Published in final edited form as:

Lab Chip. 2012 September 7; 12(17): 3055–3062. doi:10.1039/c2lc40399d.

Microfluidic platform for on-demand generation of spatially indexed combinatorial droplets

Helena Zec^{a,†}, Tushar D. Rane^{a,†}, and Tza-Huei Wang^{a,b}

Tza-Huei Wang: thwang@jhu.edu

^aDepartment of Biomedical Engineering, Johns Hopkins University, Baltimore, USA.; Tel: +1 410 5164746

^bDepartment of Mechanical Engineering, Johns Hopkins University, Baltimore, USA.; Tel: +1 410 516 7086

Abstract

We propose a highly versatile and programmable nanolitre droplet-based platform that accepts an unlimited number of sample plugs from a multi-well plate, performs digitization of these sample plugs into smaller daughter droplets and subsequent synchronization-free, robust injection of multiple reagents in to the sample daughter droplets on-demand. This platform combines excellent control of valve-based microfluidics with the high-throughput capability of droplet microfluidics. We demonstrate the functioning of a proof-of-concept device which generates combinatorial mixture droplets from a linear array of sample plugs and four different reagents, using food dyes to mimic samples and reagents. Generation of a one dimensional array of the combinatorial mixture droplets on the device leads to automatic spatial indexing of these droplets, precluding the need to include a barcode in each droplet to identify its contents. We expect this platform to further expand the range of applications of droplet microfluidics to include applications requiring high degree of multiplexing as well as high throughput analysis of multiple samples.

Introduction

Droplet microfluidic platforms are in the early stages of revolutionizing high throughput and combinatorial sample screening for bioanalytical applications^{1–4}. Droplet-based systems have many advantages over conventional microtiter techniques for combinatorial screening applications. Microfluidic droplets function as miniaturized reaction containers consist of pico- or nanolitre volumes, thereby facilitating reduced reagent consumption and background noise⁴. Furthermore, carrier fluid shields each droplet, mitigating the problems of absorption onto solid surfaces and contamination. The high surface area to volume ratio of droplets also facilitates shorter heat and mass transfer times, leading faster reaction kinetics in droplets^{1, 5}.

Recent publications in the microfluidic domain boast high throughput capability of their respective droplet-based platforms. Compartmentalization of many small droplets allows isolation of interfering molecules into separate volumes, thereby facilitating digital analysis of biological entities. Several examples have been demonstrated including: droplet platforms for high throughput single copy DNA amplification^{6–8}, droplet platforms for high throughput single cell screening^{9–11} as well as droplet platforms for single organism screening^{12, 13}. These droplet platforms are capable of high resolution screening of the

[†]These authors contributed equally to this manuscript

biological contents at the level of the fundamental unit. However, they are limited to the analysis of a single sample under a homogeneous reagent condition.

The aforementioned droplet platforms are incapable of addressing the needs of numerous applications which require high degrees of multiplexing as well as high-throughput analysis of multiple samples. Some examples include genetic fingerprinting for forensics¹⁴, single nucleotide polymorphism (SNP) analysis for crop improvement and domestication¹⁵, genotyping required for identification of genes associated with common diseases¹⁶ and generation of a blood donor genotype database for better matching between recipient and donor to prevent adverse transfusion reactions¹⁷. All these applications require multiplexed screening of a single sample with a panel of reagents (or markers) and rapid screening of a large number of samples to generate the required databases.

In recent years, there have been attempts to expand the capacity of droplet platforms for the analysis of a biological or chemical sample with multiple reagents. One of the well-tested platforms has been the droplet platform developed by RainDance Technologies, for massively parallel PCR enrichment for DNA sequencing¹⁸. This platform involves a multistep approach with generation of a large library of PCR reagent droplets by a microchip, followed by merging of these reagent droplets with sample droplets generated from a DNA sample on a second device. These sample-reagent hybrid droplets are then collected in standard PCR tubes for thermocycling, followed by fluorescence detection and sequencing. In this platform, the content of each individual droplet is unknown and is decoded only by offline nucleic acid sequencing. Therefore, it cannot be applied to other applications that require real-time detection⁴. A solution to this problem is to associate a unique optical-code with each reagent prior to mixing with the sample¹⁹. However, an optical-coding scheme based on fluorescence intensity is practically limited to a small number of 'codes' due to the small allowable number of fluorophores without spectral crosstalk and the limited dynamic range of the optical detection setup being used⁴. Furthermore, the electrocoalescence technique used in such platforms for droplet merging is susceptible to errors of no fusion caused by an excess of droplets of a reagent or unintended fusion of more than two droplets due to highly stringent synchronization requirements²⁰. A recent article demonstrated a picoinjector which can overcome this problem and be used to add controlled volumes of multiple reagents to sample droplets using electromicrofluidics²¹. However, similar to droplet platforms discussed earlier, the content of each individual droplet is unknown unless a barcode is included in each individual droplet.

