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Off-Chip Vertical Step Emulsification Droplets Preparation Device Applied for Droplet Digital PCR

*Bing Shi, Di Wu, Yangyang Jiang, Junru An, and Wenming Wu**

High efficient and stable droplets preparation is an essential technology in digital PCR (dPCR) and other analytical fields. In this paper, a microfluidic device called vertical step emulsification (VSE) device to produce emulsion droplets rapidly off-chip is developed. VSE device prepares droplets by perfluoroalkoxy (PFA) capillary and microcentrifuge tube, when the continuous water phase in PFA capillary jets into the static oil phase in the microcentrifuge tube at a certain speed, the continuous water phase is emulsified into stable droplets due to the sudden change of surface tension. In order to control the droplet size accurately, a general screw mechanism to control the gap between the PFA capillary outlet and the bottom of the inner wall of microcentrifuge tube is used. Meanwhile, dPCR tests with high dynamic range throughput droplets from VSE device are performed. The results show that the off-chip droplet preparation device developed by the authors not only gets rid of the complicated control structure, has the advantages of low cost, easy operation, and user-friendly, but also provides a sustainable development scheme for the preparation of high-throughput and stable droplets in a short time.

1. Introduction

Digital polymer chain reaction (dPCR)[1,2] is an absolute quantitative technique of nucleic acid molecules. As the latest generation of PCR technology, dPCR has smaller reaction volume, lower system noise, and higher sensitivity than traditional PCR (the first generation of PCR [termed end-point PCR],[3] the second generation of PCR [real-time quantitative PCR][4]). It can be used for high-sensitivity medical diagnosis such as HIV detection during its treatment to check the HIV treatment efficiency, and for multiplexed DNA detection with different number of copies of different DNAs such as diagnosing Down's syndrome.[5] The critical step of dPCR is to separate the independent microreaction chambers with equal volume. Nowadays, there are two main ways. One is chamber-based,[6–8] and the other is droplet-based.[9–11] The chamber-based dPCR devices need to process tens of thousands of microreactors with the same volume on substrates of different materials (such as polydimethylsiloxane [PDMS],[12] polymethyl methacrylate [PMMA],[13] fused silica,[10] silicon,[14] etc.), and then the reagents are separated into each microreactors for PCR reaction. The advantages of this kind of device are that the volume of reagents in each microreactor is absolutely equal, and it avoids cross-contamination. And the disadvantages are that the microchips cost is too high and the reagent will be wasted during injection procedure. As for droplet dPCR (ddPCR) devices, the reagents need to be separated into droplets of equal volume before performing the PCR reaction. There are two common droplet discrete devices, one is on-chip[15] and the other is off-chip.[16] No matter which device is used, the ddPCR device has the advantages of low cost, simple operation, and avoiding the waste of reagents, while its droplet preparation method limits the droplet throughput. Therefore, once the droplets throughput is improved, the ddPCR device will have a greater potential for future development.

The key point to break through the ddPCR droplet throughput is droplet preparation, we can also say the droplet emulsification, which means that one liquid (dispersed phase/water phase) is uniformly dispersed in the form of small droplets in another insoluble liquid (continuous phase/oil). T-junctions[17–19] and flow focusing[20–23] are two methods commonly used to prepare emulsion droplets. Both methods need to control the flow of dispersed phase and continuous phase precisely. The volume of the droplet depends on the flow rate of the two-phase liquid.

In recent years, the preparation of droplets by the step emulsification method[24–26] based on single-phase liquid flow has become a widely concerned research field. In this method,[27] the microfluidic device needs to be filled with continuous phase medium in advance. Then the dispersed phase is injected into the microfluidic device filled with continuous phase through a microchannel. When the height of the microchannel produces step change, the single dispersed droplet can be formed when the two-phase interface is unbalanced. Compared with the T-junctions method and flow focusing method, the step emulsification method is simple in operation, high in throughput,

Dr. B. Shi, Dr. D. Wu, Y. Jiang, Dr. J. An, Prof. W. Wu
State Key Laboratory of Applied Optics
Changchun Institute of Optics Fine Mechanics and Physics
Chinese Academy of Sciences
Changchun, Jilin 130033, P. R. China
E-mail: wuwm@ciomp.ac.cn
Dr. B. Shi, Dr. D. Wu, Dr. J. An, Prof. W. Wu
University of Chinese Academy of Sciences (UCAS)
Beijing 100049, P. R. China
The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/admi.202001074.

