Published in final edited form as:

Lab Chip. 2009 May 21; 9(10): 1344–1348. doi:10.1039/b816049j.

Reliable microfluidic on-chip incubation of droplets in delay-lines†

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Abstract

Together with droplet creation, fusion and sorting, the incubation of droplets is one of the most important and essential operations for droplet-based microfluidic assays. This manuscript concerns the development of delay-lines, which are necessary to allow incubation of reactions for precise time periods. We analyze the problems associated with creating delay-lines for incubation in the minute to hour time range, which arise from back-pressure and from the dispersion in the incubation time due to the unequal speeds with which droplets pass through the delay-line. We describe delay-line systems which resolve these problems and demonstrate their use to measure reaction kinetics over several minutes in droplets.

1. Introduction

Compartmentalization of reactions in microdroplets in emulsions has a broad range of applications in chemistry and biochemistry. Each microdroplet functions as an independent microreactor with a volume of between one nanoliter and one femtoliter, which is between 10^3 and 10^9 times smaller than the smallest working volumes in a microtitre plate well (1–2 microliters). Initially developed for directed evolution, the technique of *In Vitro* Compartmentalization (IVC)¹ of reactions in emulsions has allowed the selection of a wide range of proteins and RNAs for binding, catalytic and regulatory activities.^{2–4} However, other applications rapidly followed, notably massively parallel PCR of single DNA molecules (emulsion PCR), which is used, for example, for the Genome Sequencer FLX (Roche) and SOLiD (ABI) “next-generation” high-throughput sequencing systems.⁵ Droplet-based microfluidic systems are now further extending the range of potential applications, as they allow for the precise generation and manipulation of droplets. Microfluidic modules have been described which allow highly monodisperse droplets to be created;⁶ split;^{7,8} fused;^{7,9} sorted;¹⁰ and the contents of the droplets mixed on ms timescales;⁷ all at high throughput (typically kHz). Based on these developments a range of

†Electronic supplementary information (ESI) available: Description of the optical set-up cloning, expression and purification of β -lactamase and other strategies for reducing dispersion, as well as 5 movie files. See DOI: 10.1039/b816049j

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applications has already been transferred to microfluidic systems such as protein crystallisation,¹¹ the measurement of chemical kinetics,⁷ enzymatic assays,¹² cell based assays,^{13,14} the synthesis of monodisperse polymer beads/particles,^{15,16} the synthesis of organic molecules,^{17,18} and the synthesis of nanoparticles.^{19,20} Multiple modules can potentially be integrated into single microfluidic chips fabricated in poly-(dimethylsiloxane) (PDMS) using soft-lithography,²¹ allowing sophisticated multi-step procedures to be executed on-chip.

This manuscript concerns the development of another essential module for performing reactions in integrated microfluidic systems—the delay-line—which is necessary to allow incubation of reactions for precise time periods. For long reaction times (>1–2 h) the droplets can be incubated within an on- or off-chip reservoir and reinjected into the microfluidic device for analysis.^{13,14} But this method is not practical for shorter incubation times. For very short reaction times (<1 min) short and narrow microfluidic channels (delay-lines) have been used in which the droplets remain in single-file.¹² However, to date, it has not been possible to create a reliable delay-line for incubation times in the (extremely useful) range of 1 minute to 1 hour. The ideal on-chip incubation system should have very low back-pressure, very low dispersion of incubation times and enough flexibility so that a range of different incubation times would be accessible for a given design.

2. Materials and methods

Soft-lithography in poly(dimethylsiloxane) (PDMS, Sylgard 184, Dow Corning) was used to prepare the devices.²¹ The molds consisted of SU-8 (Microchem) with two different heights.²⁶ We therefore used the following procedure: First we spin coated the thinner SU-8 layer (25 μm) and exposed it with a mask which covers the part of the wafer designated to the deeper structures. After fully developing and baking the structures we spin coated a second higher layer of SU-8 onto the same wafer. This second layer was exposed and structured by a second mask (delay-line), which was aligned to the lower structures in a mask aligner. Designing the connectors (Fig. 1) in close proximity to each other facilitated the alignment and made it less prone to angle misalignments.

