

CENTRIFUGAL MICROFLUIDIC STEP EMULSIFICATION FOR DIGITAL DROPLET RECOMBINASE POLYMERASE AMPLIFICATION

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ABSTRACT

For the first time we show centrifugal step emulsification. It enables the fast and easy production of monodispers w/o droplets with minimal handling effort (3 pipetting steps). In contrast to previously presented centrifugal emulsification systems [1], homogenous droplets with pre-selectable diameters were generated with zero run-in time and zero dead volume. The centrifugal microfluidic step emulsification was used to perform the first digital droplet recombinase polymerase amplification (ddRPA). Compared to digital droplet PCR, the amplification time was reduced by a factor of 4 from 2 hours to 30 minutes.

KEYWORDS: Centrifugal step emulsification, Emulsion, RPA, digital, amplification, droplet

INTRODUCTION

For digital droplet amplification reactions it is important to compartmentalize the sample into many small and monodispers droplets, without wasting sample volume as dead volume. Moreover, the emulsification should be easy to use and the amplification should be fast. Most existing droplet generation techniques (e.g. flow-focusing and T-junction systems) suffer from high dead volume. Moreover, they are difficult to integrate on centrifugal microfluidic systems since the flow rate of at least two phases must be controlled tightly [2]. Centrifugal step emulsification enables very robust droplet generation with high monodispersity. Using centrifugal step emulsification, we demonstrate the first digital droplet RPA.

THEORY

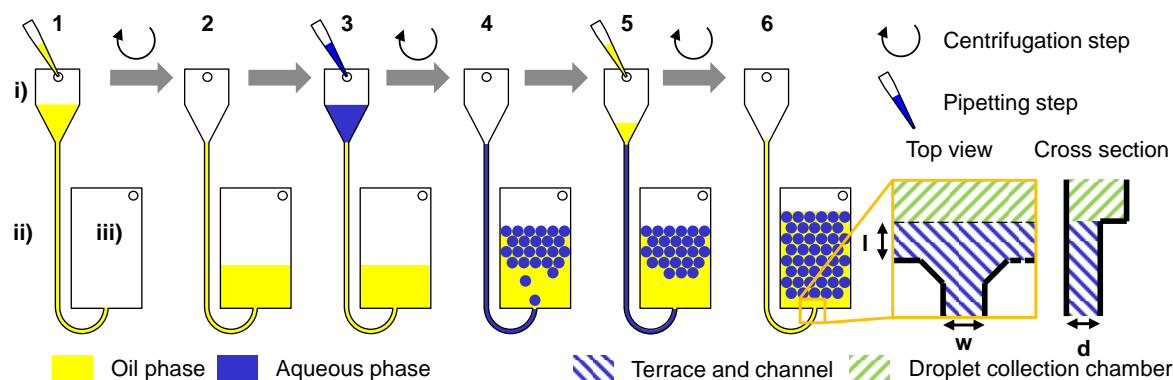


Figure 1: Principle of centrifugal step emulsification. 1) Oil is inserted into the inlet (i) 2) centrifugation forces the oil through the channel (ii) to the amplification chamber (iii). 3) Aqueous reaction mixture is inserted 4) centrifugation forces the aqueous mixture to flow through the channel, droplets are produced at the nozzles backward facing step (see inset to the right). 5) A small amount of additional oil is inserted 6) by centrifugation the remaining aqueous mixture in the channel is replaced by oil. All sample is emulsified. Reprinted with permission from [3].

Centrifugal forces are employed to control the emulsification. The system consists of an inlet chamber, an amplification chamber and a channel that connects both chambers with a nozzle at the intersection (Fig. 1). At the nozzle, the channel widens to a small plateau followed by a backward facing step. The step induces the break-up of aqueous droplets in an oil-primed surrounding.

EXPERIMENTAL

Microfluidic disks were produced by micromilling in polymethyl methacrylate (PMMA). The disks were then cleaned and sealed with pressure sensitive adhesive tape. For the experiment the disks were processed in a LabDisk Player prototype by Qiagen Lake Constance. The player allows to control spinning frequency of the disk, acceleration and deceleration rates as well as the temperature of the disk. For fluidic experiments, the structure is primed with HFE7500 containing 2 wt.-% Picosurf-1 (Dolomite, Royston, UK), followed by insertion of aqueous phase. The production of droplets was observed by using a stroboscopic camera setup. All RPA reactions were performed using RPA nfo Kit (TwistDx, Cambridge, UK) according to the manufacturer's protocol. Mg^{2+} was added immediately before the start of emulsification. *L. monocytogenes* DNA (IRMM-447 (strain 4B, NCTC 11994), Brussels, Belgium) was used in varying concentrations. The primers and probes were those from the *L. monocytogenes* kit from TwistDx. The RPA droplets were incubated at 39 °C for 25 min. A digital droplet PCR was performed for comparison using a Bio-Rad QX100 machine. For detailed procedures see [3].

RESULTS AND DISCUSSION

When the reaction mixture reaches the nozzle under centrifugation, the pressure in the reaction mix is higher than in the oil. The pressure difference can be calculated using standard hydrostatic calculations.

