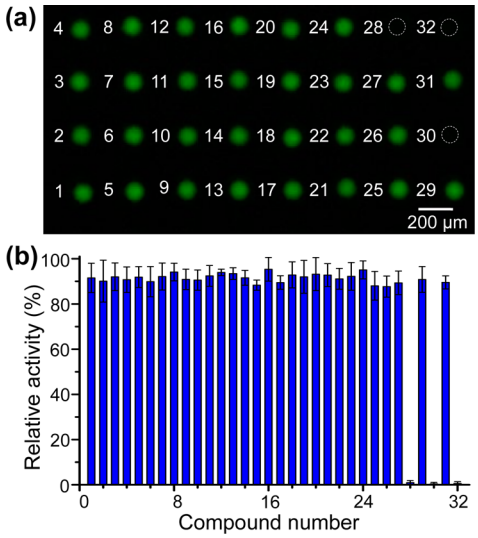

Figure 4. Picoliter-scale screening of enzyme inhibitors from a 32-compound library using the SODA system. (a) A typical fluorescent image showing the 32-compound droplets for inhibitor screening. Each droplet contains 180-pL enzyme (caspase-1, 6 mU/μL), 180-pL substrate (20 μM), and 180-pL compound solution (100 μM). (b) Quantified results of the enzyme activity in 32-compound droplet microreactors. The error bars are the standard deviation obtained from five droplets.

In the above experiments, each droplet microreactor was composed of 180-pL enzyme, 180-pL substrate, and 180-pL inhibitor solution. These volumes were chosen by combining the sample/reagent consumption and the precision of the present system. Compared with conventional 384- or 1536-well plate-based screening systems where 5−20 μL sample and reagents are commonly required in each reactor well, the sample/reagent consumption in the present system was reduced 9,200−37,000 fold.

Measurement of IC_{50} Value of Inhibitor. To characterize the inhibition efficiency of the identified inhibitors, their IC_{50} values were measured with seven different concentrations for each inhibitor ranging from 100 pM to 100 μM (Figure 5). The IC_{50} values for Nos. 28, 30, and 32 inhibitors are calculated as 31.6 ± 3.4, 39.8 ± 3.3, and 6.0 ± 0.5 nM (n = 3), respectively, using a Sigmoidal fitting method. These values agree well with those obtained from the conventional 384-well plate-based experiments as 47.9 ± 10.7, 40.7 ± 6.0, and 7.1 ± 1.2 nM (n = 3), for Nos. 28, 30, and 32 inhibitors, respectively (t test, $P > 0.05$).

To test the smallest droplet volume for screening reaction available in the present system, we performed the enzyme inhibition assay by aspirating 60 pL of enzyme, 60 pL of substrate, and 60 pL of inhibitor (No. 30) solutions to assemble each droplet. The 60 pL of aspirating volume is close to the resolution limit (30 pL) of the syringe pump with a 1-μL syringe. Figure 5d shows the dose−response curve of inhibitor No. 30 obtained using the 180-pL volume droplets. Although relatively larger standard deviations for activity measurement were obtained, the relationship between activity and concentration shows a similar trend with that of 540-pL droplets. The IC_{50} value was calculated as 30.2 ± 7.0 nM (n = 3), which has no significant difference (t test, $P > 0.05$) with that obtained with 540-pL droplets (39.8 ± 3.3 nM, n = 3).

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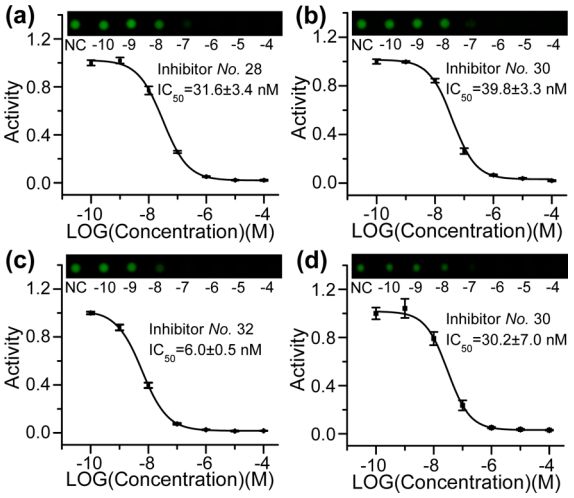

Figure 5. Measurements of IC_{50} values of the identified inhibitors. (a–c) Dose–response curves of the three identified inhibitors (No. 28, No. 30, and No. 32) obtained with a total droplet volume of 540 pL. Each droplet contains 180 pL of enzyme (caspase-1, 6 mU/μL), 180 pL of substrate (20 μM), and 180 pL of inhibitor solutions. (d) Dose–response curve of inhibitor No. 30 obtained with a total droplet volume of 180 pL. Each droplet contains 60 pL of enzyme (caspase-1, 6 mU/μL), 60 pL of substrate (20 μM), and 60 pL of inhibitor solutions. The insets show the typical fluorescent images of droplets in the measurements of the dose–response curves. The error bars are the standard deviation obtained from the data of three droplets.

