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Digital PCR Using Micropatterned Superporous Absorbent Array Chips

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Abstract

Digital PCR (dPCR) is an emerging technology for genetic analysis and clinical diagnostics. To facilitate the widespread application of dPCR, here we developed a new micropatterned superporous absorbent array chip (μ SAAC) which consists of an array of microwells packed with highly porous agarose microbeads. The packed beads construct a hierarchically porous microgel which confers superior water adsorption capacity to enable spontaneous filling of PDMS microwells for fluid compartmentalization without the need of sophisticated microfluidic equipment and operation expertise. Using large λ -DNA as the model template, we validated the μ SAAC for stochastic partition and quantitative digital detection of DNA molecules. Furthermore, as a proof-of-concept, we conducted dPCR detection and single-molecule sequencing of a mutation prevalent in blood cancer, the chromosomal translocation t(14;18), demonstrating the feasibility of the μ SAAC for analysis of disease-associated mutations. These experiments were carried out using the standard molecular biology techniques and instruments. Because of its low cost, ease of fabrication, and equipment-free liquid partitioning, the μ SAAC are readily adaptable to general lab settings, which could significantly facilitate the widespread application of dPCR technology in basic research and clinical practice.

Graphical abstract

A low-cost micropatterned superabsorbent array chip enables spontaneous fluid partitioning for digital PCR and sequencing without sophisticated microfluidic equipment.

Introduction

Polymerase chain reaction (PCR) is a workhorse biotechnology in life sciences and healthcare. As a state-of-the-art technical advance in PCR, digital PCR (dPCR)¹ has emerged as an enabling platform for genetic analysis and clinical diagnostics.^{2, 3} In contrast to the standard real-time quantitative PCR, dPCR quantifies the absolute quantity of target molecules via stochastic compartmentalization and amplification of individual DNA or RNA molecules in a massively parallel fashion. Thus dPCR affords the ability to detect and quantify rare mutations or genetic variation with superior sensitivity and accuracy.⁴⁻⁷ In addition, a broad array of applications of dPCR have been demonstrated, including single cell analysis,⁸⁻¹¹ forensic analysis,¹² and sample preparation for next generation sequencing.^{13, 14}

Recent development of dPCR techniques greatly benefit from the rapid advance in microfluidic technology as it enables effective and precise manipulation of nanoliter and smaller volumes. Currently, most commonly used methods for fluid compartmentalization in dPCR are based on droplet microfluidics, microvalve or microwell technologies. In comparison to static arrays, flow-through droplet microfluidics permits rapid and controllable generation of large number of uniform droplets to improve the analytical performance such as sensitivity, dynamic range and throughput, enabling a broad array of applications including sequencing, single cell analysis and high-throughput screening.^{6, 9, 14-17} Microfluidic droplet generation also confers flexibility to fine tune droplet sizes, even in real time.¹⁵ However, microfluidic droplet generation and detection demands sophisticated chip design, instrumentation, and expertise. Recent advance in detection technique has led to development of integrated, portable microfluidic flow-through droplet systems capable of automated, high-throughput readout of droplets.^{18, 19} Adaptation of these devices to dPCR detection remains yet to be explored. Manipulation, addressing and sorting of individual droplets remain challenging, especially in the format of continuous flow.^{14, 20, 21} Moreover, the performance statistics may vary upon operation conditions, as shown by the recent evaluation of several commercial instruments.^{4, 22}

Alternative to droplet microfluidics, the array-based technologies affords inherent compatibility with automated image registration and robotic liquid handling, while they possess a fixed number of reaction compartments which limits their analytical performance. In an array, individual reactions are spatially indexed, which is essential for distinguishing a large number of reaction conditions. Moreover, spatially defined static arrays are well suited for the applications that require real-time monitoring, physically or chemically manipulation, and retrieving of individual reactions in a high-throughput manner. Integrated PDMS microvalve allows precise control of reagent delivery and isolation of discrete reaction volumes and have been widely used in the array-based dPCR devices.^{23, 24} This approach, however, requires complex microchip architecture, sophisticated fabrication by multilayer soft lithography, and special instrument and software interface for valve control. Other microfluidic devices developed for dPCR include SlipChip,^{25, 26} centrifugal chip with a spiral microchamber array,²⁷ and the microwell chips.^{11, 28, 29} These systems feature simpler device structures and fabrication; but rely on pressure-driven sample loading and the use of oil phase to passively partition and seal aqueous solution, which could complicate the

assay operation. Several microfluidic systems in both droplet and microarray formats have been commercialized, such as RainDrop for droplet dPCR and BioMark for chip-based dPCR.³⁰ These instruments and their consumables chips are still expensive, which limits their adaptability to many applications in need, including routine use in regular research labs and point-of-care diagnosis.