Alternatively, a series of articles adopted a cartridge technique for increasing the throughput of the droplet platform²². This technique involves generation of an array of reagent plugs in a capillary (cartridge), which are sequentially introduced to a simple microfluidic device for merging with a single substrate. The reagent plugs can be further digitized into smaller droplets prior to merging with the sample. As the length of the capillary can be very long, the number of reagents to screen against the sample is virtually limitless. This technique has been applied to many applications including protein crystallization²² and study of bacterial susceptibility to antibiotics²³. Although the aforementioned droplet and cartridge platforms are capable of high throughput and multiplexed analysis, they are still limited to screening of a single sample at a time. Recently, a microfluidic platform was proposed for combinatorial chemical synthesis in picolitre droplets, where droplets of one library of reagents were fused at random with droplets containing a different set of reagents²⁰. This platform has the potential of generating a large set of possible combinations of different reagents. However, as afore-discussed, the unknown identity of the compounds within individual droplets precludes its use for many screening applications that require real-time detection.

Here we present a droplet platform capable of on-demand generation of nanolitre droplets of combinational mixtures of samples and reagents, needed for biochemical screening applications that require multiplexing and high-throughput capability. On-demand droplet generation and manipulation using pneumatic valves has been demonstrated by other groups in the past^{24–26}. However, these platforms have focused on generating multiple reagent combinations using fixed number of inputs to the device, severely limiting the number of possible sample-reagent combinations being generated on the device. The droplet platform reported in this article uses a linear array of sample plugs as an input to the device, removing the limitation imposed by the number of inputs to the device. Initially, a preformed linear array of sample plugs separated by a carrier fluid is flowed from the cartridge into the microfluidic device, wherein each plug is digitized by a pneumatic valve into smaller sample daughter droplets. The volume of the resulting daughter droplet can be precisely controlled by varying the valve opening time and the back pressure on the cartridge containing sample plugs. The daughter droplets are then directly injected with reagents in a synchronization-free manner. The microfluidic design features a robust fusion module which exploits local channel geometry for synchronization-free injection of reagents into each sample daughter droplet. After reagent injection into a sample droplet, our microfluidic device introduces additional carrier fluid containing surfactant to the channel containing the sample-reagent hybrid droplet array to prevent unwanted merging of these droplets on the device. In the proposed microfluidic device, droplets are indexed by their layout in a 1D array, enabling the identification of the contents of each droplet by spatial indexing. Spatial indexing as a means for identification of droplet content obviates the need for a limiting optical barcoding scheme.

Materials and Methods

Serial Sampling Loading System

The sample library was generated using a custom-designed Serial Sample Loading System (SSL). Fig. 2 is a schematic illustrating the functioning of the SSL system. Briefly, the SSL system was designed to be compatible with Costar 96-well plates (Corning). Initially the wells on a Costar 96-well plate are filled with the samples and the carrier fluid to be used for generating the sample plug array. An Aquapel (PPG Industries) treated silica capillary is then attached to a capillary adapter on the SSL system, which is also connected to a positive pressure input. A sample well is then interfaced with the capillary through the capillary adapter. Application of positive pressure to the sealed sample well for a controlled amount of time is then used to drive a sample plug from the well into the capillary. This sequence of steps is then repeated to load alternating sample and carrier fluid plugs into the capillary (Fig. 2b). More detailed information on the structure and operation of different components of the SSL system can be found in the Supplementary Information.

Fabrication of the Master Molds for the Microfluidic Device

The fluidic layer on the microfluidic device features five different heights of microfluidic channels (Fig. 3b and c). As a result, the fluidic mold consists of five different layers of photoresist. The fluidic channel heights in these five different photoresist layers were expected to be 25 μm , 50 μm , 100 μm , 200 μm and 360 μm . The photoresist used for the 25 μm layer was SPR 220-7.0 (Rohm & Haas), while the rest of the layers were fabricated using SU-8 3050 (MicroChem). Fabrication was performed using standard photolithography techniques. Briefly, a SPR 220-7.0 layer was spin coated on a 4 inch silicon wafer. This layer was patterned using photolithography and hard baked to generate a rounded channel cross section, required for effective valve closure, as has been described earlier²⁷. For all other layers, SU-8 3050 was spin-coated on the wafer and patterned using standard photolithography, excluding the developing step. This technique was found to be very

effective in preventing generation of bubbles and non-uniform coating of photoresist on the wafer due to the presence of features from earlier layers on the wafer. A single developing step for all four SU-8 3050 layers was used to remove excess photoresist on the wafer (Supp. Fig. 1). The control layer for the microfluidic device on the other hand consisted of microfluidic channels of a single height. As a result, the mold fabrication for the control layer was relatively simpler, with a single layer of SU-8 3050 photoresist, 50 μm in height.