DOI: 10.1002/admi.202001074

and stable in structure. However, the on-chip ddPCR device still limits the droplets throughput because of the fixed step height of the microchip and the width of nozzles, the droplets throughput has not been greatly improved, such as EDGE devices designed by Sahin and Schroën,[28] centrifugal-driven droplet generation method proposed by Li et al.,[29] and the millipede device designed by Amstad et al.[30] But the off-chip ddPCR device removes this limitation and greatly improves the droplet throughput of the ddPCR device. For instance, the cross-interface emulsification method proposed by Xu et al.,[31] the centrifuge-based step emulsification device by Shin et al.,[32] and the off-chip monodisperse droplet generation method by Chen et al.[33] Although their methods broke the limitation of low droplet throughput, the cost of their droplet preparation system is higher, and the manufacturing process is more difficult, which brings some difficulties to the inexperienced researchers in the field of system design and manufacturing.

Here, we designed an off-chip vertical step emulsification (VSE) microfluidic device with both step and nozzle adjustable. With the thrust of the mechanical pump, the continuous water phase in the capillary of VSE device is injected into the microcentrifuge tube with oil phase in advance in a certain speed, and rapidly disperses into a uniform picoliter droplet in a short time. In addition, the gap (*H* value) between the outlet end of the capillary and the inner bottom of the microcentrifuge tube can be adjusted to control the size of the emulsion droplets. The droplet diameter is in the range of 30–120 μm, and the coefficient of variation (CV) is less than 5%. When the droplet diameter is 32 μm, the maximum throughput per second is 247. In summary, VSE device uses low-cost biochemical consumable material with stable structure and simple operation to reduce the difficulty of droplet preparation enormously and provides a reliable solution for the preparation of high-throughput droplets in a short time.

2. Experimental Section

2.1. Preparation of PFA Capillary

PFA capillary (360 μm o.d., 50 μm i.d.) was purchased from IDEX Health & Science LLC (WA, USA). PFA material has similar mechanical properties,[34] thermal stability, chemical resistance, flame resistance, and radiation resistance to polytetrafluoroethylene (PTFE) material. Due to the form of PFA capillary in oil phase is hard to fix, the external part of PFA capillaries is wrapped by a steel capillary (800 μm o.d., 400 μm i.d., 5.5 cm long), which plays a role of fixing and protecting the PFA capillary.

2.2. Material for Droplet Digital PCR

Supermix (No dUTP) and fluorinated oil were purchased from Bio-rad (CA, USA). The dPCR test kit (R175H mutation of human TP53 gene) was purchased from Rainsure Sciences Co., Ltd. (Jiangsu, China). The dPCR regent was composed of a 10 μL supermix (No dUTP), 1 μL probe (FAM), 1 μL sample (R175H mutation of human TP53 gene), and 8 μL distilled water. The commercial ddPCR instrument is Rainsure (DropC-1000-2). The preparation time of 20 μL water phase is about 180 s.

3. Results and Discussion

3.1. Setup of the VSE Device and Droplet Generation

We chose the low-cost microcentrifuge tube as the "microchip" for droplet storage in order to avoid the difficulty of accurate control of two-phase liquid flow rate when using T-junctions or flow focusing in droplet preparation, and to solve the problem of expensive microchip design and manufacturing. Based on step simulation method, a microfluidic device designed with microcentrifuge tube as the component of the droplet microfluidic device consisted of steel capillaries, 1/32 in. connector (Agent Technologies Inc., CA, USA), flat-head PFA capillary, microcentrifuge tube (200 μL, Axygen, CA, USA), vital (5 mL), and adjuster (**Figure 1**a). Steel capillaries and the PFA capillary were fixed together by a connector. Adjuster was designed by Solidworks (www.solidworks.com) software, which consisted of setting screw and positioning screw (M20 × 0.5, 20 mm is a diameter, 0.5 mm is a pitch). The main function was to fix the connector by setting screw. Positioning screw was used to adjust the position of the connector. The processing of adjuster manufacture was completed by Yungongchang (Shenzhen, China). Figure S1 (Supporting Information) is the 3D model of positioning screw.

