Programmable droplet-based microfluidic serial dilutor

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\textbf{A B S T R A C T}

A programmable droplet-based microfluidic serial dilutor platform is presented, which is capable of generating a series of droplets with the scalable stepwise concentration gradient of a sample. Sequential dilution of a target molecule was automatically performed in sub-nanoliter scale droplets by synchronizing a microfluidic peristaltic mixer and a valve-assisted droplet generator. The volume of droplets dispensed from the mixer was controlled by microvalve operation, which enabled to tune the dilution with various dilution factors. After evaluation of the mixer efficiency and calibration of the droplet size at different valve operating conditions, serial dilutions of rhodamine B isothiocyanate-dextran was demonstrated, in an automated manner, at three different dilution factors. Specifically, the effect of the rhodamine B isothiocyanate-dextran concentration and temperature on variations of the fluorescent intensity was quantified. This programmable microfluidic droplet serial dilutor will open new avenues, an analytical tool, to evaluate complex chemical and biochemical reactions, especially when limited sample volume is available, for example, at the early stage of drug discovery and biochemical process developing.

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\textbf{Introduction}

Serial dilution, a series of sequential dilutions of a sample, is one of the most common and necessary processes in chemical and biological laboratories for sample preparation and experimentation. Furthermore, serial dilution is a fundamental operation to evaluate ever-increasing sample numbers in various concentration ranges to identify the dynamic interaction of target molecules with (bio)chemical libraries [1,2]. The universal technique for serial dilution relies on pipetting in a set of microtubes or in a micro-well plate, which however requires a relatively large sample volume and extensive manual handling that might result in compounding errors. Liquid handling automation, using dedicated robots, especially in combination with tests miniaturization, automation, and computational data processing, is an excellent alternative for high-throughput screening (HTS) for biochemical and biopharmaceutical applications [3]. Although liquid handling robots are well-established for these HTS purposes high-throughput screening in the pharmaceutical and biotechnology industries, these systems are costly and require considerable space for installation and operation. Advances in microfluidic technology have proposed more integrated approaches for handling complex fluid flows and producing concentration gradients in miniaturized platforms [4,5,14–23,6–13]. Microfluidic platforms offer a variety of benefits, such as having a small footprint, low sample consumption, shorter analysis time, and lower assay cost. Especially, parallelized lab-on-a-chip devices coupled with automated operations have a great potential for performing sequential biological and chemical reactions, which has relevance not only for experimental science in the laboratory but also for industrial applications.

Varying the reagent concentrations using microfluidics is essential for performing fast analytical screens which requires multiple parallel reactions, e.g., chemotaxis [24,25], haptotaxis [26], cytotoxicity [7,9,27], immunoassay [6], nucleic acid purification [12], and enzymatic reactions [14]. However, this operation is challenging in a microfluidic format because mixing relies only on diffusion. To overcome all these handling and mixing limitations, several microfluidic devices have been proposed for serial dilution...
to obtain concentration gradients of a sample, using networks of continuous flows, large-scale integration with microvalves [10–16], and microdroplets [17–23], as also reviewed by Kennan et al. [28].

By interconnecting multiple microfluidic T-mixers in continuous flow branches, a Christmas tree-shaped channel network was developed for generating linear sequential concentration gradients [4,5]. To enhance mixing performance on a reduced footprint, a variety of mixers and designs have been integrated, such as a herringbone mixer [6], 3D fluidic channel connections [7], a serpentine Tesla mixer [27], and high volume mixing channels [8,9]. Such microfluidic platforms all support the formation of a linear, complex, and combinatorial concentration gradient in continuous fluid flows, which is beneficial for analytical assays where reagent perfusion is necessary, e.g., cell-based screening. However, high-throughput screening for quantitative analysis, especially when limited resources are available, remains challenging because creating a concentration gradient in a continuous flow consumes a relatively large amount of sample.

