Supplementary: Automated Dynamic Inlet Microfluidics System: Cost-effective Biaxial Nanoliter Droplet on Demand Generation Platform and Its Application in Agglutination assays

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1. WHEN THE VOLUME OF ANALYTE GETS LOW IN EPPENDORF TUBE

As the Eppendorf tube sits on top of the oil, the oil rises into the tube to a certain height. In this study, the Eppendorf tube is lowered a few millimeters into the oil, which means the oil rises a few millimeters through a 0.7mm Eppendorf tube. Due to this, the level of the analyte has to be maintained above a minimum amount, otherwise, oil seeps into the Eppendorf tube pushing the analyte up and leading to a shift in the oil and analyte interface. The change in the interface lead to droplet size reduction, as shown in Fig. S1. After droplet number 16 there is a drastic decrease in the droplet's volume, due to low analyte volume and interface shifting. For this study, the minimum analyte volume limit is 30μL.

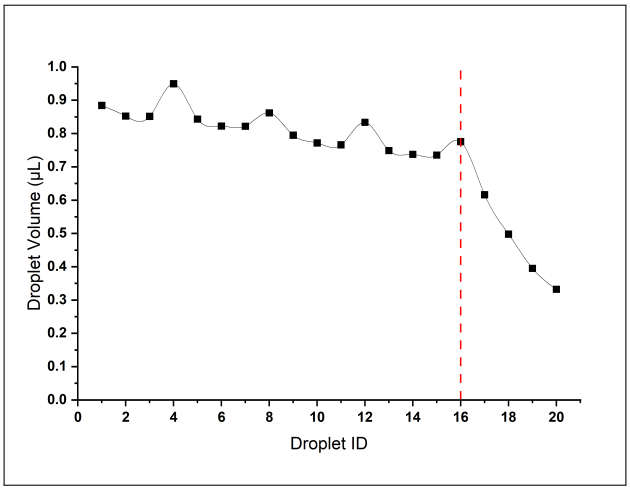

Fig. S1. Train of 100 droplet of 1 μL target volume is generated at 35μL/min.

2. MINIMUM DROPLET VOLUME AT 35 μL/MIN

The MATLAB script moves the syringe needle tip from oil (origin) up into the analyte taking t_{up} ms, waiting for the calculated delay (t_{delay}), and then taking t_{down} ms to bring the tip back down into the oil (origin) to generate a droplet. The G4 command is used to give a delay between t_{up} and t_{down}. When no delay is required between t_{up} and t_{down}, no command is written, however, if G4 P0 is written, it should give 0ms delay. Few experiments are conducted with G4 P0 commands which introduce a 125 ms delay between the commands instead of 8.3 ms, as per Table 2. The extra ≈100 ms leads the minimum volume achieved at 35 μL/min to be 0.4 μL rather than 0.3 μL, as shown in Fig. S2.

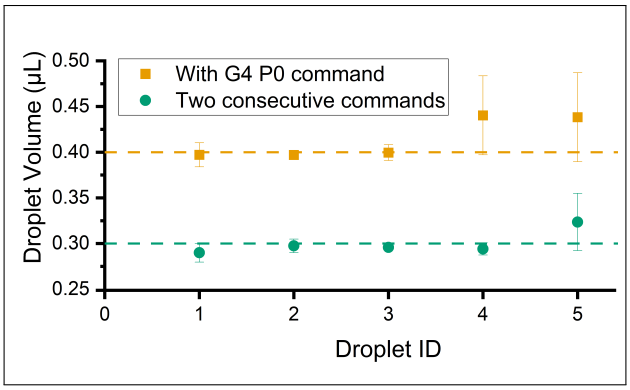

Fig. S2. 5 droplet of generated at 35μL/min to show the minimum droplet volume possible when t_{delay} is equal to zero compared to when G4 P0 command is used for t_{delay}.

3. LONG TRAIN OF 100 DROPLETS OF 1 μL

To show the developed system's capability to generate a long train of droplets, a train of 100 droplets is generated at 35μL/min, shown in Fig. S3. As the Eppendorf tube is open from the top, it can be refilled continuously, and therefore the length of the train can be extensively long.