The connection between VSE device and syringe (1 mL) was made by PTFE tube (900 μm o.d., 600 μm i.d.) (Figure 1b). The syringe was fixed on mechanical pump (Longer Precision Pump Co., Ltd., Baoding, China). To ensure the normal operation of mechanical pump, ≈100 μL continuous phase (Bio-rad, CA, USA) was injected into the syringe in advance. Then 20 μL dispersed phase (water phase or sample) was pumped in the front of syringe at a constant slow speed for droplets generation. This device is an off-chip open system. When droplets are generated, the gas column will be discharged in the form of bubbles, which will not affect the preparation of droplets. Therefore, when pumping the dispersed phase, it is not necessary to deliberately avoid the inhalation of the micro gas column. For more information, please refer to Movie S1 (Supporting Information).

In order to prevent the droplets from being damaged by the inner wall of the microcentrifuge tube, 20 μL continuous phase was injected into the microcentrifuge tube before VSE device started to prepare droplets (Figure 1c). The volume of continuous phase could be adjusted according to the experimental requirements only to ensure that the oil level exceed the outlet end of PFA capillary, which is the nozzle. The size of droplet was controlled by general screw mechanism by the way that we used positioning screw to control the gap (*H* value) between PFA capillary and microcentrifuge tube (Figure 1c). The corresponding *H* value is calculated as shown in Equation (1)[35]

$$H = P \times \frac{\varphi}{2\pi} \tag{1}$$

where *H* is the gap height (axial displacement of positioning screw), *P* is the pitch of the positioning screw, and ϕ is the rotating angle. In general, the screw mechanism is based on the principle of screw drive. It uses the screw pair composed of screw and nut to realize the transmission requirements, mainly to change the rotating motion into linear motion. It has the advantages of compact structure, uniform transmission,