To facilitate the widespread application of dPCR, there are continuous efforts being invested in improving the technology and reducing the cost. For instance, various microwell-based devices have been reported to achieve equipment-free or spontaneous fluid compartmentalization driven by different mechanisms, including the self-priming chip using vacuum packaging,³¹ the monosaccharide pre-coated microwell chip for dissolution-guided wetting,³² and the self-digitization chips based on passive two-phase flow manipulation by microfluidic geometry.²⁹ Paper-based devices, such as provide a suitable platform for convenient fluid transport and manipulation based on spontaneous wetting.^{33, 34} For instance, a SlipPAD device has been developed to synergistically marry the ability of the SlipChip for high-throughput, multiplexed sensing with the advantages of the paper-based chips in simple fabrication and spontaneous fluid transport.³³ While dPCR on the glass SlipChip has been demonstrated, the applications of the SlipPAD to dPCR have not been demonstrated, presumably due to the difficulty in device sealing to prevent evaporation during thermal cycling and the limitation in feature sizes that can be fabricated on paper. Here we developed a new micropatterned superporous absorbent array chip (μ SAAC) which takes advantages of superior water adsorption capacity of superabsorbent gels to drive spontaneous filling of microwells for dPCR. In contrast to regular hydrogel absorbents that require a long time to reach equilibrium swelling from the dry state due to slow water absorption by diffusion, engineered superporous hydrogels confer fast swelling and superabsorbent properties and thus have received broad applications in daily life, industry and medical care.^{35, 36} Compared to the aforementioned microchip-filling methods, our approach has several advantages: first, it does not demand special microfluidic design and deliberate control of two-phase flow system; second, its fabrication and operation are simple and robust; third, it avoids the possible sample evaporation under vacuum or introduction of contaminants along with the coating materials; lastly, it is applicable to different polymer materials than PDMS. Using large λ -DNA as the model target, we validated the μ SAAC for stochastic partition and quantitative detection of DNA molecules. Furthermore, as a proof-of-concept, we conducted dPCR detection and single-molecule sequencing of the chromosomal translocation t(14;18), a mutation prevalent in blood cancer, which demonstrates the feasibility of the μ SAAC for analysis of disease-associated mutations. These experiments were carried out using the standard molecular biology techniques and instruments. Owing to its low cost, ease of fabrication, and simple operation, the μ SAAC should provide a dPCR technique that is readily adaptable for genetic analysis on a routine base in general lab settings. Complementary to the advanced dPCR systems that are commercially available, this simple technique hold the potential to help facilitate the widespread application of dPCR technology in basic research and clinical practice.

Experimental

Reagents and materials

All solutions were prepared with deionized water (18.2 MV-cm, Thermo Scientific). 4% non-crosslinked agarose beads (20–50 μm in size) preserved in 20 % ethanol was purchased from Agarose Bead Technologies. The following reagents were used as received: 2-propanol (IPA) (99.5%; Sigma-Aldrich), ethanol (100%, Decon Laboratories Inc.), Fluorinert® FC-40 (3M), epichlorohydrin (ECH, 99%, Sigma-Aldrich), sodium borohydride solution (12% Wt. NaBH_4 in 14 M NaOH solution, Sigma-Aldrich).

Microfabrication

The PDMS microwell array chips were fabricated by standard soft lithography. New Si wafers were cleaned in Piranha solution (a mix of concentrated sulfuric acid and 30% hydrogen peroxide with a ratio of 3 to 1) for 15 min, rinsed by deionized water and dried with nitrogen. The thick SU-8 molds were fabricated using SU-8 2050 (MicroChem). Briefly, a cleaned Si wafer was spin-coated with SU-8 2050 at 500 rpm for 15s and 1500 rpm for 30 s, prebaked at 65 °C for 8 min and 95 °C for 28 min, and exposed for 15 s for total exposure energy of 110 mJ cm^{-2} . The wafer was then post-baked at 65 °C for 5 min and at 95 °C for 20 min, followed by a 5 min development and hard-baking at 150 °C for 2 h. The SU-8 molds were finally silanized with trimethylchlorosilane under vacuum for 4 h. Negative PDMS replicas were made by pouring a 10:1 mixture of PDMS base with the curing agent over the wafer, followed by curing at 70 °C overnight. Cured PDMS was then peeled off the mold and cut into individual chips. PDMS microwell chips were used without any surface treatment.