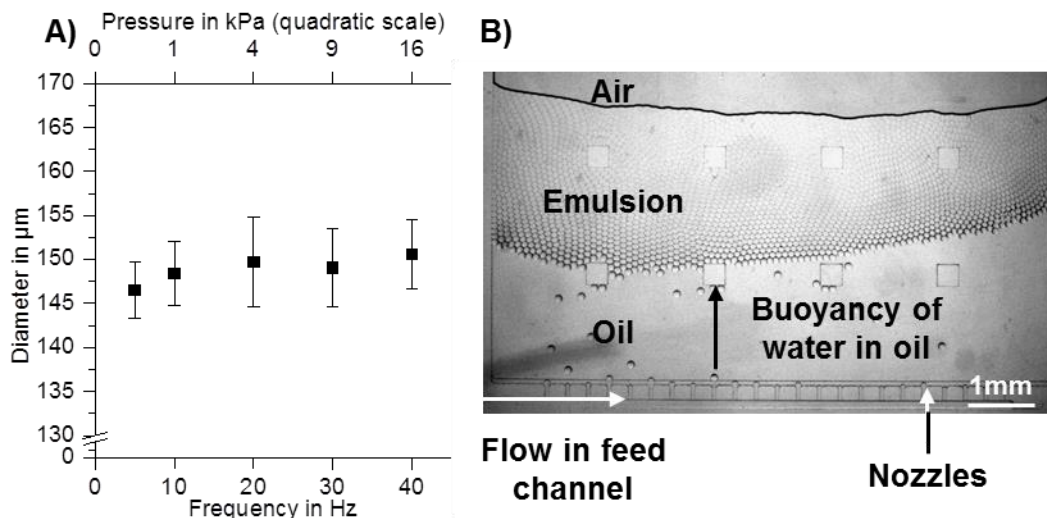


Figure 2: A) Droplet diameter is independent of pressure difference or spinning frequency. B) Parallelized centrifugal step emulsification. Aqueous phase is flowing into the feed channel and is emulsified upon reaching the nozzles (all branching off the same feed channel). Right part reprinted with permission from [3].

Due to this pressure difference, a flow across the backward facing step is induced and droplets are formed. However, the droplet size is independent of the pressure difference over two orders of magnitude and consequently independent of the spinning frequency (Fig. 2A). The droplet size's coefficient of variation was 2-4%. The droplet generation rate could be tuned by changing the speed of rotation from 1 to 500 droplets/s for one single nozzle. To further increase the droplet generation rate, multiple nozzles branch off from a shared feed channel (Fig. 2B).

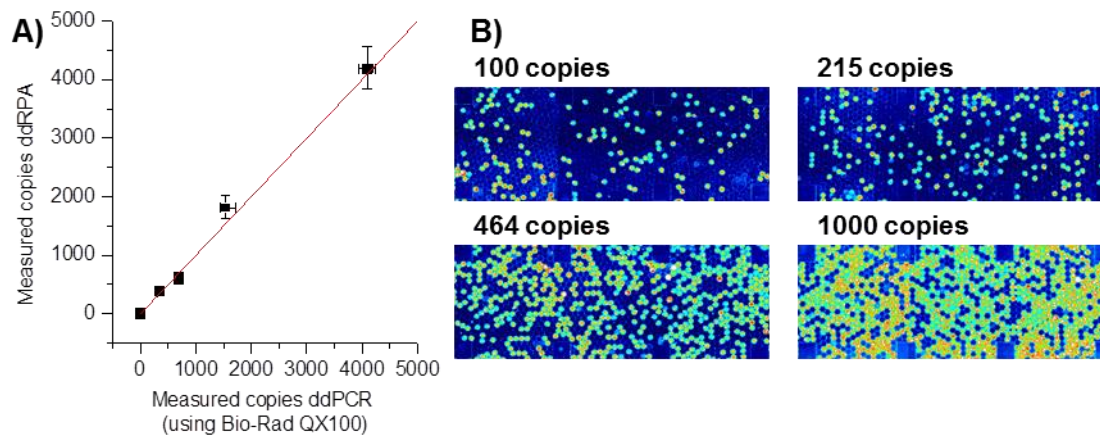


Figure 3 A) Results from droplet digital RPA (ddRPA) correspond well with results from ddPCR. B) false color fluorescent image of droplets after RPA. Reprinted with permission from [3].

The copy numbers counted with ddRPA were compared to copy numbers measured with digital droplet PCR performed in a Bio-Rad QX100 instrument and showed very good concordance (Fig. 3A). Speed of analysis was increased from 2 hours for a ddPCR experiment to 30 minutes for a ddRPA experiment.

CONCLUSION

The introduction of step emulsification to centrifugal microfluidics combines the advantages of droplet based microfluidics (thousands of individual reactions) with unique features of the centrifugal approach (easy handling, no pump interfaces, separation by buoyancy). The droplet generation on the disk is reliable, fast and proceeds with a low CV. The disk is easy to fabricate with scalable manufacturing technologies such as thermoforming or injection. Digital droplet RPA allows very fast quantification of DNA. As next steps, the combination of centrifugal step emulsification with existing unit operations may enable dead volume free sample-to-digital-answer analysis with minimal user interaction.

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