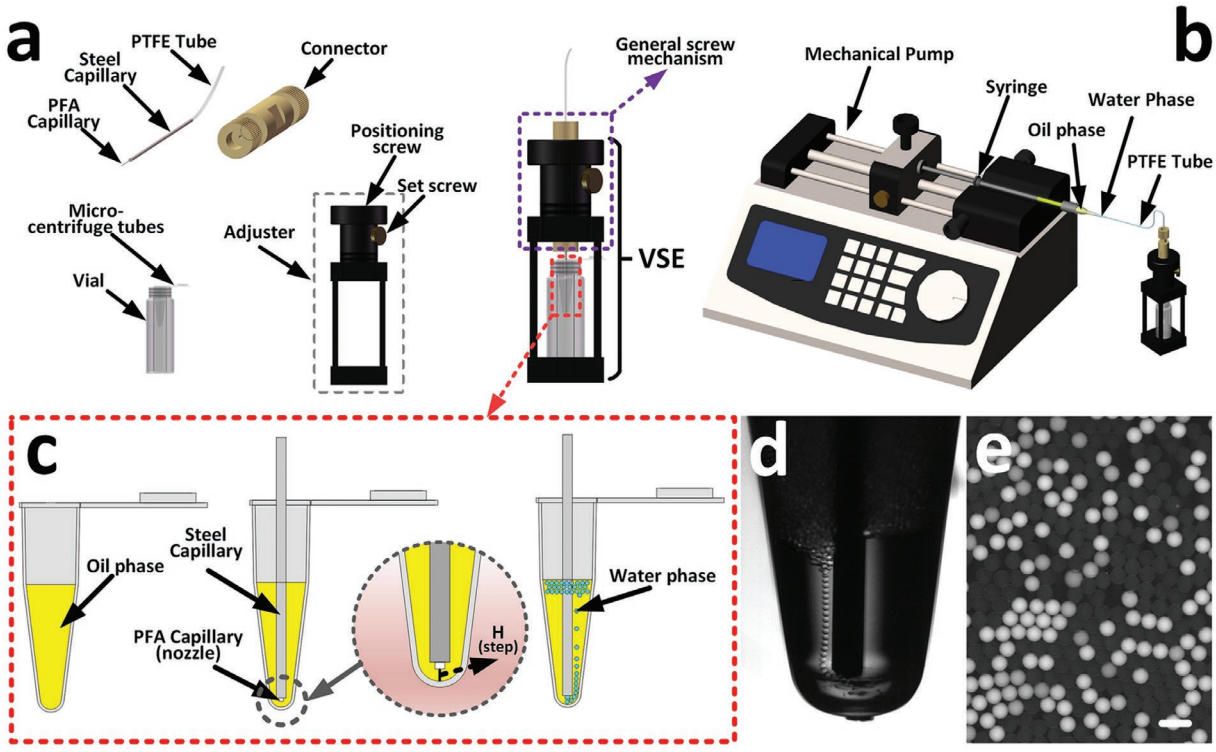

Figure 1. a) VSE device. b) The system of droplets preparation. c) Schematic of droplets generation. d) The process of droplets generation from high-speed camera (Dimax HS4, PCO, Germany). e) The digital amplification within the droplet (Scale bar = 100 µm).

accuracy, stability, and easy self-locking. Thus, by adjusting the rotating angle of positioning screw, it can control the *H* value precisely. After adjusting the VSE device, the droplets will gather in the upper layer of oil level (Figure 1d) during the process of droplets preparation because the density of oil phase is greater than that of water phase.

3.2. Adjustment of Droplet Size

Most of the previous work focused on the effect of the opening angle of nozzles,[31] the viscosity of oil phase,[36] and the nozzle geometry[29] to the uniformity of droplets. However, VSE device is an off-chip microfluidic system, which can adjust the droplet size by adjusting the inner diameter of the PFA capillary, the rotating angle of positioning screw, and the speed (*V* value) of mechanical pump, respectively. But the diameter of the droplet is relatively large when the inner diameter of the PFA capillary is large. At the same volume of the dispersed phase, the larger the droplet volume, the less the droplet total amount, and the lower the sensitivity of the dPCR. Therefore, we only discussed VSE device with 50 µm inner diameter of PFA capability, and other PFA capability with larger inner diameter in Figure S2 (Supporting Information).

3.2.1. Effect of Mechanical Pump Speed on Droplet Size

The speed of dispersed phase injected into continuous phase is decided by the speed of different mechanical pumps. When *H* value was 50 µm, a series of speed *V* value (100, 200, 300, 400, 500, 600, 700, and 800 µL h^{-1}) of mechanical pumps is shown in **Figure 2**a. With the increase of *V* value, the diameter of the droplet increases significantly (Figure 2b), which is shown as a positive correlation (R^2 = 0.9874) and conforms to the principle of step emulsification.[37] In order to measure the distribution of droplet diameter at the same *V* value, 20 µL dispersed phase was used for ten repeated tests. At last, the CV values of droplet diameter were less than 5% (Figure 2c). In the meanwhile, the total time and the total number of droplets prepared by 20 µL dispersed phase at different *V* values were recorded, as shown in **Table 1**. Furthermore, the droplets preparation ability of VSE device at different *V* values was obtained in Figure 2d. When *V* value was 100 µL h^{-1}, the number of droplets per second was about 250. When *V* value was higher than 100 µL h^{-1}, the number of droplets per second was about 150.

3.2.2. Effect of Step Height on Droplet Size

A series of step height *H* values (50, 125, and 250 µm) is shown in **Figure 3**a. In addition, in order to compare the effect of the speed of mechanical pump on droplet size under different *H* values, a series of *V* values (100, 300, 500, and 700 µL h^{-1}) was selected. Under two conditions, we observed the droplet size distribution. As shown in Figure 3b, at different *H* values, the droplet diameter still increases with the increase of mechanical pump speed. However, when the *H* value was 125 µm, the droplet size distribution was the most uniform and the linearity was the best (R^2 value = 0.9971).