Microvalve technologies provide accurate control of multiple fluid flows and active peristaltic mixing of solutions in large-scale systems with integrated reactors [10,11]. Sequential and/or combinatorial concentration gradients have been generated by metering, combining, and mixing multiple reagents in parallelized nanoliter reactors [12–15]. Also, the in-line arrangement of multiple peristaltic mixers enables the serial dilution of a target reagent by mixing stored solutions in each dilution stage sequentially [16]. Although valve-assisted devices have proven to be particularly promising for increasing the integration potential for reactor-based screening, the combination of reagents and the resulting concentration gradients have so far been predetermined by the device design, e.g., the size of reactors and/or the channel configuration.

Droplet-based microfluidics has provided a new paradigm to conduct reactions in a rapid and robust manner, with great flexibility in the size of the reactors, down to extremely tiny volumes [17–22]. In this discrete configuration, in a continuous flow network, target substances in solutions are injected into an immiscible carrier fluid to form nano-to-picoliter droplets, and concentration gradients were for instance obtained by mixing solutions before droplet formation [18], by merging already formed droplets [19,20], by injecting diluent droplets into an existing sample droplet [17], or by passing a sample droplet through diluent droplets [21] in droplet trapping units. However, in practice, it is difficult to obtain fast and stable changes in flow-rates of multiple fluids, even when using precision syringe-pumps. To address this challenge, microvalves have been integrated with droplet-microfluidic devices for precise and programmable droplet manipulation [22,23]. Microvalve-assisted droplet generators provide more flexibility by mechanical cutting of a dispensed flow and injecting new droplets into formed droplets to eventually create a concentration gradient. However, a disadvantage of the droplet-merging-based dilution approach is to keep the final volume of the droplets constant, which requires continuous multiple droplet size control and synchronized merging at each droplet generating process are required.

Here, we report an automated droplet-based microfluidic serial dilutor, in which a series of droplets is generated with excellent control on their size, to create a concentration gradient of a target molecule with flexible dilution scales. The microfluidic platform comprises a peristaltic mixer and a droplet generator to dilute the target reagent in sub-nanoliter water droplets in an oil carrier fluid. The performance of the peristaltic mixing unit was first optimized by varying the operating conditions of the microvalves. Also, the droplet size was calibrated by changing the applied pressures for the water and oil phases as well as the dispensing time to program the dilution factor of the serial dilution. By synchronizing the mixer and the droplet generator, a microfluidic serial dilution of rhodamine B isothiocyanate-dextran (RD) was automated with dilution factors of 7.69, 5.32, and 2.70, and the influence of both the RD concentration and the temperature on the fluorescent intensity was evaluated. The programmable droplet-based microfluidic serial dilutor might be useful for performing complex chemical and biochemical reactions in experimental sciences and engineering, particularly where tiny sample consumption is preferred, e.g., biochemical process development and drug discovery and screening.

**Materials and methods**

**In-line serial dilution with flexible dilution factors**

A conventional serial dilution method follows a sequential dilution procedure where each step includes the preparation of a diluent, the transfer of an aliquot of stock solution, and actual mixing (Fig. 1A). In contrast to this stepwise process, the microfluidic droplet-based serial dilution was conceptualized to conduct all the required procedures in-line with on-demand dilution factors. The design of the microfluidic droplet serial dilutor included a mixer, a closed reactor that was initially fully

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**Fig. 1.** The concept of serial dilution. (A) Conventional manual serial dilution procedure. (B) In-line automated serial dilution with modifiable dilution factors.
occupied with a sample solution, connected to an inlet and an outlet (Fig. 1B). The inlet and outlet are equipped with valves that were interconnected to synchronize the opening and closing times. Hence, when a certain volume of a diluent was introduced into the mixing reservoir, the same volume of the solution was dispensed from the mixer, consequently resulting in a solution concentration decrease in the mixer. The governing equation for calculating the concentration change of the solution in the mixer after adding the diluent \( C_{n+1} \) is:

\[
C_{n+1} = \frac{V_0 C_{in} + (V_0 - V_{out}) C_n}{V_0}
\]  

where \( C_n \), \( C_{in} \), \( V_0 \), \( V_{in} \), and \( V_{out} \) are the concentration of the solution in the mixer, the concentration of the diluent introduced into the mixer, the volume of the solution in the mixer, the volume of the diluent added into the mixer, and the volume of the solution dispensed from the mixer, respectively. When the input and output volumes are the same, and the concentration of the diluent is zero, the equation can be simplified as:

\[
C_{n+1} = \frac{V_0 - V_{in}}{V_0} C_n
\]  