Pretreatment of agarose beads

Prior to use, the 4% non-crosslinked agarose beads were first treated by freeze/thaw cycles to improve the porosity and then chemically crosslinked to enhance the mechanical strength. Briefly, 18 ml of beads was frozen at –12 °C for 24 h and thawed at the room temperature for several times. Then 1 ml sodium borohydride solution (12% NaBH_4 in 14 M NaOH solution) and 20 μL ECH were added into the bead suspension, mixed for 30 min, and then incubated at 55 °C for 6 hours with a stirring speed of 100 rpm. Crosslinked beads were then washed with a large volume of water. To completely remove non-crosslinked agarose and unreacted chemicals, the beads were washed by 600 ml boiling water for at least 6 times, incubated in ethanol for 48 hours, and finally re-suspended in IPA. A small volume of the treated agarose beads was left to dry slowly in air prior to use.

Fabrication of μSAAC

The process of preparing μSAACs is illustrated in Fig. 1A. First, a bead paste was prepared by priming ~30–50 μL dried beads with 5–10 μL IPA and then loaded onto the surface of a PDMS microwell chip. A clean glass slide was used to scrape the beads across the entire surface several times to fill the microwells. This process was repeated twice to ensure all the wells are fully packed with the beads that are dried due to IPA evaporation during packing.

Excess beads were scraped off the surface and the ready-to-use μ SAAC was completed by sealing the chip with a small piece of adhesive film cut from that for 96-well PCR plate.

Digital PCR on μ SAAC

All oligonucleotides used in this study were ordered from IDT. PCR reagents were obtained from Life Technologies (Carlsbad, CA). PCR mix was prepared freshly to contain 1 $\times$ Platinum® Taq DNA polymerase buffer with 3 mM MgCl₂, 0.2 mM dNTPs, 0.4 mg mL⁻¹ heat inactivated BSA, 0.4 μ M each of primers, 0.15 U μ L⁻¹ Platinum® Taq DNA polymerase, 0.5 $\times$ EvaGreen™ dye (Biotium, Hayward, CA) and DNA template. λ -DNA standards were 10-fold serial dilutions of the 0.54 μ g mL⁻¹ stock solution (New England Biolabs) with the concentrations ranging 5.4 pg mL⁻¹ to 54 ng mL⁻¹. 1 μ L of DNA solution was added into 39 μ L PCR mix prior to dPCR assays. A pair of primers (forward: 5'-CGCGATATGCTGCGCTTGCT-3', reverse: 5'-TAAGCACGAAGCTCAGCCAGAACGA-3') were used to detect λ -DNA. Genomic DNA purified from a t(14;18) positive RL lymphoblasts (CRL 2261) in our previous work was used for the proof-of-concept demonstration of mutation analysis.⁶ An established primer pair was adopted to target t(14;18): forward (5'-CTATGGTGGTTTGACCTTTAGAGAGTT-3') and reverse (5'-ACTCACCTGAGGAGACGGTGAC-3'). To perform digital PCR using the μ SAAC, 10- μ L PCR mix was pipetted onto the chip surface and spread out to cover the entire microwell array. The chip was then placed in a closed petri dish with a water dampened KimWipe for brief incubation (10 min) until the microgels in the PDMS microwells fully absorb the PCR solution. Excess PCR mix and beads were then scraped by a PDMS slab and the microwell chip was sealed firmly against the oil-coated glass slide a thin layer of fluorocarbon oil Fluorinert® FC-40 to completely isolate individual microwells. The top of PDMS chip was covered by another glass slide to prevent water evaporation from the PDMS microwells during thermal cycling. Finally, the chip assembly was mounted on a PCR MasterCycler nexus thermocycler (Eppendorf) using an in-situ adapter for PCR amplification. Thermal cycling was carried out with a 2 min hot start at 95 °C, 34 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s and a final elongation step at 72 °C for 5 min. Microchips were directly used for fluorescence imaging after thermal cycling. Bright field and fluorescence images were taken using a Zeiss Axiovert A1 inverted fluorescence microscope equipped with a LED excitation light source (Thorlabs, Newton, NJ). The digital images were analyzed with Image J (NIH, <http://rsbweb.nih.gov/ij/>) to determine the percentage of fluorescent wells. The results were compared to that obtained using the microfluidic droplet dPCR technology that we established previously (see Supporting Information for details).^{6, 9, 15}