The above enzyme inhibition experiments demonstrate the high reliability and stability of enzyme reactions in picoliter-scale droplets with volumes 3–5 orders of magnitude lower than those of conventional multiwell plate systems. This could be attributed to the accurate droplet manipulation and the good chemical and biological compatibility of the droplet/oil interface.

Screening of Synergistic Effect. Recent studies suggested that using two or more drugs together could produce a greater effect than using them individually, which is termed as "synergistic effect".[38] Benefiting from the high flexibility of the SODA system in droplet manipulating, we preliminarily investigated the synergistic effect of the identified inhibitors (Nos. 28, 30 and 32) and a known caspase inhibitor (Z-VAD-FMK) by assembling two of these inhibitors into each droplet with orthogonal combination method. Such an orthogonal combination experiment with multiple different samples is difficult to perform using a microfluidic system under continuous-flow mode. The screening results are shown in Figure 6 and Table S4 in SI. The inhibition effects of two combined inhibitors are commonly between the individual inhibition effects of the two inhibitors, indicating no evident synergistic effect is observed among these inhibitors. Even so, considering the extremely low sample/reagent consumptions of the SODA system over routine systems, the above results proved the present system could provide a potential powerful tool for synergistic effect screening with large number of compound combinations.

Complex Droplet Manipulation for Serial Dilution. The SODA system can perform complicated and multistep droplet manipulation using the ADM operation mode, which could be used as a versatile picoliter-scale liquid handling platform. We demonstrated the versatility of the SODA system by performing serial dilution experiments in nanoliter-scale droplets involving multistep droplet manipulations. Serial dilution was conducted by moving the x–y–z stage to allow the capillary tip to insert into the parent droplet, aspirating the solution into the capillary tip (Figure 2b1), and injecting the solution to the daughter droplet (Figure 2b2) (also see Movie S3 [si_004.avi] in SI). To evaluate its performance, we first prepared an array of 4.8 nL droplets of sodium fluorescein with six different concentrations ranging from 100 nM to 10 mM. Excellent slope (0.9958) and linear relationships (R^2 = 0.9996) are obtained between the theoretical concentrations and the actual measured concentrations of fluorescein in the droplets (Figure 7a). The RSD of each concentration is lower than 1.9% (n = 5). Then, we applied this method to prepare inhibitor solutions in droplets with wide range concentrations spanning 6 orders of magnitude for IC_{50} profiling. Figure 7b shows the dose–response curve of inhibitor No. 28. The calculated IC_{50} value (41.7 ± 5.4 nM, n = 3) agrees well with that obtained with large-volume dilution experiments (31.6 ± 3.4 nM, n = 3) (P > 0.05). Using the serial dilution technique, the primary drug screening step can be combined with the secondary inhibition efficiency (IC_{50}) profiling step, which provides a total solution for the miniaturized screening. The inhibitor consumption for the whole six-orders-of-magnitude serial dilution and IC_{50} profiling experiments is only 5.28 nL.

The above serial dilution experiment involves various droplet manipulations with picoliter-scale precision, such as droplet metering, generation, indexing, sampling (splitting), transferring, and fusion. To the best of our knowledge, such an

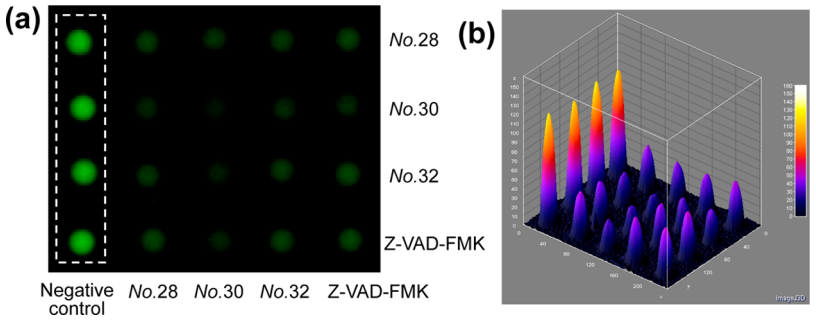

Figure 6. Screening of synergistic effect of the three identified inhibitors (Nos. 28, 30 and 32) and a known caspase inhibitor (Z-VAD-FMK). The experments were performed by assembling two of these inhibitors into each droplet in an orthogonal combination fashion. (a) Typical fluorescent image and (b) the corresponding 3D surface plot showing the results of synergistic effect screening. Each droplet contains 180 pL of enzyme, 180 pL of substrate, 180 pL of inhibitor 1, and 180 pL of inhibitor 2 soluntions; Enzyme concentration: 6 mU/μL; Substrate concentration: 20 μM. The concentrations of inhibitors No. 28, 30, 32, and Z-VAD-FMK are 31.6, 39.8, 6, and 186 nM, respectively.