Microfluidic Device Fabrication

The microfluidic devices were fabricated using multilayer soft lithography techniques²⁷. The protocol differed slightly from our standard protocol^{28–30} due to the need for proper functioning of push-down valves³¹ while accommodating tall features (up to 360 μm) on the fluidic layer. The thickness of the polydimethylsiloxane (PDMS) membrane separating the control layer and the fluidic layer in a microfluidic device needs to be less than ~50 μm , for complete valve closure at reasonable pressure (~30 PSI). However, the presence of fluidic regions as tall as 360 μm on the fluidic layer mold precluded the possibility of covering the entire fluidic layer mold with PDMS, while maintaining the thickness of the PDMS layer to a value less than 50 μm in the regions of the device containing valves. To overcome this problem, a modified three-layer fabrication process was developed. Detailed description of the fabrication process is included in the supplementary material (Supp. Fig. 2).

Capillary-to-Chip Interface

Following the microfluidic device fabrication, a silica capillary was attached to the ‘capillary inlet’ on the microfluidic device (Fig. 3b). The 360 μm tall channel region at the capillary inlet accommodates a silica capillary with an OD of 360 μm . A 10 mm section of silica capillary is inserted horizontally into this tall channel on the device until it is flush with the 200 μm tall fluidic channel on the device. To seal the capillary to the chip and prevent leakage, PDMS was dispensed around the capillary at the interface between the capillary and the device. The PDMS tended to crawl into the 360 μm channel and surround the capillary, effectively sealing the capillary-to-chip connection. The final assembly was baked for at least 2 hours at 80°C before usage.

Device control

All the inputs on the device were kept under constant pressure, with independent input pressure for 1) carrier fluid input, 2) all four reagent inputs and 3) carrier fluid with surfactant input. The pressure applied to the capillary input was controlled directly by the pressure controller used for the SSL system. All the valves on the device were controlled by an array of off-chip solenoid valves, as has been demonstrated earlier²⁸. We developed Matlab (Mathworks, Natick MA) software for computer control of the valve array. This software allowed us to execute a predetermined sequence of valve actuation with independent time control for each actuation. The opening of a valve corresponding to an input on the device led to the release of a droplet of fluid from that inlet into a central channel on the device. The volume of this droplet could be controlled through variation of the opening time of the valve.

Reagents

All the devices and capillaries were treated with Aquapel to render their surface hydrophobic. The testing of our platform was performed using food dyes (Ateco, Glen Cove, NY) to mimic different samples and reagents for easy visualization. The carrier fluid used to maintain the separation between sample plugs consisted of a perfluorocarbon (FC-3283) and a non-ionic fluorosoluble surfactant (1H,1H,2H,2H-Perfluoro-1-octanol)

mixed in a ratio of 8:1 by volume. The carrier fluid with surfactant consisted of FC-40 (3M) and 2% 'EA' surfactant (Raindance Technologies) by weight.

Sample plug and droplet volume estimation

We estimated the volume of sample plugs and sample droplets generated using the SSL system and the microfluidic device respectively. This volume estimation was performed by processing the images of these sample plugs or droplets using the software ImageJ³². Specifically, for sample plug volume estimation, a series of sample (blue food dye) plugs were generated in a silica capillary using the SSL system. A colour image of these plugs was taken against the white background of a 'letter' sized sheet of paper using a standard Digital Single-Lens Reflex (DSLR) camera. This image was imported in ImageJ and the length scale was set to true length using the known length of the letter sized paper in the image. The lengths of the sample plugs were then manually measured for each plug using the 'Measure' function in ImageJ. The plug lengths could be converted to plug volumes with the known cross sectional area of the capillary.

For sample droplet volume estimation, we generated droplets made of blue food dye using one of the four reagent inlets on the microfluidic device, until the whole incubation region on the device was full of droplets. The whole device was then imaged using a DSLR camera. The image was imported in ImageJ and cropped to obtain an image of the incubation region on the device. This image was then converted to a binary image using colour thresholding to identify droplets over the background image. An estimate of the droplet area for each droplet in the image was then obtained using the 'Analyze Particles' function. This analysis was limited to particle areas larger than a lower threshold to exclude any particles and occasional satellite droplets from the analysis. The droplet areas thus estimated were then converted to droplet volume using the known depth of the incubation channel region (200 μm).