Hence, the dilution factor is determined by the volume of the introduced diluent at a fixed mixer size. For example, logarithmic, half-logarithmic, and quarter-logarithmic dilutions could be obtained by adding \( 10^{-1} \), \( 3.16^{-1} \), and \( 1.78^{-1} \) of the initial volume of the solution in the mixer \( V_0 \).

**Design of the microfluidic droplet-based serial dilutor**

The device comprised a microfluidic mixer, a valve-assisted droplet generator, and two droplet incubation stages. The chip architecture included two layers, a top fluidic channel layer and a bottom valve-control channel layer. The designs of channel networks, solution inlets and outlets, and control ports are shown in Fig. 2A. The flow channels for loading a carrier fluid (grey color),

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**Fig. 2.** The design and operation of the microfluidic droplet-based serial dilutor. (A) CAD design of the chip. (B) Microscopic image of a fabricated device. (C) Operation of the device. On-chip serial dilution was processed by repeating three steps, (a, d) pushing the solution in the mixing unit with a diluent, (b, e) droplet formation, and (c, f) peristaltic mixing of the diluent and the sample solution. Photos were extracted from a recorded video. Scale bars, 300 \( \mu \)m. (For interpretation of the references to colour in the text, the reader is referred to the web version of this article.)
a diluent (light grey color), and a sample solution (blue color) were connected to the inlet and outlet in the fluidic layer. The channels to control the shut-off (red color) and mixing valves (magenta color) were connected to dedicated ports in the control layer. The mixing unit connected to the sample-, diluent-, and carrier fluid channels and shut-off valves were located at the channel junctions to open and close the connections (Fig. 2B). Fig. 2C illustrates the process flow of repeated droplet-based serial dilutions. Initially, the sample solution was loaded into the mixer by opening a valve for the reagent channel, while the valves near the diluent channel and oil channel were closed. Then, all valves were closed to isolate the mixer, and a droplet was dispensed into the carrier fluid flow by opening the valves near the diluent and oil channels (Fig. 2C (a)). The size of the droplet was determined by controlling the valve opening time. After forming the droplet (Fig. 2C (b)), three mixing valves were sequentially actuated to generate a fluid flow for enhanced mixing of the sample solution and diluent (Fig. 2C (c)). By repeating the processes of droplet formation and peristaltic mixing (Fig. 2C (d)–(f)), a serial dilution of the sample solution was achieved in a series of homogeneously-sized droplets, and all procedures were automated through proper programming of the valve operating sequences. Supplementary Video S1 presents the automated processes of the microfluidic droplet-based serial dilution.

Device validation (peristaltic mixing and droplet generation)

A food dye solution filtered with a 0.2-μm syringe filter (Whatman PLC, Sigma-Aldrich, Zwijndrecht, The Netherlands) and deionized water from Milli-Q filtration system (Millipore Co.) were used for the characterization of peristaltic mixing in the device. Mineral oil containing 1.5% (w/w) Span 80 (all from Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) was used as the carrier fluid for the formation of the water-in-oil (w/o) droplets.