Single-molecule sequencing

dPCR amplification of RL gDNA was performed at an equivalent concentration of ~1 copy per well (cpw) on a μ SAAC with 120 μ m $\times$ 120 μ m microwells, following the method described above. After that, the glass substrate was carefully removed and the microwell chip was visualized under the fluorescence microscope. Amplicon-containing beads in individual positive microwells were manually pipetted from the chip into PCR microtubes and re-amplified using the PCR conditions described in the last section. Re-amplification products were run on a 1.5% agarose gel; the separated DNA band was then excised,

extracted with a QIAquick Gel Extraction Kit (QIAGEN), and sequenced by the standard Sanger sequencing.

Results and Discussion

Working Principle and Device Fabrication

Our device is essentially a low-cost microwell array chip fabricated with PDMS. Compared to the commonly used microwell chips fabricated on glass or silicon wafer, the PDMS device eases fabrication and substantially reduces cost. Three different devices were designed, which consist of square microwells of $60\ \mu\text{m} \times 60\ \mu\text{m}$, $120\ \mu\text{m} \times 120\ \mu\text{m}$, and $240\ \mu\text{m} \times 240\ \mu\text{m}$ dimensions, respectively. The dimensions of the fabricated PDMS microwells showed less than 5% deviation as measured by microscopic imaging. The height of the microwells, which is controlled by the thickness of the SU8 mold, was measured to be $112 \pm 5\ \mu\text{m}$ across the entire wafer. Because of high surface hydrophobicity, filling PDMS microwells on the nanoliter or smaller scales poses a significant technical challenge to achieving effective liquid dispensing for accurate and reproducible dPCR. To address this challenge, we attempted to use highly porous microgels to enable spontaneous wetting and filling of PDMS microwells without surface modification. As shown in Fig. 1A, a superporous absorbent microgel array is fabricated by fully packing the PDMS microwells with dry agarose gel microbeads. The loaded PDMS chip is then sealed with a small piece of adhesive film to construct the ready-to-use μSAAC . Our device allows one to perform digital PCR following a simple protocol without the need of sophisticated sample loading equipment (Fig. 1B). Briefly, a PCR mix of $10\ \mu\text{L}$ containing DNA targets is pipetted onto the chip to cover the entire microwell array. The microgels absorb the PCR solution to fully fill the PDMS microwells. Excess PCR mix is removed and the microwell chip is sealed against a glass slide pre-coated with a thin layer of fluorocarbon oil to isolate individual nanoliter microwells. Finally, the chip will be placed on a thermal cycler for PCR amplification, followed by the fluorescence detection to digitally count the copy number of targets.

The key component of the μSAAC is the superabsorbent material. It should meet the following criteria for the application to dPCR: first, it is able to rapidly absorb water to drive spontaneous wetting and filling of untreated PDMS microwells; second, it possesses high porosity with large pore size to ensure stochastic partition of target molecules; third, it is compatible with PCR reaction; finally, its fabrication should be simple and inexpensive. To satisfy these requirements, we chose to fabricate the micropatterned superporous microgels with agarose microbeads. Agarose gel is a natural and biocompatible polymer with relative large pores. It has been shown that agarose gel has no inhibitory effects on PCR even at high concentrations.^{10, 37} Agarose microbeads with various sizes and chemical modifications can be readily synthesized in research labs and commercial products are extensively available. In this work, a commercial 4% non-crosslinked agarose beads of 20–50 μm in size were chosen as this gel concentration provides large pore size and reasonable mechanical strength.

Using the method described in the Experimental section, the PDMS microwells were fully packed with dry agarose beads to ensure uniform packing across the entire chip (Fig. 2A). The agarose microbeads packed inside a microwell construct a highly porous microgel with