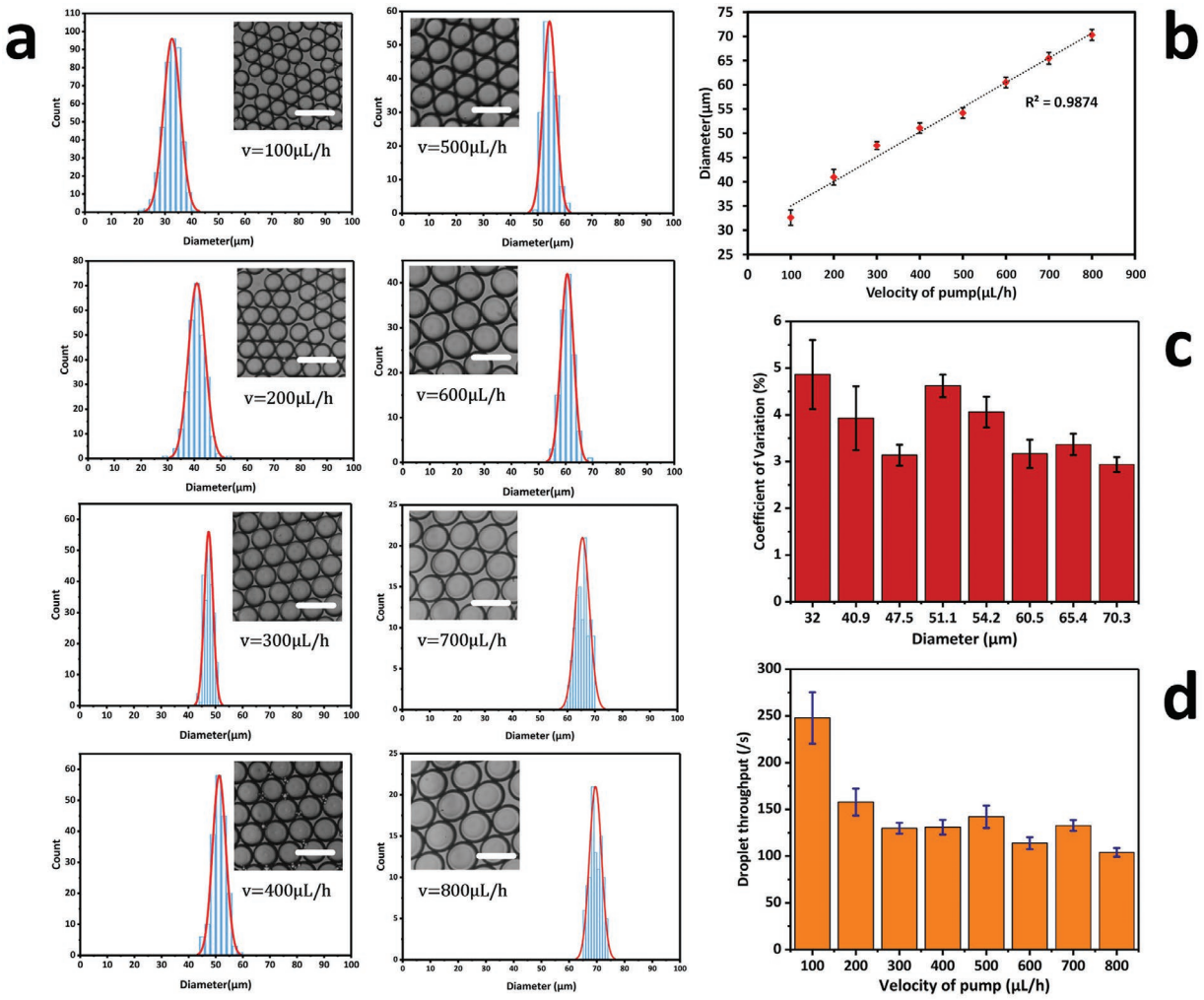

Figure 2. a) With the increase of *V* value, the droplet diameter increase (*H* value is 50 μm). b) A positive correlation between *V* value and droplet diameter. c) The CV value of different droplet diameter. d) With the increase of *V* value, the droplet throughput changes (Scale bar = 100 μm, error bars are the standard deviations).