Results and Discussion

Overall Work Flow

Fig. 1 is a schematic illustrating the functioning of the platform. Initially a cartridge (capillary) is loaded with a library of sample plugs forming a serial sample plug array: plugs are separated from each other by an immiscible carrier fluid. This cartridge is interfaced with a microfluidic device featuring multichannel architecture and pneumatic microvalves. The microfluidic device digitizes sample plugs into smaller daughter droplets on. Each sample daughter droplet then moves to the downstream fusion region where a specific reagent is injected into the sample daughter droplet. The reagent droplets are injected into the sample daughter droplet through controlled actuation of valves corresponding to the reagent inlets. Supp. Video 1 shows this sequence of events. No strict synchronization or droplet detection module is necessary for fusion of sample and reagent to occur as the sample droplet is elongated in the fusion area (Supp. Video 2), exploiting the local channel geometry. The resulting sample-reagent droplet undergoes mixing and travels downstream to the incubation region on the device. After reagent injection, additional carrier fluid containing surfactant is released into the central channel on the device to stabilize sample-reagent hybrid droplets. The sequence of droplets is maintained throughout the device, precluding the need for a complicated barcoding scheme to identify the contents of each individual droplet.

Capillary-to-Chip Interface

Our prototype platform necessitated the capillary-to-chip interface design to allow for sample plug introduction on chip. This objective presented a unique challenge, since proper

functioning of the platform requires smooth transition of sample plugs from the large ID of the capillary to shallow channels on the device in the valve regions. There have been demonstrations of capillary-to-chip interfaces in the past for introducing sample plugs from a capillary to a microfluidic device. However, the devices used don't face this problem as they typically feature large channels with a valveless design^{22, 23}. The capillary interface we designed (Fig. 3b and c) between the capillary and microfluidic chip was found to be effective in minimizing plug break up as plugs moved from the high ID (200 μm) of the capillary to the shallow channels on chip (25 μm). This transition consisted of 5 different channel sections with gradually reducing channel heights of 360 μm , 200 μm , 100 μm , 50 μm and 25 μm . This gradual transition minimizes the shear stress on the sample plug as it traverses from a capillary to the shallow channels on the chip, preventing its breakup in transit.

Droplet uniformity using mechanical valve based droplet generation

We examined the performance of the mechanical valves on our microfluidic device for their capability to control the droplet size generated. To conduct this experiment, we primed the incubation channel on the device with the carrier fluid. We then used one of the reagent inlets on the device for generating droplets made of blue-colored food dye into the incubation channel region. The two parameters which could be used to control the droplet size generated from a reagent inlet are 1) Input pressure to the reagent inlet (P_{reagent}) and 2) The opening time of the valve corresponding to the reagent inlet (T_{open}). Initially, we fixed the value of P_{reagent} and generated droplets on the device for different values of T_{open} . Droplet generation was continued for each condition tested, until the incubation region on the device was completely full of droplets. We then estimated the volume for all these droplets using the image processing technique discussed in the 'Materials and Methods' section. The mean and standard deviation of fifty droplets generated for each condition was plotted against T_{open} in Fig. 4b. This experiment was repeated for three different fixed values of P_{reagent} . As expected, the linear relationship between droplet volume and T_{open} indicates excellent and predictable control of the device over droplet volume. Small standard deviation observed on the droplet volume also indicates excellent droplet uniformity for identical droplet generation conditions. This result is very important to ensure the capability of the device to generate droplets of various compositions on-demand.

Sample Digitization

We examined the capability of our device to digitize a set of sample plugs being supplied to the device into smaller sample daughter droplets. To conduct this experiment, we generated a set of sample plugs into a silica capillary using the SSL system. These sample plugs were delivered to the microfluidic device through the capillary inlet, under pressure provided by the pressure controller on the SSL system. For this experiment, the repeating sequence of steps executed on the device was as following: 1) Generate small droplet from a sample plug in the central channel, 2) Move the droplet towards incubation region with carrier fluid 3) Release small amount of carrier fluid with surfactant in the central channel. Repeating this set of steps led to generation of an array of sample droplets generated through digitization of sample plugs on the device. Examples of unmerged sample daughter droplets are shown in Fig. 6a (Sample droplets A, B, C and D). The order of the sample plugs in the capillary is consistently maintained on the device, even after the digitization operation. One shortcoming of this operation is the generation of non-uniform droplets towards the beginning and the end of the sample plugs. This is because the valve actuation sequence is continuously executed without any sensing of sample plug arrival on the device. However, the sample droplet uniformity is maintained throughout the rest of the sample plug. As the droplets generated on the device are stabilized with surfactant, undesirable merging of non-uniform droplets originating from the front- or back-end of plugs is avoided on the device.