large interstitial space formed between the beads, as seen in Fig. 2B. Since the beads occupy a large fraction of the microwell volume, it is important to increase the porosity of agarose microbeads in order to enhance the ability to absorb water and the intraparticle accessibility for DNA molecules. Therefore the non-crosslinked agarose beads were subjected to freeze/thawing for several cycles prior to use. Freezing-based methods are widely used to engineer the porosity and micro-structures of hydrogels. By controlling freezing conditions, ice crystallization in hydrogels can create macroscopic porous structures for a variety of applications, including DNA extraction, chromatographic separation, biosensing, tissue culture, and drug delivery.^{38–42} In this work, freezing of the beads was performed at a relatively high temperature (-12°C) for 24 hrs in order to form large ice crystals that penetrate the polymer network to yield large open pores.^{38, 43} It was found that the freeze/thawed agarose beads were fragile and damaged during the packing process. Thus the agarose beads were chemically cross-linked to improve their mechanical strength. The treated agarose beads were dried by critical drying and then examined using scanning electron microscopy (SEM). As shown in Fig. 2B, the freeze/thawed beads display a much more porous surface and internal structure than the untreated beads. Such increase in porosity caused by freeze/thawing has been found to augment the swelling capacity and speed of hydrogels in aqueous solution.⁴⁴ Overall, our method presents a new route to fabricate arrayed superabsorbent microgels with a hierarchical pore structure for dPCR.

Nanoliter Fluid Dispensing and Compartmentalization

The μSAAC was assessed for dispensing and compartmentalization of a large array of nanoliter liquid volumes without the aid of vacuum or complex pumping/valving system. It was observed that the dry microabsorbents can be wetted and swollen by water quickly to spontaneously fill the PDMS microwells. The filling speed obtained with freeze/thawed agarose beads (< 2 min.) was found to be significantly faster than that of the untreated beads (~ 10 min). This is consistent with previous experimental observations and can be attributed to the extremely high porosity of freeze/thawed agarose beads and the large interstitial pores among the beads, which permits fast flow into the agarose beads to expedite water absorption.^{35, 44} Rapid filling of the microwell chip is crucial for accurate dPCR because it minimizes water evaporation during sample loading which can cause significant concentration change and perturbation on random partitioning of DNA across the chip. Preventing evaporation is especially critical for handling a few microliter liquid on a relative large open chip surface compared to micrometer-scale enclosed channels.⁴⁵ We observed that much faster chip filling using the freeze-thawed beads significantly reduces water evaporation compared to the untreated beads. Therefore, we constructed the μSAAC with highly porous treated agarose beads for the subsequence studies in order to achieve stochastic DNA partitioning and to improve the accuracy and precision of dPCR.

Our method works well even for the partially packed microwells, as displayed by a time-lapse image sequence in Fig. 3A. When loaded with water, air bubbles were trapped in the partially packed microwells, resulting in Cassie-state wetting on the surface (Fig. 3A1). Swelling of the microgels upon water absorption can overcome the interfacial tension to wet the hydrophobic PDMS surface and expel air bubbles trapped in the microwells to achieve Wenzel-state wetting of the entire chip (Fig. 3A2–4). This finding indicates the robustness of

our method for liquid dispensing in PDMS microwells. When saturated with water, the originally opaque microgels (Fig. 2A) becomes transparent due to match of refractive indexes between the hydrogel and water, indicating complete filling of interparticle pores and the microwells (Fig. 3B). A small portion of the beads in the swollen superabsorbent microgels came out of the microwells and were removed with excess PCR solution before sealing the microwells, which does not affect liquid dispensing and quantitative detection of dPCR, as shown in Figs 4 to 6.

We then assessed the performance of filling entire large microwell array chips using a fluorescent dye solution. Our results demonstrated the ability of the micropatterned superabsorbents to fill thousands of discrete subnanoliter microwells with 100% efficiency and good uniformity (Fig. 3B and C). This fluid compartmentalization performance is critical to archiving stochastic partition of DNA molecules and thus quantitative dPCR, as further discussed below. Several spontaneous fluid compartmentalization methods have been reported for dPCR, including vacuum packaging,³¹ dissolution of pre-coated monosaccharides,³² or passive two-phase flow manipulation by microfluidic geometry.²⁹ Compared to these methods, our approach affords several advantages: first, no special geometrical design of microstructures and deliberate control of two-phase fluid system are required; second, it eases device fabrication and assay operation; third, it avoids the possible sample evaporation under vacuum or introduction of contaminants along with the coating materials; lastly, it is applicable to different polymer materials than PDMS as it does not rely on the gas permeability of PDMS. Therefore the μ SAAC is amenable to the well-established polymer chip manufacturing to further reduce the chip cost.

