FULLY INTEGRATED DILUTION SERIES GENERATION ON A LABORATORY CENTRIFUGE

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ABSTRACT
We present a novel approach for the fully automated generation of dilution series using a disposable microfluidic cartridge. Fluids are metered, mixed and routed without active valving by defined rotational speed only. As a processing device, a standard lab centrifuge can be used. The dilution ratio can be freely choosen by loading the corresponding volume of sample into the cartridge without the need for changes in the microfluidic layout. Dilution series of Fluorescein in PBS buffer with ratios of 1:3 and 1:5 are shown with each dilution series comprising 5 single dilution steps. Reproducibility was determined by fluorescence measurement.

KEYWORDS
Lab-on-a-Chip, dilution series generation, centrifugal microfluidics

1. INTRODUCTION
Manual pipetting of dilution series is a time consuming, error-prone procedure conducted multiple times in daily laboratory work. Typical application examples include dilution of bacteriological samples e.g. for subsequent counting, dilution of DNA to create PCR standards or dilution of inhibitors for dose-response measurements. For high throughput applications, pipetting robots offer an acceptable solution for automation. However, in the mid- and low-throughput range, only a few approaches offer an alternative to automated or manual pipetting [1-3].

C.–Y. Chen et al. demonstrated a PDMS chip with five inlets and magnetically driven valves. Depending on the valve position, dilution of tetraethylammonium (TEA) in a buffer by log10 steps over 5 orders of magnitude was possible. At the outlet the effect of the diluted TEA on ion channels of patched HEK cells was monitored [1].

A pressure driven microfluidic system for the automated generation of serial dilutions was presented by *J. Koehler* et al. Different substrates for enzymekinetic and dose-response measurements were diluted and temporal changes in fluorescence signals were measured in a standard plate reader [2].

Samsungs patent application US 2008/0193336 A1 introduces a centrifugal microfluidic system for the generation of discrete dilutions by mixing liquids in a central mixing chamber and then routing the mixture into separate chambers. Volumes of the mixed liquids were defined by a complex system of laser actuated wax valves that open and close fluidic pathways [3].

Limitations of these systems are the application of manufacturing technologies lacking mass production capability like PDMS casting [1]. Presented systems require active valving e.g. with lasers [3] or additional equipment for precise liquid actuation [2].

We present a novel method, not limited to those shortcomings, by using a disposable thermoplastic cartridge. Instead of applying syringe pumps for fluid actuation, centrifugal forces generated by a standard laboratory centrifuge are used to transport fluids. Active valving is not required throughout the complete process.

2. MATERIALS AND METHODS

Prototyping of the cartridges
Microfluidic structures are designed using 2D CAD software AutoCad (Autodesk, Germany). The layout is then micromilled into standard 4 mm thick PMMA plates (Maertin, Germany). Inlet- and ventilation holes are fabricated by drilling. To remove residues from manufacturing, the cartridges are cleaned thoroughly with isopropanol and water and finally sealed with pressure sensitive polyolefine foil (#900320, HJ Bioanalytik, Germany). *Figure 1* depicts a cartridge with two independent microfluidic structures for dilution series generation.

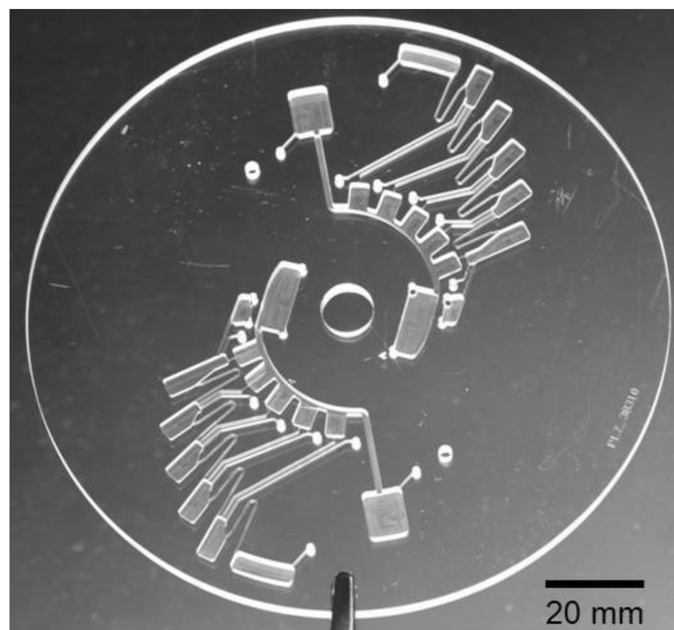

Figure 1: Cartridge featuring two independent microfluidic structures for generation of dilution series.

Working principle

The microfluidic layout is composed of a set of standard microfluidic unit operations including aliquoting structures, capillary siphons and hydrophobic valves *(Figure 2)*.

Each dilution structure features two separate inlet chambers for dilution buffer (inlet 1) and sample (inlet 2) respectively. Inlet 1 is connected to five aliquoting fingers with defined volume and a waste chamber. This structure enables for splitting of the initial amount of dilution buffer into five aliquots while excess liquid is gated into a waste chamber [4].