After casting the mold in PDMS and binding it to a glass slide (after activation in an oxygen plasma) the channels were made hydrophobic using a commercial surface coating agent (Aquapel, PPG Industries). The flow rates were controlled by syringe pumps (PHD2000; Harvard Apparatus). In all experiments flow rates of 400 $\mu\text{L h}^{-1}$ for the oil phase and 100 $\mu\text{L h}^{-1}$ in total for the aqueous phases were used to create 53 μm droplets (78 pL) at a 50 μm nozzle.

For the dispersion characterization experiments the oil phase consisted of a perfluorocarbon oil (FC40 - 3M) containing 2.5% (w/w) of a surfactant, made of the ammonium salt of a perfluorinated polyether (PFPE) (Krytox FSL - Dupont).²⁷ For the kinetic measurements with β -lactamase the oil phase consisted of “R” oil with 1% (w/w) “EA” surfactant (both from Raindance Technologies). One aqueous phase for the co-flow consisted of PBS with 0.1% BSA, 20 μM Fluorocillin and 10% DMSO. The other aqueous phase consisted of PBS with 0.1% BSA and 20 nM (80 nM) β -lactamase. At a flow rate ratio of 1 : 1 this led to a

final concentration in each droplet of 10 nM (40 nM) β -lactamase, 10 μ M Fluorocillin, 0.1% BSA, 5% DMSO in PBS.

More details about the optical set-up as well as cloning, expression and purification of β -lactamase are described in the ESI.[†]

3. Results and discussion

3.1 Solutions to avoid pressure problems

Two simple equations are necessary to characterize the behavior of delay-lines. Eqn (1) estimates the delay time t , whereby Q is the flow rate and l , w and h represent the length, width and height of the channel. Eqn (2) estimates the pressure drop P along a channel, whereby c is a constant depending on the w/h ratio (eqn (3)) and η is the viscosity. Eqn (2) is accurate to within 0.26% for any rectangular channel with $w/h > 1$, provided that the Reynolds number is below ~ 1000 and no bubbles, droplets or obstructions are present.²² It remains difficult to calculate P exactly for two phase microfluidic flow.²² However, the estimate of the pressure drop given by eqn (2) is sufficient to guide the design of delay-lines. The following example shows that the pressure over long channels can easily surpass the working limits of the pumps (~ 33 bar) and of the device (delamination at ~ 3 bar).²³ To obtain 10 s of delay at a total flow rate of 500 μ L h⁻¹ in a channel of width $w = 50$ μ m and height $h = 25$ μ m (suitable for 30–50 μ m droplets in single-file) a channel length of $l = 1.1$ m is necessary.

According to eqn (2) this leads to a back-pressure of over 100 bar ($\eta = 3.4$ mPa s for the oil and $c = 17.5$). A solution to the pressure problem can be found by inspecting eqn (2). All the parameters affect the pressure drop linearly except for the smallest channel dimension (usually the channel height), where the pressure drop is inversely proportional to the cube of the channel height. This means that increasing the channel height will significantly reduce the pressure drop.

$$t = \frac{lwh}{Q} \quad (1)$$

$$P = c\eta \frac{l}{wh^3} Q \quad (2)$$

$$c = 12 \left[1 - \frac{192}{\pi^5} \frac{h}{w} \tanh \left(\frac{\pi w}{2h} \right) \right]^{-1} \quad (3)$$

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Therefore, the use of delay-lines with deep, wide channels allows longer droplet incubation times without any back-pressure problems. However, to create and manipulate picoliter volume droplets the channels need to have dimensions similar to the droplet size (20–50 μm). A solution to satisfy both criteria is to create a device with narrow, shallow channels where the droplets are created, split, fused, analyzed and sorted, followed by a second part to incubate droplets with deep, wide channels to avoid pressure problems and to increase delay times. An example of such a device is presented in Fig. 1 and its fabrication is described in the experimental section.

An additional approach to increase delay times and to reduce the pressure drop is to decrease the total flow rate Q . However, the aqueous flow rate can not be reduced since it determines the throughput (droplets/second) and an oil flow rate of at least the same magnitude as the aqueous is also necessary to create well defined droplets.²⁴ A solution is to extract oil after the droplets have been formed. The device shown in Fig. 1 allows the creation of droplets at any flow rate and the subsequent oil extraction (of up to 92% of the oil) leads to a reduction of the total flow rate. With this approach the delay time increases proportionally with the volume of oil extracted and delay times of 12 min are easily achievable even with the relatively short ($l = 40\text{ cm}$, $w = 1\text{ mm}$, $h = 75\text{ }\mu\text{m}$) delay-line shown in Fig. 1. By further increasing the channel dimensions, even longer delay times were achieved; the longest tested ($l = 1\text{ m}$, $w = 1\text{ mm}$, $h = 150\text{ }\mu\text{m}$) reached incubation times of up to 69 min without any back-pressure problems.