Digital PCR Using μ SAAC

To demonstrate the application to quantitative DNA detection, we evaluated the μ SAAC for dPCR detection of λ -DNA. Although water occupies the most of volume in a swollen 4% agarose gel, mass transport of DNA molecules can be slowed down by the gel fibers or sterically excluded by the small pores in the hydrogel network. Therefore, it is important to investigate how size exclusion affects the stochastic partition of DNA molecules into individual microwells for accurate digital detection. To this end, λ -DNA was chosen as the model molecule because it is a 48.5 kbp double-stranded DNA (dsDNA) that is larger than the sizes of genetic templates normally used for PCR amplification. Fig. 4 shows representative fluorescence images of dPCR detection of a 10-fold dilution series of λ -DNA standards with concentrations ranging from 0.0054 to 5.4 ng/mL. 10 μ L PCR mix was applied to the chip, which contains a total of ~25 to 25,000 copies of λ -DNA. The chips used in these experiments contain 50×50 microwells with the average dimensions measured to be $62 \mu\text{m} \times 62 \mu\text{m} \times 112 \mu\text{m}$ (i.e., 0.43 nL). Thus the final concentrations of λ -DNA spiked in PCR mix were equivalent to be 0.001 to 1 copy per well (cpw). It was observed that the negative control displayed nearly no fluorescent wells (positive) due to non-specific amplification and the number of positive wells increases along with the target concentration. These results indicate good specificity of PCR amplification in the agarose bead-packed microwells.

To assess the quantitative performance of dPCR using the μ SAAC, we quantified and plotted the percentages of positive wells as a function of equivalent DNA concentration in a log-log calibration curve presented in Fig. 5A. The plot shows a good linearity at diluted λ -DNA concentrations (0.1 cpw) and levels off as the DNA concentration increases. The experimental values were compared with the theoretical microwell occupancy calculated for our device using the Poisson distribution^{5, 9} in order to quantitatively evaluate the effectiveness of DNA compartmentalization. Very good agreement was observed between the experimental results and the theoretical curve (Fig. 5A), suggesting good detection accuracy. This observation indicates that large λ -DNA molecules were stochastically distributed and encapsulated into discrete 0.43 nL microwells. This finding could be attributed to the fact that the PCR solution was absorbed into the microwells by microgels, carrying randomly dispersed DNA molecules along. Therefore, partitioning of DNA molecules from the bulk into the microwells is predominantly driven by the hydrodynamic flow and dependent on the overall solution volume absorbed by the microgels rather than the volume fraction accessible to DNA. As a result, in our method size exclusion does not significantly affect the stochastic partition of DNA. Furthermore, since the gel fibers accounts for only a very small volume fraction (< 2 %) in the swollen superporous microgels assembled by 4% freeze/thawed agarose beads, the volume of PCR solution dispensed into a microwell is nearly the same as the microwell volume, which allows us to accurately calculate the concentration of DNA targets in samples using the Poisson statistics.

We compared the dPCR performance of μ SAAC to that of droplet dPCR (ddPCR) measurement of the λ -DNA standards (0.054 and 0.54 ng/mL) using a microfluidic ddPCR technology which has been well established in our lab.^{6, 9, 15} The average concentrations and their expanded uncertainties at a confidence level of 95% were calculated following the approach described by Pinheiro, et al. for evaluating two commercial dPCR instruments,⁴⁶ which permits direct comparison with their results. As presented in Fig. 5B, the average concentrations with an expanded uncertainty obtained with the μ SAAC were determined to be $(2.19 \pm 0.19) \times 10^4$ copies/mL and $(2.44 \pm 0.17) \times 10^5$ copies/mL for two λ -DNA standards, respectively, which match closely with the results of the droplet dPCR, $(2.27 \pm 0.09) \times 10^4$ copies/mL and $(2.55 \pm 0.09) \times 10^5$ copies/mL. This comparison confirms the good detection accuracy of μ SAAC, which supports the stochastic distribution of λ -DNA molecules across the microwell chip. The relative expanded uncertainty of the microfluidic ddPCR was found to be comparable with that of the commercial ddPCR instrument (Bio-Rad) for λ -DNA quantification (see Table S1). The relative expanded uncertainty of the μ SAAC was relatively larger than those of the droplet dPCR measurements and slightly lower than that reported for the BioMark system (Fluidigm) using a 765-unit array chip⁴⁶ (Table S1). This can be attributed to the fact that the accuracy and precision of dPCR is dependent on the number of reactions analyzed, which has been observed in many studies.^{26, 31, 46} Thus our device can be scaled up to improve its accuracy and precision. Another factor that can affect the reliability of dPCR is manual sample loading on the μ SAAC prototype. In this work, we conducted manual sample loading in order to demonstrate the adaptability of our method to the routine applications in the regular biology lab settings. While this manually operated chip could result in analysis variation when compared to the commercial systems, it provides a point-of-use tool for applications that