Generation of droplets of combinatorial mixtures

In this section we demonstrate proof-of-concept generation of combinatorial mixtures from sample plugs and reagent droplets on our device. For discernibility, we chose to use different food dye solutions to simulate different samples and reagents. Fig. 5 shows how reagent injection operations are performed in the fusion region on our device (also shown in Supp. Video 1). First, a sample plug travels from the capillary on to the microfluidic device. This plug is then chopped into a smaller sample daughter droplet. This droplet is then moved to the downstream fusion zone through release of carrier fluid in the central channel on the device. As every single input on the device is controlled with an individual valve, the device functions like an assembly line with complete temporal and spatial control over every single operation, as against typical droplet generating devices where the carried fluid flow is continuous. This level of control also implies that the operation of the device can be paused and resumed with a completely new valve actuation sequence, on demand without affecting the existing droplets on the device. None of the droplet devices reported in literature so far has this capability to the best of our knowledge. A reagent droplet is then injected directly into the sample daughter droplet (Fig. 5a). The volume of reagent solution injected into the sample droplet can be controlled through variation of the opening time for the valve corresponding to the reagent inlet. Supp. Video 2 provides a close-up view to how reagent injection is performed. The fusion zone is designed such that the sample daughter droplet is sufficiently elongated within a region, which overlaps with all the injection ports of the interrogating reagents. This elongated droplet state removes the need for strict positioning accuracy requirements on the sample droplet for reliable injection of reagent into the sample droplet. In addition to demonstrating injection of a single reagent in a sample droplet, we have demonstrated injection of up to four reagents into a single sample droplet as shown in Fig. 5b. The concept of droplet elongation to aid reagent injection can be easily scaled to accommodate tens of reagent inlets, if desired. Post reagent injection, no unwanted mixing of reagents was observed with subsequent sample plugs. Occasional residual reagents residing between the activated valve and central channel at a reagent inlet are encapsulated by a sheath of carrier fluid which prevents fusion with the next sample droplet.

After reagent injection, the sample-reagent droplet is driven further downstream with the help of carrier fluid. Following this, a small plug of carrier fluid with surfactant is released in the central channel for stabilizing the droplets in the incubation region. Using this scheme we can simultaneously take advantage of a surfactant-free zone in one area of the chip to promote sample-reagent merging while deliberately using surfactant in another area to increase droplet stability and prevent unwanted droplet merging. In addition, the backpressure on the carrier fluid inlets was used to control flow velocity of the droplets. For the results presented in this paper, the flow velocity of droplets was ~5 mm/second. However, the flow velocity can be easily tuned by controlling the back pressure on the central carrier fluid channel.

Fig. 6 demonstrates the reliability of the fusion mechanism on our device. Fig. 6a is a table of 16 different sample-reagent combinations generated on a single chip through all possible merging combinations of four different sample daughter droplets (A: blue, B: yellow, C: green, D: water) with four different reagents (Reagent 1: orange, Reagent 2: water, Reagent 3: blue, Reagent 4: yellow) with the condition of merging exactly one sample with one reagent.

The micrographs in Fig. 6b show a repeating sequence of the sample-reagent hybrid droplets in the incubation region of the chip. The chip is operated such that the sample daughter droplets are merged with a repeating sequence of four different reagents. As a result a repeating sequence of four possible combinations generated through mixing a single sample with four different reagents can be seen in each individual micrograph. Once a sample plug

is exhausted, the sample daughter droplets generated from the next incoming sample plug start merging with the same repeating sequence of reagents generating a repeating sequence of a new set of four different sample-reagent combinations in the incubation region on the chip.

The droplet monodispersity as well as the uniform spacing between droplets is clearly visible in these micrographs. The inset in Fig. 6b displays zoomed-in view of these micrographs of the incubation region illustrating two repeats of each sequence in the incubation region. These images also demonstrate the capability of the device to maintain the order in which droplets are generated, throughout the incubation region on the device. We have demonstrated 16 combinations in this instance, but by employing multiple (2, 3 or 4) reagent merging with sample daughter droplets, as demonstrated in Fig. 5b, many more combinations can be generated using our prototype device.

Conclusion

In summary, we have demonstrated a platform capable of preparing droplets from combinational mixtures of a large number of samples and reagents. This is accomplished by synchronization-free and detection-free fusion of sample daughter droplets and reagents. A key benefit of this architecture is the ability to scale this device to analyze N samples against M reagents ($N \times M$) where N can range from hundreds to thousands without accompanying increase in device complexity. Additional reagent set multiplexing can be accomplished analogously by introducing linear arrays of reagent set plugs similar to sample introduction. Furthermore, this design allows for spatial indexing, by maintaining the sequence of droplets from generation throughout incubation, precluding the need for barcoding.