3.2 Dispersion of incubation times

Whereas wider and deeper channels resolve the pressure problem, the order of droplets is not necessarily maintained in these channels. A well known phenomenon in microfluidic channels is the so-called Taylor dispersion of reagents due to the parabolic flow profile within the channels (Poiseuille flow).²⁵ As a consequence, the flow rate in the center of the channel is higher than the flow rate close to the walls. Therefore, as soon as a channel is wide enough for droplets to overtake each other these different flow rates over the cross section affect the droplet flow. The central droplet stream can flow faster than the streams close to the walls, thereby leading to significant differences in the incubation times of individual droplets.

Using the device in Fig. 1 we investigated the dispersion of droplets in a delay-line. For this purpose we used a stream of highly fluorescent droplets followed by a stream of low fluorescent droplets. We achieved this by co-flowing two streams of phosphate buffered saline (PBS) with and without 20 μM fluorescein into the nozzle. By switching the co-flow ratio from 4 : 1 to 1 : 4 two droplet populations containing different fluorescein concentrations were created directly after each other with a transition time of about 10 s. The transition time is defined as the time between the arrival of the first droplet with low fluorescence and the arrival of the last droplet with high fluorescence (followed by a continuous sequence of at least 10^6 low fluorescence droplets). A LabView program controlled the flowrates, and recorded the fluorescence intensity of individual droplets at the end of the delay-line. The recording was started when the ratio of the co-flow was switched so that the start of the transition corresponds to the delay time and the duration of this

transition is the transition time. The percentage of high fluorescent droplets F was evaluated in packages of 100 droplets. The corresponding values were plotted into a time trace (see Fig. 2a) and no dispersion could be assumed if the transition time was still within 10 s at the end of the delay-line.

However, under many conditions much longer transition times were observed (see Fig. 2a). A systematic analysis showed that the droplet density has a strong effect on the dispersion. For this analysis, droplets were generated under identical conditions (resulting in a constant droplet volume of 78 pL and a diameter of 53 μm) while changing the droplet density by extracting different volumes of oil (Fig. 2a). At low droplet densities (oil/aqueous ratio of 3) a sharp transition (no dispersion) was observed. With increasing droplet density the transition became longer. In this regime, typically about 50–60% of the highly fluorescent droplets population passed through almost at the same time while the rest was significantly retarded. For example at an oil/aqueous ratio of 1.25 some droplets needed 6 min to pass the delay-line while others needed up to 11 min. Finally, at very high droplet densities the transition time decreased again.

These observations can be explained by inspecting Fig. 2b and the ESI Movies M1, M2 and M3.[†] At low droplet densities most of the droplets remain in the fastest streamlines in the middle of the channel and flow at almost equal speeds. At medium densities droplets get pushed outwards to the walls where they experience lower flow rates and are overtaken by the more central droplets. At very high densities the droplets adopt a crystal-like packing, making overtaking almost impossible, and move as one block through the channel.

Fig. 2c summarizes the dispersion ratio R (transition time/delay time ratio) of the droplets at different oil/aqueous ratios. The dispersion is very important for themid-range of oil/aqueous ratios with values of R as high as >90%. In this regime any quantitative analysis of reaction kinetics becomes impossible since the incubation times vary almost over a 2-fold range. In the low density regime (right part of the graph) the dispersion is low ($R \approx 10\%$), but the delay time may not be sufficiently long. Therefore, the high density regime would be desirable since both the delay time is long and the dispersion low ($R \approx 15\%$). However, it remains difficult to run the system in this regime. The slope of the curve is very steep and small changes in the volume fraction of the extracted oil can increase the dispersion by minutes. Furthermore, the system is not very flexible, since only the lowest oil/aqueous ratio can be used, limiting the spectrum of accessible delay times for a given design.