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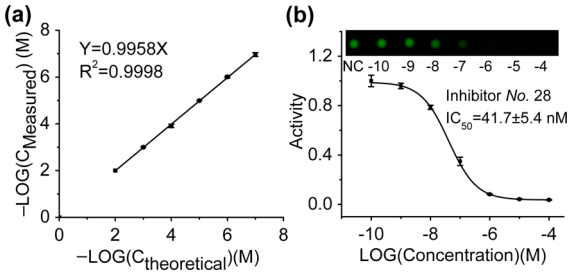

Figure 7. (a) Linear regression curve of the theoretical log concentrations and the actual measured log concentrations in 4.8-nL droplets produced in the serial dilution experiment using sodium fluorescein as model sample. The error bars are the standard deviation obtained from five droplets. (b) Dose−response curves of inhibitor No. 28. The inhibitor solutions are prepared using the nanoliter-scale serial dilution method in 4.8-nL droplets. The error bars are the standard deviation obtained from three droplets. Each droplet contains 180-pL enzyme (caspase-1, 6 mU/μL), 180 pL of substrate (20 μM), and 180 pL of inhibitor solutions.

experiment has not been realized in other previously reported droplet systems.

CONCLUSION

In summary, the SODA system is proved to be a promising platform for picoliter-scale liquid handling, analysis, and screening. It provides a novel droplet manipulation method with picoliter precision, using the programmable combination of the capillary-based liquid aspirating−depositing and the moving of an oil-covered droplet array. This ADM-based droplet manipulation method is different from the previously reported microfluidic droplet systems based on continuous-flow mode or digital microfluidics. Flexible droplet manipulations could be automatically achieved including droplet generation, indexing, sampling, transferring, splitting, and fusion in picoliter-scale precision. This endows the present system with ultralow sample/reagent consumption and substantial versatility in analysis and screening for multiple different samples. Compared with the usual consumption of 5 μL in conventional 1536-well plate-based screening systems, the consumption of samples and reagents in the SODA system is reduced 9,200−27,600 fold. Even compared with other droplet-based screening systems including the DropLab system,[18,20,21,23,24] the consumptions for multiple sample screening are also reduced 10−20 fold. The versatility of the SODA system was successfully demonstrated in performing multiple different types of analysis and screening using one simple device and different ADM operation programs, including screening of enzyme inhibitors, wide-range serial dilution of inhibitors, measurement of IC_{50} values of inhibitors, and screening of synergistic effect of multiple inhibitors. These applications usually require different sample/reagent combinations and different operation procedures, which are difficult to be fully realized with other single droplet systems.

Since all of the liquid handling operations were performed by the robot in an automated fashion under control of the computer, this platform can be easily embedded into current large-scale screening systems without excessive modification of hardware. Although the screening throughput of the present system is relatively lower than those of commercial high-throughput screening systems based on robots, there is compensation in the greater flexibility in droplet manipulation. In the present system, the liquid handling throughput is mainly limited by the low moving speed (initial velocity: 6 mm/s; acceleration: 10 mm/s^2; uniform velocity: 10 mm/s) of the $x-y-z$ stage available in the authors' laboratory. If high-speed automated stages are used, the time for composite droplet generation could be shortened to several seconds. Another efficient and widely used method to increase the liquid handling throughput is the use of multichannel devices, i.e. multiple capillary probes. The related work is ongoing in the authors' laboratory.

The SODA system is particularly suitable for ultralow-consumption analysis and screening of multiple different samples. Although only enzyme inhibitor screening is demonstrated, the present system could also be applied in other analysis and screening for multiple samples, such as single cell analysis, protein crystallization screening, combinatorial chemistry, clinical diagnosis, and proteomic studies. Although no evident cross contamination was observed in the enzyme inhibitor screening experiments due to the use of inert mineral oil, one potential problem of the present system in other applications is that some molecules may pass through the surrounding oil and enter the adjacent droplets, leading to possible cross contamination. This could be solved by choosing suitable inert oil to meet different applications, or using deep picowells with independent covering oil to eliminate the cross contamination through the oil.

ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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