This platform presents a novel design with several important components: a unique SSL system which uses pressure to inject uniform volumes of sample into a capillary directly from an industry standard multi-well plate. This capillary is then interfaced with a microfluidic device using a novel capillary-to-chip connection. The microfluidic device is capable of combinatorial screening operations. Robust synchronization-free reagent injection is performed on the device based on a design which capitalizes on droplet elongation in the fusion zone on the device. In our prototype design up to 4 reagent droplets can be fused with a single sample droplet. However, by employing the same concept many more reagent inlets can be introduced on chip to perform merging operations. In addition, we have demonstrated a technique for reagent injection in droplets that capitalizes on controlling droplet surface chemistry by controlling surfactant concentration at different regions on the chip. That is, we have demonstrated a surfactant-free environment in the fusion zone on the device, thereby promoting reagent injection in sample droplets while the droplets are stabilized by surfactant in the incubation region.

For the microfluidic chip design, several areas can be explored to further enhance the operation of the chip. To make the transition of sample plugs from a capillary to the microfluidic device more gradual a photolithography process employing a grayscale mask could be used. This approach can generate very gradual reduction in channel cross section from a large capillary to shallow microfluidic channels on the device as against the 5 step reduction demonstrated in the current version of the device. Furthermore, reagents may be loaded in cartridge format to further enhance multiplexing capabilities. We expect the platform described here to be a promising candidate for combinatorial screening applications using droplet microfluidics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Dong Jin Shin for his help with writing the LabVIEW software for controlling the SSL system. We are thankful to A.P. Lee, M. Simon and R. Lin at University of California Irvine and W.C. Chu and H. Sullivan at Pioneer Hi-Bred International, Inc. for helpful discussions. We also thank the funding support from DARPA (Micro/Nano Fluidics Fundamentals Focus (MF3) Center), Pioneer HiBred International, Inc., and NIH (R01CA155305, U54CA151838).

Notes and References

1. Teh SY, Lin R, Hung LH, Lee AP. Lab Chip. 2008; 8:198–220.10.1039/b715524g [PubMed: 18231657]
2. Huebner A, Sharma S, Srisa-Art M, Hollfelder F, Edel JB, Demello AJ. Lab Chip. 2008; 8:1244–1254.10.1039/b806405a [PubMed: 18651063]
3. Pompano RR, Liu W, Du W, Ismagilov RF. Annu Rev Anal Chem (Palo Alto Calif). 2011; 4:59–81.10.1146/annurev.anchem.012809.102303 [PubMed: 21370983]
4. Guo MT, Rotem A, Heyman JA, Weitz DA. Lab Chip. 2012.10.1039/c2lc21147e
5. Song H, Chen DL, Ismagilov RF. Angew Chem Int Ed Engl. 2006; 45:7336–7356.10.1002/anie.200601554 [PubMed: 17086584]
6. Kumaresan P, Yang CJ, Cronier SA, Blazej RG, Mathies RA. Anal Chem. 2008; 80:3522–3529.10.1021/ac800327d [PubMed: 18410131]
7. Beer NR, Hindson BJ, Wheeler EK, Hall SB, Rose KA, Kennedy IM, Colston BW. Anal Chem. 2007; 79:8471–8475.10.1021/ac701809w [PubMed: 17929880]
8. Kiss MM, Ortoleva-Donnelly L, Beer NR, Warner J, Bailey CG, Colston BW, Rothberg JM, Link DR, Leamon JH. Anal Chem. 2008; 80:8975–8981. [PubMed: 19551929]
9. Huebner A, Srisa-Art M, Holt D, Abell C, Hollfelder F, deMello AJ, Edel JB. Chem Commun (Camb). 2007; 12:1218–1220.10.1039/b618570c [PubMed: 17356761]
10. Clausell-Tormos J, Lieber D, Baret JC, El-Harrak A, Miller OJ, Frenz L, Blouwolff J, Humphry KJ, Koster S, Duan H, Holtze C, Weitz DA, Griffiths AD, Merten CA. Chem Biol. 2008; 15:427–437.10.1016/j.chembiol.2008.04.004 [PubMed: 18482695]
11. Baret JC, Miller OJ, Taly V, Ryckelynck M, El-Harrak A, Frenz L, Rick C, Samuels ML, Hutchison JB, Agresti JJ, Link DR, Weitz DA, Griffiths AD. Lab Chip. 2009; 9:1850–1858.10.1039/b902504a [PubMed: 19532959]
12. Shi W, Qin J, Ye N, Lin B. Lab Chip. 2008; 8:1432–1435.10.1039/b808753a [PubMed: 18818795]
13. Shi W, Wen H, Lu Y, Shi Y, Lin B, Qin J. Lab Chip. 2010; 10:2855–2863.10.1039/c0lc00256a [PubMed: 20882233]
14. Sobrino B, Brion M, Carracedo A. Forensic Sci Int. 2005; 154:181–194.10.1016/j.forsciint.2004.10.020 [PubMed: 16182964]
15. Gupta PK, Rustgi S, Mir RR. Heredity (Edinb). 2008; 101:5–18.10.1038/hdy.2008.35 [PubMed: 18461083]
16. Ragoussis J. Annu Rev Genomics Hum Genet. 2009; 10:117–133.10.1146/annurev-genom-082908-150116 [PubMed: 19453250]
17. Veldhuisen B, van der Schoot CE, de Haas M. Vox Sang. 2009; 97:198–206.10.1111/j.1423-0410.2009.01209.x [PubMed: 19548962]
18. Tewhey R, Warner JB, Nakano M, Libby B, Medkova M, David PH, Kotsopoulos SK, Samuels ML, Hutchison JB, Larson JW, Topol EJ, Weiner MP, Harismendy O, Olson J, Link DR, Frazer KA. Nat Biotechnol. 2009; 27:1025–1031.10.1038/nbt.1583 [PubMed: 19881494]
19. Brouzes E, Medkova M, Savenelli N, Marran D, Twardowski M, Hutchison JB, Rothberg JM, Link DR, Perrimon N, Samuels ML. Proc Natl Acad Sci U S A. 2009; 106:14195–14200.10.1073/pnas.0903542106 [PubMed: 19617544]