The effect of concentration and temperature on the fluorescence intensity

1000 mg L⁻¹ rhodamine B isothiocyanate-dextran (RD, average molecule weight ~10,000, Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands) prepared in Milli-Q water (Millipore Co.) was used as a sample solution for characterizing the influence of the temperature on the fluorescence signal. The dextran conjugate was selected to prevent the penetration of rhodamine B molecules into PDMS channels [34]. The RD solution was introduced in the mixing unit in the chip and diluted with Milli-Q water to obtain a serial dilution in a series of droplets. The droplets were collected in the incubation stages, and the RD fluorescent signal of the droplets was quantified by N 2.1 filter cube (excitation: BP 515−560 nm; emission: LP 590 nm). An indium tin oxide (ITO) heater and a controller were obtained from Cell MicroControls (Norfok, VA, USA) and calibrated to vary the temperature in the incubation stages in the microfluidic device [32]. Note that the temperature was changed after performing a serial dilution in a series of droplets, so potential changes in solution viscosity or PDMS elasticity due to the temperature change, which could affect droplet size and dilution, is avoided. Furthermore, the fluorescent intensity was analyzed in the whole area of the droplet, which means that the total amount of fluorescent molecules is recorded. Hence, any droplet size change due to temperature will not affect the obtained fluorescent intensities of the RD droplets.

Results and discussion

Validation of the mixing capability of the peristaltic mixer

Microfluidic peristaltic mixing, one of the essential functions of the microfluidic droplet-based serial dilutor, was first evaluated to optimize the mixing performance as well as to synchronize the mixing and droplet generation processes. Fig. 3A depicts the process flow of peristaltic mixing in the device. First, a filtered blue food dye solution was loaded into the mixer through the sample channel and isolated by closing all valves near the mixer (Fig. 3A (a)). Then, Milli-Q water was introduced by simultaneously opening two valves near the diluent and the carrier fluidic channels (Fig. 3A (b)). Since a constant pressure of 200 mbar and 220 mbar was applied on the backside of the water and oil phases, respectively, the volume of Milli-Q water injected into the mixer was determined by controlling the opening time of the valves. By loading Milli-Q water with a valve opening time of 200 ms, half of the mixer volume was filled with Milli-Q water. After loading Milli-Q water, the solutions were mixed, and the average brightness value of the solution in the total area of the mixer was monitored over time (Fig. 3A (c) and (d)). Fig. 3B (left) presents the brightness value change in the mixer without the actuation of the mixing valves. The average brightness value decreased with the mixing time until a constant value was reached when mixing was completed. Using diffusion only, the mixing of two solutions was completed after 10 min.

To enhance the mixing performance, three valves were actuated with the sequence (100), (110), (010), (011), (001), and (101), where
0 means that the valve is open, and 1 that it is closed. In the closed loop-shaped mixer, the peristaltic pumping generated the rotation of fluids and stretched the parabolic profile of the Poiseuille flow [11]. The long stretched interface between the fluids enabled the enhanced diffusion of molecules in the fluids. A previous study on a microfluidic peristaltic mixer reported that a large valve area, a small distance between valves, and a fast operating frequency increased mixing efficiency [35]. Hence, the microfluidic serial dilutor included mixing three valves with a surface area of $100 \times 150 \text{ mm}^2$ and an inter-valve distance of 75 $\mu$m, which were identified as the optimal dimensions for this particular device design and fabrication. Fig. 3B (right) illustrates the complete mixing time at various valve operating frequencies ranging from 1 to 40 Hz. The complete mixing time was found to decrease exponentially with the increasing valve operation frequency, and solutions were mixed in 2 s at operating frequencies above 5 Hz. Following to this mixer characterization, a valve operating frequency of 20 Hz and a mixing time of 2 s were used for programming an in-house built automated operation software for the serial dilution chip. Mixing solutions in the device at various valve operating frequencies are presented in Video S2 in supplementary materials.