3.3 Reducing the dispersion of incubation times

To address the problem of dispersion we tested two different approaches. The first approach consisted of preventing the droplets from overtaking each other by dividing the channel into multiple narrow channels (see ESI[†]). This design did not reduce the dispersion sufficiently to enable reliable kinetic experiments (R still reaches values of over 50%). The second strategy consisted of repeatedly shuffling droplets by introducing constrictions every 3 cm along the delay-line (Fig. 3a). These constrictions reduce the channel width to the dimension of a droplet and result in a repeated mixing of the droplets over the channel cross section, preventing the same droplets from remaining in the same (faster or slower) flow lines. This

random re-distribution was verified by analyzing high speed movies as shown in the ESI Movie M4.[†]

Indeed, after testing several different constriction designs (see ESI), a significantly reduced dispersion ($R = 10\%$) was found (Fig. 3b) compared to the delay-line without constrictions (Fig. 2c). Furthermore, the shape of the transition changed. For a delay-line without constrictions the slow droplets lead to a long ‘tail’ as can be seen in Fig. 2a (*e.g.* oil/aqueous ratio of 1.5 or 1.0) and the transition is non-symmetrical. In contrast, for the delayline with constrictions the shape of the transition becomes symmetrical (Fig. 3c). The incubation times of individual droplets in the delay-line are equally distributed around a mean value and the transition can be perfectly fitted with a logistic function, which corresponds to a Gaussian distribution of the incubation time. This Gaussian distribution is obtained at all droplet densities and the width of the distribution (which is a measure for the dispersion) scales proportionally with the incubation time of the droplets in the delay-line. With this improvement, the whole system becomes more stable and reproducible and opens up an important range of new applications.

3.4 Measurement of enzyme kinetics

As a first demonstration of the delay-line reliability, we measured the kinetic of an enzymatic reaction. The turnover of the fluorogenic substrate Fluorocillin by the enzyme β -lactamase was detected over a range of several minutes in the delay-line. For this purpose, we introduced an additional feature into the layout of the delay-line. Whereas the geometry of the delay-line in Fig. 1 only allowed a single measurement at the end of the delay-line, now several additional measurement points were introduced between the inlet and the outlet (Fig. 4a). These measurement points were designed within the narrow and shallow channels to obtain sufficient spacing between the droplets and also to confine them laterally for the fluorescence detection. Droplets therefore moved back and forth between the deep channels for incubation and the narrow channels for measurements (see ESI Movie M5[†]).

When performing measurements at several points along the delay-line the droplet density is an important factor. If the droplets are packed too densely the fluorescence signal of individual droplets cannot be resolved anymore. Therefore, these experiments need to be carried out at an oil/aqueous ratio that provides a good compromise between delay times and droplet spacing. This ratio corresponds exactly to the intermediate regime where the dispersion in a delay-line without constrictions is the highest. In contrast, for the delay-line with constrictions this medium packed regime is practically accessible without dispersion. Furthermore, since the dispersion is no longer influenced by the droplet density a whole range of incubation times can be reached by varying the amount of oil extracted.

Fig. 4b shows the fluorescence signal of the β -lactamase reaction measured at different time points. At each point the distribution is Gaussian as expected and the standard deviation is directly proportional to the mean fluorescence (data not shown). Furthermore, the measured kinetics follows exactly the same trend as in the assay performed in a cuvette (inset, Fig. 4b), showing that the system is fully biocompatible and accurate. These results clearly show that the improved delay-line layout is a very well-suited system to analyze enzymatic reactions in a fast, convenient and reliable way.

4. Conclusions

In summary, this article analyses the problems associated with designing delay-lines to allow on-chip incubation time 1 minute for droplet-based microfluidics systems. Moreover, the article provides solutions to two fundamental problems, namely the problems of pressure and unequal incubation times of droplets in the delay-lines (dispersion). The back pressure of the system can be reduced by using a two depth device with wide, deep channels for droplet incubation and narrow, shallow channels for the generation and manipulation of droplets. The extraction of oil directly after droplet generation further reduces the back pressure and facilitates even longer incubation times, which may easily reach the hour range. In addition, the extraction of oil broadens the range of incubation times, accessible for a given delay-line design. A general solution to the dispersion problem is the use of constrictions that redistribute the droplets repeatedly along the delay-line. This repeated shuffling of droplets leads to a significant reduction in the dispersion of incubation times and distributes these times equally (Gaussian) around a mean value. These improvements allow the creation of integrated droplet-based microfluidic systems for a wide range of (bio)chemical reactions, containing multiple modules, including delay-lines which allow reaction times of 1 min to >1 hour. Finally, we validated our delay-line system by measuring the reaction kinetics of the enzyme β -lactamase on-chip: the reactions kinetics were identical to a conventional cuvette-based assay.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank J.-C. Baret, D. A. Weitz, D. Link and the members of Raindance Technologies for their support as well as A. Plückthun for providing the plasmid pAK400, H. Roesch for the video editing and A. Salomon for her help with the SEM. L. F. was supported by the EC FP6 Marie Curie Research Training Network, ProSA. K. B. acknowledges support from longterm fellowships from FEBS and HFSP. This work was also supported by the Ministère de l'Enseignement Supérieur et de la Recherche, CNRS and ANR (ANR-05-BLAN-0397).