need fast and low-cost DNA/RNA quantification on a routine base. It is worth noting that the measurement uncertainty of our device compares favorably with that reported by another equipment-free, point-of-care dPCR chip that relies on vacuum to drive self-filling of microwells.⁴⁷ For applications that requires better detection accuracy and reproducibility, it is possible to combine our chip with an automated sample loader, similar to the commercial OpenArray® AccuFill™ sample loader (ThermoFisher), to minimize the errors induced by the manual operation. It is also well documented that the sensitivity and dynamic range of dPCR is largely dependent on the total number of reactions that can be analyzed. Compared to the droplet dPCR technologies, the μ SAAC has preset capacity (i.e., number of microwells) for single-copy PCR, just like other microarray systems, which limits the detection sensitivity, accuracy, and dynamic range.^{26, 46} As a proof of concept, here we used a moderately sized μ SAAC device with 2500 wells to demonstrate the application of our method for dPCR. This 2500-well chip was able to detect as low as 5.4 pg/mL (0.001 cpw) λ -DNA with a dynamic range up to 5.4 ng/mL (1 cpw), as seen in Fig. 5A. The sensitivity and the dynamic range of our device can be readily customized to meet the need of specific applications by scaling up the microwell number on the chip.

Detection and Sequencing of Genetic Mutation

The μ SAAC developed here adopts a simple open microwell format. Compared to the enclosed microwell structure commonly used in microfluidic dPCR devices,^{24, 28, 29, 31} this format enable convenient liquid dispensing and recovery from individual nanoliter or picoliter volumes. Thus nano-/microwell systems have been widely used for high-throughput analyses in biology and medicine, including chemical screening,⁴⁵ single cell analysis,^{48, 49} and sequencing.⁵⁰ To demonstrate the potential applications of the μ SAAC to biomedical research and clinical studies, we tested it for detection and single-molecule sequencing of a cancer-associated genetic abnormality, the chromosomal translocation t(14;18), using the PCR assay established previously.⁶ The chromosomal translocation t(14;18) is a mutation prevalent in many blood cancers, including 85–90% of cases in follicular lymphoma,^{51, 52} and has also been found in healthy individuals.^{53–55} Thus t(14;18) holds potential clinical value for risk assessment and early diagnosis of lymphoma.

The experimental process is illustrated in Fig. 6A, in which the μ SAACs were prepared as the off-the-shelf chips and only standard molecular biology equipment and techniques were employed. Briefly, dPCR was first performed to detect the t(14;18) and the amplicons yielded in individual positive microwells were manually sampled by pipetting under a microscope and re-amplified by standard PCR to yield sufficient template for DNA sequencing. The re-amplification product was run by gel electrophoresis and the DNA band was excised from the gel and sequenced using the standard Sanger sequencing. As a proof of concept, dPCR detection of t(14;18) was performed using a standard sample of gDNA purified from RL cells at the mutation concentration of $\sim 6 \times 10^5$ copies/mL, equivalent to ~ 1 cpw. The on-chip dPCR detection yielded $\sim 65\%$ positive wells (Fig. 6B), which agrees well with the prediction of the Poisson distribution. The measured concentration was calculated to be 6.3×10^5 copies/mL, in good agreement with the expected value (5% deviation). Gel separation of the re-amplification product showed a single DNA band (Fig. 6C), verifying the specificity of the dPCR assay. A typical sequencing electropherogram was presented in

Fig. 6D, which recovered the sequences from both Chromosomes 14 and 18 and also identified a unique short sequence inserted at the junction. The chromosome breakpoint locations and the insertion sequence were found to match the unique t(14;18) translocation “fingerprint” of RL lymphoblast sequence.⁵⁶ Overall, these results should have demonstrated the applicability of the μ SAAC for quantitative detection and single-molecule sequencing of genetic alterations.