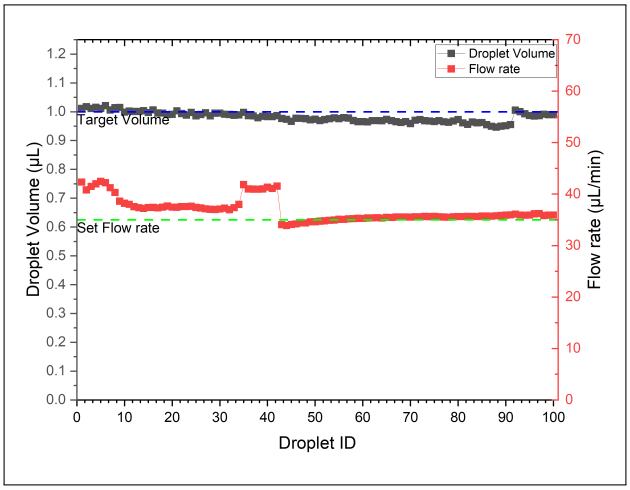

Fig. S3. Train of 100 droplet of 1 μL target volume is generated at 35μL/min.

4. LOWEST VOLUME: TRAIN OF 12nL DROPLETS

The lowest target volume tested for the developed system is 12nL at 1.5μL/min with 1 μL syringe in the syringe pump. The variation is the volume of droplet generated is high, however, the cyclic fluctuations due to the lead screw of syringe pump can be seen in Fig. S4.

5. TYGON TUBE FOULING

Repeated use of Tygon tubing showed signs of degradation causing the water droplets to adhere to the walls, leading to satellite droplets latching to the walls and droplet breakage. Therefore, the tubing has to be replaced periodically. PTFE tubing does not show degradation after use, however, the inner vortices of the droplet are limited leading to limited mixing. In agglutination assays in Lab-in-Tubing the mixing inside the droplet plays a crucial role in incubation. Limited mixing leads to longer incubation time. Fig. S6 is an example of this. This means that Tygon tubing captures molecules from agglutination assays causing cross-contamination. The agglutination assays are carried out in ascending order of concentration to avoid contamination of the previous assay on the current.

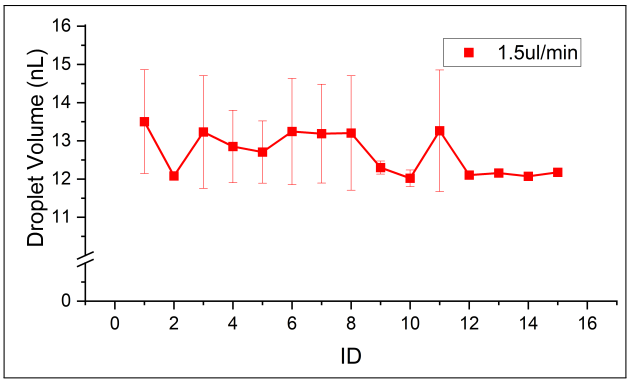

Fig. S4. Train of 15 droplets of 12 nL target volume is generated at 1.5μL/min. Repeated experiment three (3) times

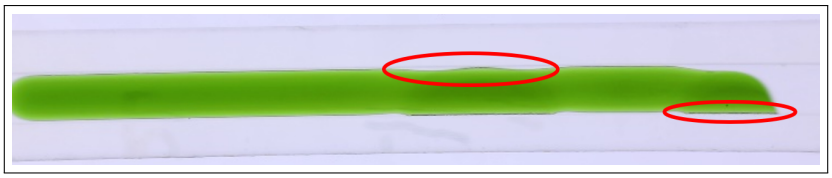

Fig. S5. Encircled parts show water droplets adhering to the Tygon tubing wall

6. INCREMENT OF DELAYS AND CONSEQUENT INCREASE IN VOLUME

Prusa Mini+ printer allows the user to enter delay using the G4 command in milliseconds (P) and seconds (S). G4 P100 means a delay of 100ms, whereas G4 S100 means a delay of 100 seconds. Table 2 in the article shows that there is a difference between programmed delay and actual delay executed by the printer. Fig. S6 shows a train of 20 droplets, with varying delays (100ms, 125ms,150ms, and 200ms). Experiments are repeated multiple times. The volumes generated for 100ms and 150ms are not discernable from each other. However, the volume generated at 100ms and 200ms can be distinguished clearly. Few droplets, e.g. first droplet of P200, show large variation between repetition of experiments. This occurs as the descending syringe needle pulls the analyte out of the orifice, generating a droplet with larger than the anticipated volume.