20. Theberge AB, Mayot E, El Harrak A, Kleinschmidt F, Huck WT, Griffiths AD. *Lab Chip*. 2012; 12:1320–1326.10.1039/c2lc21019c [PubMed: 22344399]
21. Abate AR, Hung T, Mary P, Agresti JJ, Weitz DA. *Proc Natl Acad Sci U S A*. 2010; 107:19163–19166.10.1073/pnas.1006888107 [PubMed: 20962271]
22. Li L, Mustafi D, Fu Q, Tereshko V, Chen DL, Tice JD, Ismagilov RF. *Proc Natl Acad Sci U S A*. 2006; 103:19243–19248.10.1073/pnas.0607502103 [PubMed: 17159147]
23. Boedicker JQ, Li L, Kline TR, Ismagilov RF. *Lab Chip*. 2008; 8:1265–1272.10.1039/b804911d [PubMed: 18651067]
24. Zeng S, Li B, Su X, Qin J, Lin B. *Lab Chip*. 2009; 9:1340–1343.10.1039/b821803j [PubMed: 19417898]
25. Guo F, Liu K, Ji X, Ding H, Zhang M, Zeng Q, Liu W, Guo S, Zhao X. *Appl Phys Lett*. 2010; 97:233701–3.
26. Wang H, Liu K, Chen KJ, Lu Y, Wang S, Lin WY, Guo F, Kamei K, Chen YC, Ohashi M, Wang M, Garcia MA, Zhao XZ, Shen CK, Tseng HR. *ACS Nano*. 2010; 4:6235–6243.10.1021/nn101908e [PubMed: 20925389]
27. Unger MA, Chou HP, Thorsen T, Scherer A, Quake SR. *Science*. 2000; 288:113–116. [PubMed: 10753110]
28. Puleo CM, Wang TH. *Lab Chip*. 2009; 9:1065–1072.10.1039/b819605b [PubMed: 19350088]
29. Puleo CM, Yeh HC, Liu KJ, Wang TH. *Lab Chip*. 2008; 8:822–825.10.1039/b717941c [PubMed: 18432356]
30. Rane TD, Puleo CM, Liu KJ, Zhang Y, Lee AP, Wang TH. *Lab Chip*. 2010; 10:161–164.10.1039/b917503b [PubMed: 20066242]
31. Melin J, Quake SR. *Annu Rev Biophys Biomol Struct*. 2007; 36:213–231.10.1146/annurev.biophys.36.040306.132646 [PubMed: 17269901]
32. Abramoff MD, Magalhaes PJ, Ram SJ. *Biophotonics International*. 2004; 11:36–42.