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Fig. 1.

Layout of a two-depth device with a delay-line. The 50 μm wide nozzle for droplet generation and the fluorescent read-out channel after the delay-line have a channel depth of 25 μm (light grey). In addition, this part contains two fork-like structures shortly after the nozzle which allow for the extraction of oil (top right image). The delay-line itself (dark grey) consists of channels with a depth of 75 μm, length of 40 cm and a width of 1000 μm. The droplets pass from a 25 μm to a 75 μm deep channel at the connection point (bottom left image). The SEM image of the connection point (bottom right image) shows the robustness

of this fabrication technique. Even if the channels are not perfectly aligned and overlap, the fluidic connection remains intact and no lifting of the structures occurs.

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Fig. 2.

Dispersion of droplets at different droplet densities (oil/aqu. ratio). (a) The delay-line is filled with highly fluorescent droplets ($F = 100\%$), followed by a second population of droplets with lower fluorescence ($F = 0\%$) with an initially sharp transition time (~ 10 s) at the beginning of the delay-line. At the end of the delay-line the spreading of this transition is an indicator for the dispersion at different droplet densities (oil/aqu. ratios). (b) At low densities (top; oil/aqu. > 3) the droplets do not have contact with the channel walls and all flow at roughly equal speed. At medium droplet densities (middle; oil/aqu. ratio of ~ 0.7 – 3) some droplets travel in the slow flow lines close to the walls and are overtaken by droplets following in the faster more central streamlines. At very high densities (bottom; oil/aqu. ratio < 0.7) this effect is reduced, due to the dense packing. The corresponding movies are

available as movies M1,M2 and M3 in the ESI.† (c) Measurements of the transition parameters for different droplet densities. The transition time is defined as the difference between the start (●) and the end (∇) of the transition. A fit to the start of the transition (●), which corresponds to the delay time, returns the volume of the delay-line (26.6 μL) in good agreement with the calculated value (28.2 μL). The inset shows the relative dispersion ratio R defined as the transition time/delay time ratio.

Fig. 3.

Reducing dispersion in delay-lines. (a) Droplets are stochastically redistributed (mixed) at each constriction, preventing the same droplets from remaining in the same (faster or slower) streamlines (see ESI Movie M4†). (b) Results of the dispersion measurements. The constrictions significantly reduce the dispersion (compared to Fig. 2c) for all droplet densities. The transition time is never higher than 45 s corresponding to a relative dispersion ratio R lower than 10%. (c) Due to the mixing effect the shape of the transition becomes logistic, corresponding to a Gaussian distribution for the incubation times.

Fig. 4.

(a) Layout of a device designed for kinetic measurements including eight measurement points within the shallow channels (50 μm wide, 25 μm deep; light grey). The first point is located just after the droplet creation, followed by six loops (shown in the microscope image and the ESI movie M5†) and one last measurement point before the outlet. The deep channels (1000 μm wide, 75 μm deep; dark gray) contain constrictions every 3 cm. (b) Enzyme kinetics of μ -lactamase. Histograms of fluorescence intensities of droplets containing 10 nM β -lactamase and 10 μM Fluorocillin were collected at the 8 measurement

points (time points) in the delay-line. As expected for a delay-line with constrictions all the distributions (each containing 20 000 droplets) appear to be Gaussian. The inset shows the mean droplet fluorescence values plotted against the corresponding times for each measurement point. At an enzyme concentration of 10 nM the reaction remains linear whereas it starts to saturate at 40 nM. Both measurements are in good agreement with the cuvette-based measurements (solid lines).

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