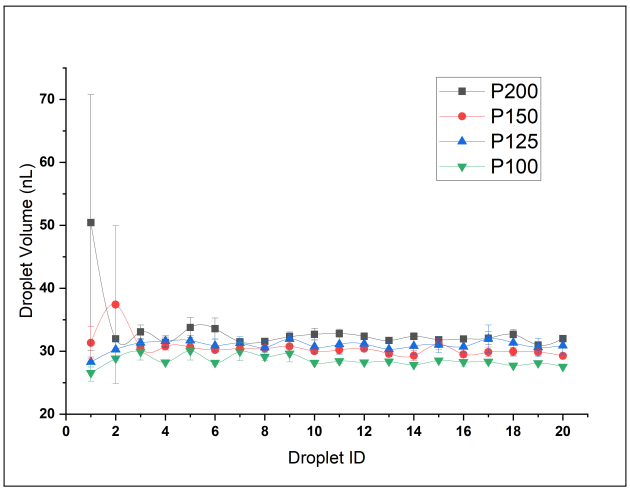

Fig. S6. Train of 20 droplets 0f 30 nL with varying delay. Delays of 100ms, 125ms,150ms, and 200ms are programmed.

7. DROPLET MERGING

The timing between two consecutive droplets is very important. When generating trains from two different analytes, it can be observed that the droplets would merge if the interval between them is kept too short (the minimum time of 1.385s). Droplet merging is very useful in microfluidics, as the start time of two analyte's mixing is known exactly, therefore, the time for incubation can be calculated with high accuracy. However, the droplet merging in this system is unpredictable, making it more of a liability than an asset.

The ability of a microfluidic system to merge droplets passively is very useful in applications such as concentration gradients, chemical and biological reactions. In protein, enzymes, and chemical reactions, the initialization moment which occurs at the time of droplet merging allows for a better incubation time assessment. However, in the developed system, the droplet merging occurs sporadically, with a higher likelihood when there is no delay between switching neighboring analyte Eppendorf tubes on the 3D printed rack atop the well. Fig. S7 shows the result of three (3) experiments where 100% of 10 droplets of 0.3 μL from two different analytes resulting in 10, 0.6 μL droplets.

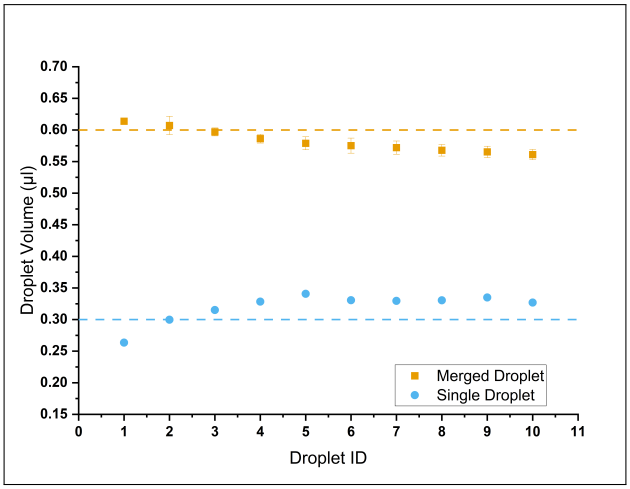

Fig. S7. Train of 10 droplets 0f 0.3 μL from two different analytes is generated with minimum timing between changing the analyte resulting in droplet merging. Results of three (3) experiments where 100% is achieved is shown

Media: video file titled 'DropletMerging.mp4'

The video titled DropletMerging.mp4, in the supplementary files, demonstrates an instance where two droplets merge. The different dyes allow for the visualization of streamlines within the droplets.

8. ANALYTE SELECTION AND THE RELATED ISSUES

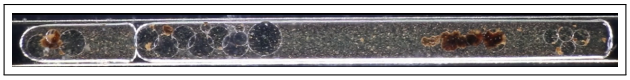

Fig. S8. C1 Streptavidin Beads set is used with biotinylated BSA, resulting in leakage through 0.7 mm orifice in Eppendorf tube, resulting in irregular droplet size and oil droplets emulsification within each droplets

The analyte can leak from the orifice in the Eppendorf tube and sink to the bottom of the well if the density of the analyte is comparable to or higher than the density of the silicone oil (0.97 g/cm^3) used in this study. The water density is comparable to the density of the silicone oil but the surface tension and capillary forces keep the water in the Eppendorf tube.