Conclusion

To facilitate the widespread application of digital PCR, we have developed a new μ SAAC which is a low-cost PDMS chip with an array of microwells packed with superporous absorbent microgels. The micropatterned superabsorbent gels enable spontaneous filling of PDMS microwells for compartmentalization of target molecules without the need of sophisticated microfluidic sample loading equipment and operation expertise. Using large λ -DNA as the model template, we have characterized the μ SAAC for stochastic partition and quantitative digital detection of DNA molecules, which matched well with the theoretical prediction by the Poisson distribution. Furthermore, we have demonstrated dPCR detection and single-molecule sequencing of the chromosomal translocation t(14;18), suggesting the ability of the μ SAAC for genetic analysis of diseases. These experiments were carried out by following the protocols that are compatible with standard biological research lab setting. Because of the simple fabrication process, the μ SAAC device holds the potential for mass production of the off-the-shelf product, which could significantly facilitate the adaptation of dPCR technology in general research labs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Digital PCR using micropatterned superporous absorbent array chip (μ SAAC). **(A)** Schematic illustration of fabrication of ready-to-use μ SAAC. A PDMS microwell array chip is used to pack dry agarose microparticles to construct an array of hierarchically porous microgels. **(B)** Workflow for digital PCR using μ SAAC. A PCR mix containing DNA targets is pipetted to cover the surface of a μ SAAC. The microgels in the PDMS microwells fully absorb the PCR solution after brief incubation. Excess PCR mix are removed and the microwell chip is sealed against a glass-slide coated with a thin layer of fluorocarbon oil to isolate individual DNA copies into nanoliter compartments. Finally, the chip will be placed on a thermal cycler for PCR amplification, followed by the fluorescence detection to count the copy number of targets.

Figure 2.

Fabrication of the μ SAAC. **(A)** Bright-field microscopic image showing an array of bead-packed microwells. The arrayed superabsorbent microgels appear dark in the dry state due to the scattering of light by the highly porous structure. Well size: $62\ \mu\text{m} \times 62\ \mu\text{m}$. **(B)** SEM images of cross-linked 4% agarose beads without (left) and with (right) treatment of several freeze/thaw cycles before cross-linking.

Figure 3.

Spontaneous filling of μ SAAC with aqueous solution. (A) Bright-field microscopic images showing wetting and swelling of the microgels composed of packed agarose beads by water to fill the untreated hydrophobic PDMS microwells. Well size: $240\ \mu\text{m} \times 240\ \mu\text{m}$. (B, C) Bright-field and fluorescence images showing uniform filling of the μ SAAC by the fluorescent dye solution. Well size: $62\ \mu\text{m} \times 62\ \mu\text{m}$.

Figure 4. Representative fluorescence images of digital PCR detection of λ -DNA using μ SAAC. A 10 $\times$ dilution series of λ -DNA standards (0.0054 to 5.4 ng/mL) were assayed, which is equivalent to the final concentrations ranging from 0.001 to 1 copy per well (cpw). The averaged dimensions of microwells were 62 μ m $\times$ 62 μ m $\times$ 112 μ m.

Figure 5.

Quantitative dPCR detection of λ -DNA using μ SAAC. (A) Log-log plot of the percentage of positive wells as a function of the equivalent DNA concentration measured from the dPCR analysis of λ -DNA using μ SAAC shown in Figure 4. The solid curve is the theoretical plot calculated by Poisson statistics. The experimental data were obtained by detecting λ -DNA samples across a concentration range from 0.0054 to 54 ng/mL, which is equivalent to approximately 0.001–10 copies per well (cpw). Error bars represent standard deviation ($n = 3$). (B) Comparison of μ SAAC dPCR and droplet dPCR for absolute quantification of λ -DNA concentration. Two DNA standards with the concentrations of 0.054 and 0.54 ng/mL were measured. Error bars represent the expanded uncertainty at a confidence level of 95% ($n = 3$).

Figure 6. Single-molecule PCR detection and sequencing of genetic mutations using μ SAAC. (A) Illustration of the workflow for dPCR detection and subsequent sequencing of a chromosomal translocation t(14;18). (B) Representative section of a fluorescence image of detecting t(14;18) at a concentration of $\sim 6 \times 10^5$ copies/mL (equivalent to ~ 1 cpw) using a μ SAAC with 120 μ m microwells. (C) Gel image showing negative control (lane 1) and a band of DNA (lane 2) re-amplified from the amplicon sampled from a positive well visualized in (B). The gel band was cut off and the DNA product was extracted as the template to conduct Sanger sequencing reaction. (D) The electropherogram of sequencing the junction region across the breakpoint of the t(14;18) translocation amplified by dPCR on μ SAAC. The sequences from both chromosomes 14 and 18 with a unique breakpoint insertion sequence were identified as indicated. The binding site of the reverse primer on Chromosome 14 is underlined.