Control on the droplet size

The dilution factor in the microfluidic droplet-based serial dilution is determined by the volume of the diluent loaded into the mixer, which is equal to the volume of dispensed sample solution volume. Hence, the capability of the device to form monodispersed droplets with a well-defined size is essential to achieve accurate serial dilution with tunable dilution factors of the dilution factors. The size of the droplets formed by a valve-assisted droplet generator typically depends on the flow-rate ratio of the aqueous phase and the carrier fluid as well as the dispensing (valve opening) time [22]. To calibrate the droplet size as a function of the device operating conditions, droplets were generated at various pressures applied for the water and oil phases as well as different dispensing times, and analyzed. The filtered food dye solution and the mineral oil supplemented with 1.5 % (w/w) Span 80 were used as the aqueous phase and carrier fluid, respectively, for generating w/o droplets. The dye solution was introduced into the diluent channel and dispensed into the oil phase by opening two valves, one located between the diluent channel and the mixer, and the other one between the mixer and the oil channel, concurrently. The valve opening time for dispensing the water phase into the oil
phase was varied from 40 to 200 ms. A constant pressure of 200 mbar was applied for loading the oil phase, while various pressures ranging from 160 to 260 mbar were applied for the aqueous phase to vary the pressure ratios for the water and oil phases from 0.8 to 1.3. The droplet formation process at these various pressure ratios, while using a constant dispensing time of 100 ms, is presented in Fig. 4A: a linear increase in the droplet size was observed when increasing the applied pressure for loading the water phase. Next, while maintaining the same applied pressures of 200 mbar for both the aqueous and carrier fluid flows, the dispensing time was increased, which as shown in Fig. 4B results in the production of larger droplets. For calibrating the volume of the formed droplets according to various operating conditions, the droplets were collected from the outlet of the device and placed on a cavity slide (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) to ensure that the droplets retained a spherical shape. The measured volume of the droplets was summarized in the graphs in Fig. 4C. The droplet volumes ranged from 0.19 ± 0.01 nl to 3.82 ± 0.02 nl at different operating conditions. The influence of both the applied pressure ratios for the water and oil phases and the dispensing time on the droplet volume is summarized in Fig. 4C. The linearity of the fitting lines and the coefficient of determination (R² ≥ 0.986, n = 20) highlight the robustness of our device to produce monodispersed droplets. Since the mixer volume was maintained constant and equal to 5 nl, the dilution factor in the device could be programmed on-demand by selecting the operating conditions, e.g., applied pressures for the different fluid flows and the dispensing time, based on these calibration

**Fig. 4.** Controlling the droplet size by varying the operating conditions. Droplets of different sizes were formed by (A) varying the ratios of the pressures applied for the water and oil phases at a constant dispensing time (100 ms), and (B) varying the dispensing time at a constant pressure ratio for the water and oil phases (1.0). Scale bars, 200 μm. (C) Relationship between the droplet volume and the pressure ratio (left) and the dispensing time (right) (n = 20).
curves. The droplet generation process at various dispensing times is shown in Video S3 in supplementary materials.

**Serial dilution of rhodamine-dextran (RD): effect of the temperature**

Concentration gradients of RD were created in a series of droplets as a proof of concept to demonstrate on-demand serial dilution in the device. Before starting the microfluidic serial dilution, the fluorescence intensity of the RD droplets was calibrated as a function of their concentration. RD solutions with various concentrations, 1000 mg L\(^{-1}\), 500 mg L\(^{-1}\), 250 mg L\(^{-1}\), 125 mg L\(^{-1}\), 62.5 mg L\(^{-1}\), 31.25 mg L\(^{-1}\), and 15.625 mg L\(^{-1}\), were prepared in Mill-Q water and dispensed from the diluent channel into the carrier fluid (mineral oil containing 1.5 % (w/w) Span 80) in the oil channel to form w/o droplets, and the fluorescence intensity of the droplets was quantified. As shown in Fig. 5A, the RD fluorescence intensity in the droplets varies linearly as a function of the RD concentration in the tested range (n = 10).

For performing droplet-based microfluidic RD serial dilutions, 1000 mg L\(^{-1}\) of the RD solution was loaded into the mixer, followed by Mill-Q water, which pushed the RD solution to form RD droplets in the carrier fluid. The applied pressure for both the water and oil phases was 200 mbar, and three different dispensing times, 66.7 ms, 100 ms, and 200 ms, were tested for generating droplets having volumes of 0.65 ± 0.01 nl, 0.94 ± 0.01 nl, and 1.85 ± 0.02 nl, respectively (n = 3). For these conditions, the dilution factors, as derived from the measured droplet volume, were 7.69, 5.32, and 2.70, respectively. The relationship between the RD droplet index and the RD concentration using the different dilution factors is presented using linear (Fig. 5B) and logarithmic (Fig. 5C) scales (n = 3). The initial concentration of the RD solution, 1000 mg L\(^{-1}\), was lowered about 100 times over 35, 24, and 11 droplets by 7.69-fold, 5.32-fold, and 2.70-fold serial dilutions.