Occasionally the piercing action of the syringe needle tip causes excessive water to leak out, leading to larger first droplets mentioned in section 3.2. M270 Streptavidin Dynabead (2.7μm diameter) also faces this issue, but it is not excessive. Whereas when C1 Streptavidin (1μm diameter) beads are used the droplets have a large number of oil droplets emulsified inside the droplet (Fig. S7). The larger beads could induce more shear-thickening under the stress of the syringe needle piercing, effectively reducing the leakage due to the suspension with smaller beads. Additionally, the use of a premixed solution containing Streptavidin Dynabead and biotinylated BSA, which initiates a reaction when combined, suggests that the agglomeration of the larger 2.8 μm beads might partially obstruct or diminish the orifice's effective cross-sectional area more significantly than the smaller 1 μm beads, thereby decreasing the leakage.

When a premixed solution consisting of C1 Streptavidin microbeads (1μm) and biotinylated bovine serum albumin (bBSA) is used in the developed system, it leads to analyte leakage through the Eppendorf tubes. The leakage leads to irregular droplet size, however, more importantly, there is a large amount of oil droplet emulsification inside the analyte droplet as shown in Fig. S8. Images for these bead tests are taken in a dark-field setting for better visualization of emulsification.

If the analyte's density is around or above 0.97 g/cm^3, then the user can select oil like Fluorinert™ FC-40 from Sigma-Aldrich with a density of 1.870 g/cm^3 or HFE-7500 oil (3M) with 1.614g/cm^3 density. Oil with higher density would result in the analyte in the Eppendorf tube not leaking through its orifice at the bottom. Both oils are extensively used in literature related to droplet microfluidics.

9. ADAPTING THE DEVELOPED ADIM SYSTEM

If the 3D printer is changed then the Z-axis feedrate will be different and that has to be taken into account when calculating t_{up}, t_{down}, and t_{delay}. The first step should be to find the difference between the programmed delay and the actual delay for the new 3D printer by video analysis, the methodology discussed in Section 2.1. All "axes' movements and the delay can be evaluated by analyzing the movement of the syringe needle tip captured in a video." The movements should be for different delay times listed in Table 2. Next, the user should set up the whole system, the tubing running from the syringe needle tip to the syringe pump. If additional components are to be added in line with the system, they must be added at this stage, e.g. droplet cartridge or flow rate sensor. The user has to find the t_{system} after the system is set up, previous publication [21] discusses the methodology for this in detail. If the syringe needle is programmed to travel x mm in the analyte when the feed rate on the Z axis of the printer is y mm/min, t_{up}, t_{down} can be easily calculated. With all the parameters evaluated, the desired droplet volume can be achieved using Equation 1.

At a given flow rate, there is a limit to the minimum droplet volume due to the feed rate of the 3D printer in the Z-axis. For instance, section 3.2 (Train of Droplets with Constant Volume of Single Analyte) states: "The minimum volume possible at 35 μ L/min flow rate is 0.3 μ L with CV% of 6.3%...". This is because in this study the syringe needle tip moves 1.5 mm into the analyte, therefore the t_{up} and t_{down} are calculated to be 250 ms combined. This leads to the minimum volume of 0.3 μ L. The user should lower the flow rate, which in turn lowers the minimum droplet volume. A faster 3D printer would also lower the minimum possible droplet volume, at a given flow rate.

To generate droplets smaller than the volumes outlined in this manuscript, the user must change both the tubing and the source of negative pressure. With tubing with a smaller inner diameter and a stable low-flow rate syringe pump, the mechanism developed here can generate much smaller droplet volumes. The limiting factor in this study is the New Era Systems NE-1000 syringe pump, which gives fluctuations at low flow rates.

As the Eppendorf tube is open from the top, it can be refilled continuously, and therefore the length of the train can be extremely long. However, if the user wants to generate droplets with large volumes, there is a limit on the analyte volume the Eppendorf tube can hold before the analyte starts to leak from the orifice at the bottom. This is mainly because the density of silicon oil and water is comparable, as discussed in Section 8 of this supplementary. For the Eppendorf tube, changing the oil to FC-40 would increase the volume of analyte that can be stored in the tube before it leaks. The weight of the analyte has to be less than the force required to break the oil-analyte interface at the 0.7 mm hole at the bottom of the Eppendorf tube. This principle will apply even if the user uses a larger analyte container e.g. 50mL Falcon tubes.