Figure 1. Schematic of the sample screening platform

Step 1: A cartridge is loaded with a library of sample plugs separated by an immiscible carrier fluid. This cartridge is interfaced with a microfluidic device. **Step 2:** On-demand digitization of incoming sample plugs into smaller daughter droplets. The volume of individual daughter droplets can be controlled by valve opening time and back pressure on the cartridge. **Step 3:** By exploiting the cross sectional area of the central channel, the sample daughter droplet is stretched in the “Fusion Region”. This approach allows for robust, synchronization-free injection of up to four reagents simultaneously directly into the daughter droplet. The volume of reagent injected is controlled through modulation of back pressure and valve opening time corresponding to the reagent inlet. **Step 4:** Once sample and reagent have been combined into one droplet, the droplets are stabilized with carrier fluid containing surfactant to prevent unwanted droplet coalescence downstream. **Step 5:** Sample-reagent droplets travel to an incubation channel. **Step 6:** The sample-reagent droplets are incubated while maintaining their sequence in downstream incubation channels. This approach allows for droplet identification through spatial indexing in a 1-Dimensional array.

Figure 2. 'Serial Sample Loading' (SSL) system

a) Schematic: A custom SSL system was designed which employs positive pressure to inject a sample plug into a microcapillary from a standard multi-well plate. A custom capillary adapter in the SSL system provides an interface between a microcapillary and a multi-well plate. This adapter can seal a well on the multi-well plate, thus generating a temporary pressure chamber inside the sample well. A pressure input on the adapter can then be used to apply controlled pressure to the fluid inside the sample well. This positive pressure drives a small plug of sample from the well into the microcapillary. Following this, the seal is broken and the multi-well plate is moved to seal another sample well with the capillary adapter. This sequence of steps is repeated to generate a sample library into the microcapillary. b) An image of a food dye sample plug array generated using the SSL system. Each sample plug is separated from each other by an immiscible carrier fluid.

Figure 3. Microfluidic Device Design

a) Photograph of a prototype device. The microfluidic device has a multichannel architecture: 1) The central channel with fusion region and incubation region (purple), 2) Capillary inlet, 3) Reagent inlets: reagent 1 (pink), reagent 2 (orange), reagent 3 (green), reagent 4 (turquoise) and surfactant oil inlet (yellow). The valves on the device (V1–V7) are indicated by a turquoise dye. b) A scan of the capillary inlet region, indicating the height difference between different sections of the capillary inlet to facilitate smooth sample plug transition from the large ID of the capillary to the shallow channels on the microfluidic device.

Figure 4. Control of droplet volume and droplet uniformity using mechanical valve based droplet generation

a) Micrograph of the incubation region on the microfluidic device filled with reagent droplets generated using sequentially increasing valve opening times ($T_{on} = 0.3, 0.4, 0.5, 0.6, 0.7, 0.8$ seconds) for a fixed back pressure on the reagent inlet ($P_{reagent} = 5$ psi). This resulted in a linear array of repeats of a sequence of six droplets, with each droplet increasing in volume. b) A plot of droplet volume versus the valve opening time (T_{on}) for a valve corresponding to a reagent inlet for three different values of back pressures applied to the reagent inlet ($P_{reagent} = 2.5, 5$ and 7.5 psi). The droplet volumes plotted are an average of volumes estimated from 50 droplets for each condition. The error bars in the volume data are too small to be seen on the plot.

Figure 5. Demonstration of reagent injection in sample daughter droplets merging with reagents
 a) Time series of images indicating reagent injection into sample daughter droplets at the 'Fusion zone' on the device. A sample daughter droplet (yellow) is released from the capillary inlet and is halted in the 'Fusion zone' by actuating a valve upstream which controls carrier fluid injection into the central channel on the device. A reagent (blue) is released and injected directly into the sample droplet. The elongated configuration of the sample daughter droplet in the 'Fusion zone' ensures robust reagent injection operation on the device without the need for precise sample droplet positioning. b) A series of photographs demonstrating injection of different numbers of reagents into a sample daughter droplet simultaneously.

Figure 6. Photographs of the incubation region indicating multiplexing capability of the device

a) Table with micrographs indicating combinatorial mixture droplets generated on the device using four different samples [Sample A (blue), Sample B (yellow), Sample C (green), Sample D (water)] and four different reagents [Reagent 1 (orange), Reagent 2 (water), Reagent 3 (light blue), Reagent 4 (yellow)]. Droplets 1A–4A, 1B–4B, 1C–4C, and 1D–4D are each generated from a combination of the sample and reagent in the corresponding column and row respectively. For example, Droplet 1A is the combination of sample A with reagent 1. b) Left panel shows four different micrographs showing repeating sequences of sample daughter droplets generated from a single sample plug merged with four different reagents on a single device. The right panel shows zoomed in version of a small section of the micrographs from the left panel for easy visualization of the droplet sequence. The droplet identification codes in this panel are the same as those used in subfigure a. Note: The sequence of droplets seems to be going in opposite direction in alternate channels due to the changing direction of flow in serpentine channels.