In Figure 3c, it could be observed from the change of CV value (all less than 5%) that when *H* value was 125 μm, CV value decreases steadily with the increase of mechanical pump speed. This illustrates that the standard deviation of droplet diameter is low, which proves that the distribution of droplet diameter tends to be stable with the increase of mechanical pump speed. In Figure 3d, we can observe that when *H* value is 125 μm, with the increase of mechanical pump speed, the droplet throughput is also increasing. On the contrary, when *H* values are 50 and 250 μm, the throughput of droplets decreases with the increase of mechanical pump speed. Although the droplet size distribution at *H* value of 125 μm is better than that of 50 and 250 μm, in order to generate 20 000 pL droplets with uniform size and suitable for ddPCR in a short time, the best *H* value is 50 μm.

Table 1. Total time and total droplet number of droplets for 20 μL dispersed phase preparation.

Velocity [μL h^{-1}]	Time [s]	Droplet number
100	433	107 181 ± 11 934
200	345	54 436 ± 4970
300	274	35 577 ± 1587
400	216	28 286 ± 1688
500	171	24 287 ± 2051
600	151	17 179 ± 976
700	124	16 335 ± 712
800	105	10 964 ± 484

3.3. Amplification of Nucleic Acids in Droplets

To demonstrate that the off-chip device based on step emulsification method can be used in ddPCR based on the above data analysis, VSE device adopted a PFA capillary with a diameter of 50 μm as nozzles, step height of 50 μm, and mechanical pump speed of 500 μL h^{-1} to prepare droplets for ddPCR. We selected four groups of different concentration gradients of the same sample to perform ddPCR test. The sample concentration ranges from several