To characterize the effect of the temperature, a series of droplets with RD concentrations varying from 1000 to 19 mg L\(^{-1}\), generated by a 5.32-fold droplet-based serial dilution, was collected in the incubation stage in the device. Thereafter, the temperature was increased from 25 °C up to 50 °C with steps of 2.5 °C and decreased again to 25 °C. At each temperature, the RD fluorescence intensity of the droplets was measured and compared with the initial intensity. The fluorescence images of these RD droplets with various concentrations at different temperatures are shown in Video S4 in supplementary materials, and Fig. 6 illustrates the effect of the temperature and the RD concentration on the droplet fluorescence intensity. The RD fluorescence intensity was found to decrease as the temperature increased, and this decrease was more pronounced for higher RD concentrations, reaching a plateau of 1.96 %/°C. The concentration-dependent decrease rate might have resulted from the reabsorption of emitted light or collisional quenching behavior of fluorescence molecules at higher densities [36]. This maximum decrease rate is in close agreement with values reported in the literature of 1.90 %/°C, which was measured for rhodamine B in water using a spectrophotometer equipped with a temperature control module [37]. The reliable fluorometric measurement of molecules in droplets associated with the fast heat transfer in such small volumes, providing exquisite control on the temperature is of great interest to monitor the temperature-dependent kinetics of reactions with fluorescence markers for biotechnological studies, e.g., protein aggregation [32,38], and enzyme kinetics [14,23].

![Fig. 5. Droplet-based serial dilution of RD with various dilution factors. (A) Relationship between the concentration and fluorescence intensity of RD (n = 10). (B) Droplet index and RD concentrations of serially diluted RD droplets plotted on a linear scale (n = 3). (C) Logarithmic regression of the droplet index and the RD concentration. Dashed lines represent calculated values. 1000 mg L\(^{-1}\) of RD solution was diluted in a series of droplets in the device at a constant applied pressure ratio (P\(_\text{water}/P\text{oil}\)) of 1.0 and using various dispensing times.](image-url)
Fig. 6. Influence of the concentration and the temperature on the fluorescence signal of RD droplets. (A) Fluorescence intensity as a function of the temperature at various RD concentrations. (B) Rate of fluorescence intensity decrease (%) per temperature (°C).

Conclusion

In conclusion, we have developed a programmable droplet-based microfluidic serial dilutor for creating on-demand concentration gradients of a sample solution. The microfluidic peristaltic mixer and the valve-assisted droplet generator were first calibrated and synchronized to achieve fast mixing and reliably control the droplet size for droplet-based serial dilution. Next, serial dilutions of rhodamine B isothiocyanate-dextran (RD) was demonstrated at various dilution factors. The automated programmable serial dilution in a series of nanoliter droplets will open new avenues to evaluate chemical and biochemical reactions in high-throughput screening processes.

Conflict of interest

The authors have no conflict to declare.

CRediT authorship contribution statement

Hoon Suk Rho: Conceptualization, Methodology, Software, Validation, Data curation, Formal analysis, Investigation, Visualization, Writing - original draft, Writing - review & editing. Yoonsun Yang: Conceptualization, Data curation, Formal analysis, Investigation, Writing - review & editing. Leon W.M.M. Terstappen: Funding acquisition, Writing - review & editing. Han Gardeniers: Methodology, Writing - review & editing. Séverine Le Gac: Methodology, Writing - original draft, Writing - review & editing. Pamela Habibovic: Methodology, Supervision, Funding acquisition, Writing - original draft, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi: https://doi.org/10.1016/j.jiec.2020.08.004.

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