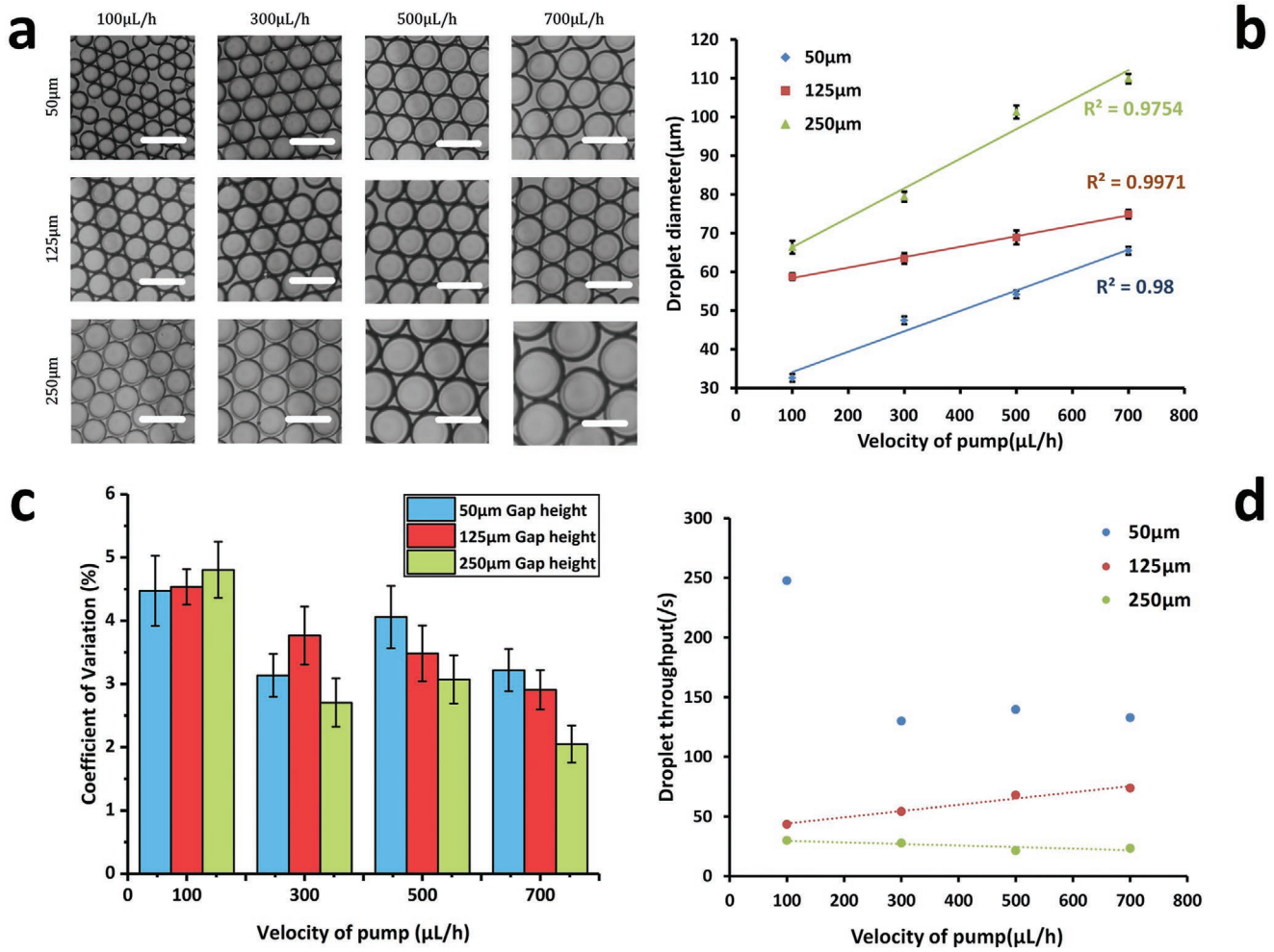

Figure 3. a) The images of droplet with different *V* values and *H* values. b) A positive correlation between *V* value and droplet diameter under different *H* values. c) The CV value under different *H* and *V* values. d) Under different *H* values and with the increase of *V* values, the droplet throughput changes (Scale bar = 100 μm, error bars are the standard deviations).

copies per μL to 3000 copies μL^{-1} (**Figure 4**a), and each concentration of samples was tested four times. Four groups of samples to be tested with same volume were, respectively, used by VSE device and commercial dPCR instrument (Rainsure) to prepare droplets. The prepared droplets were transferred to microchip by a pipette. Finally, four groups of samples were put into commercial dPCR instrument (Rainsure) for PCR amplification at the same time. The results of the ddPCR tests are shown in Figure 4b,c. After four repeated tests, the data detected by VSE device were 3.5955, 30.7425, 327.757, and 3457.742 copies μL^{-1}, respectively, and the detection data obtained by Rainsure were 2.8455, 29.5535, 273.6425, and 3257.742 copies μL^{-1}, respectively. The results of the two methods are consistent, and the VSE device is faster and easier to operate. It shows that VSE device provides a potential method for off-chip droplets preparation.

4. Conclusion

In conclusion, we developed a novel droplet emulsification device VSE, which has the ability to generate high-quality droplets and the characteristics of stable structure, simple operation, high throughput, and low cost. Compared with the two droplet generation methods, traditional T-junctions and flow focusing, our method greatly reduces the difficulty of fluid control and microfluidic system design and producing and avoiding the pretreatment of chip materials. What's more, in our method the process of microchip design and manufacturing is eliminated in comparison with recent popular on-chip step emulsification microchip. The width of nozzle can be easily changed by replacing different diameters of the PFA capillary. The height of step can be accurately positioned by the screw mechanism. Moreover, VSE device can be used for high dynamic range ddPCR test. So this low-cost device with simple operation method seems to be adopted by other laboratories. However, there is still a bottleneck in this method that droplets will be exposed to the air in the process of droplet transfer causing unnecessary pollution. At the same time, the detection scheme of ddPCR is not ideal. Therefore, we will try to explore a more simple and accurate detection scheme to a void unnecessary pollution to be used with VSE device in the future.

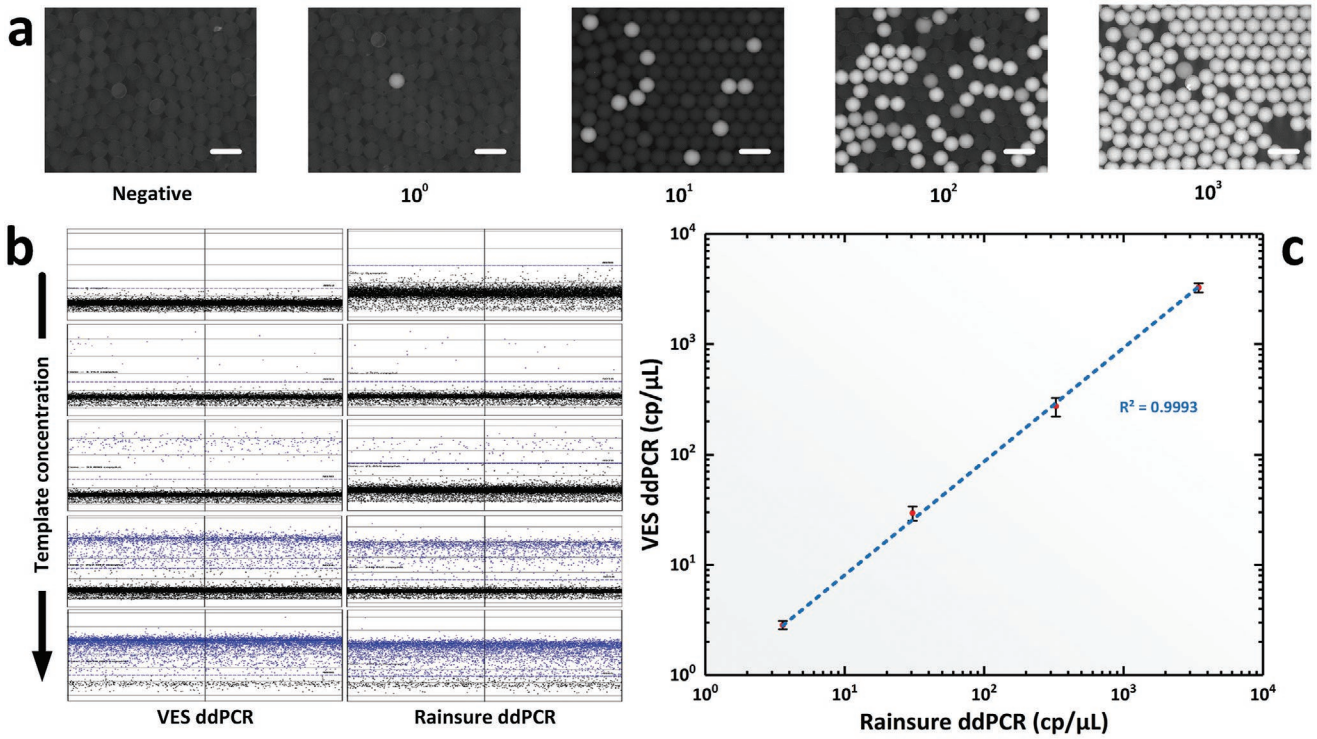

Figure 4. ddPCR analysis with droplets generated by VSE device. a) The ddPCR test within the droplets (picture from Rainsure analytical equipment). b) The results of ddPCR performed using VES device, compared to the result using Rainsure platform. Each dot presents an effective droplet readout. If its fluorescence intensity is above a threshold, a droplet is identified as positive (blue), otherwise negative (gray). c) The concordance between the result from two approaches (Scale bar = 100 μm, error bars are the standard deviations).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

This project was supported by the CAS Pioneer Hundred Talents Program, the National Natural Science Foundation of China (No. 61704169), the Natural Science Foundation of Jilin Province (20180520112JH), and the Talent Project of Jilin Province.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

capillary, droplet digital PCR, microcentrifuge tubes, screw mechanism, step emulsification

Received: June 16, 2020
Revised: August 14, 2020
Published online: September 28, 2020

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