Every single finger of the aliquoting structure is connected to a dilution chamber via a hydrophobic constriction (150 µm width x 100 µm depth). Each constriction is coated with 0.2 µL of a 0.5% w/w Teflon AF 1600 solution (DuPont, USA) dissolved in Fluorinert FC-77 (3M, USA). Due to the change in surface property and geometry, the constrictions act as closed valves at frequencies below a certain breakthrough frequency

Adjacent dilution chambers are connected by capillary siphons (400 µm width x 200 µm depth). At high rotational frequencies, the centrifugal force prevents liquid from capillary priming of the siphon while at lower frequencies capillary forces are dominating and the siphon primes. Once the siphon is primed completely, reacceleration leads to a fluid flow to the radially outward direction. The siphons are connected to the dilution chambers in a way that they only transfer a defined liquid volume into the adjacent chamber. In order to obtain hydrophilic surfaces and to enhance capillary priming, all siphons are coated with 3 µL polyethylene glycol (PEG) dissolved in methanol (conc. = 5 mg/mL).

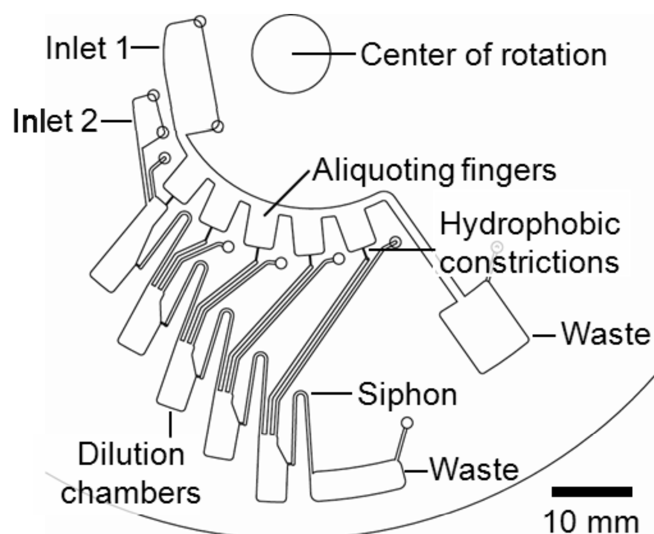

Figure 2: Each dilution structure features two inlets for dilution buffer and sample, five aliquoting fingers to meter dilution buffer and five dilution chambers. Aliquoting fingers are connected to dilution chambers via hydrophobic constrictions acting as valves. Capillary siphons transfer a defined amount of fluid from one dilution chamber into the adjacent chamber.

Experimental

The disk is directly mounted on the axis of the laboratory centrifuge (Sigma 1-15, Sigma GmbH, Germany) and fixed by a screw. Rotational frequencies were set manually. However, an RS 232 interface for complete automation of the microfluidic protocol is available.

The microfluidic protocol consists of two parts (*Figure 3*)

(1) 200 µL dilution buffer (phosphate buffered saline - PBS) are loaded into inlet 1 and rotation is started. Initial acceleration to 15 Hz forces the dilution buffer into the aliquoting structure, filling each aliquoting finger with approximately 30 µL PBS while the supernatant is gated into the waste chamber. At this spinning frequency, the PBS doesn't pass the hydrophobic constrictions since the hydrodynamic pressure is too low. After acceleration to 50 Hz, the 30 µL PBS aliquots are gated into the dilution chambers. The rotation is then stopped.

(2) The volume V in µl of liquid loaded into inlet 2 defines the dilution ratio V / (V + 30 µL). In our experiment 15 µL and 7.5 µL Fluorescein, for preparation of 1:3 and 1:5 dilution series respectively, are used as sample reagent.

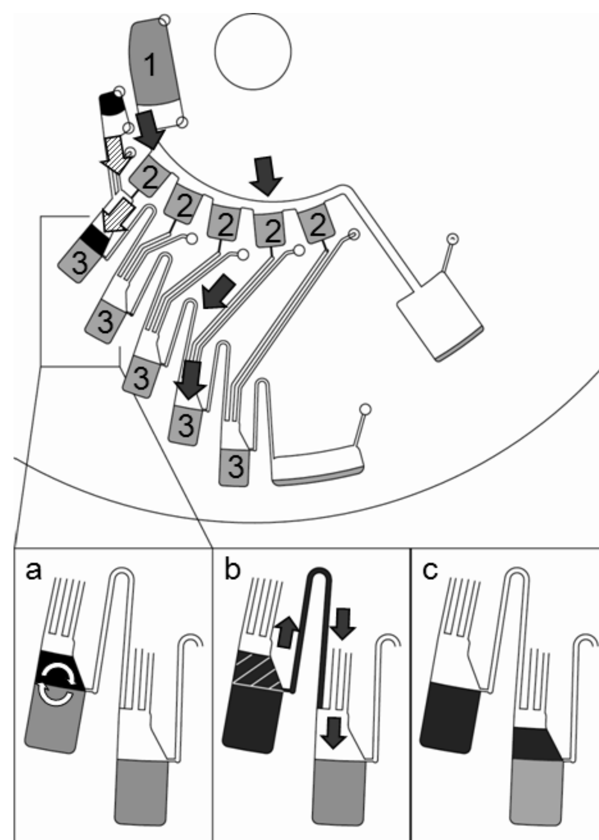

Figure 3: 200 µL PBS from inlet 1 (1) are divided into five 30 µL aliquots (2) at 15 Hz. Supernatant is gated into the waste. At 50 Hz, the aliquots are routed into dilution chambers (3) (arrows). After the disk stops, 15 µL (7.5 µL resp.) of Fluorescein are loaded into inlet 2. At 50 Hz, Fluorescein is gated into first chamber (dashed arrows). (a) Fluorescein and PBS are mixed by alternating spinning frequency between 15 Hz and 50 Hz. (b) Disk is stopped and siphon primes due to capillary forces. 15 µL (7.5 µL resp.) (dashed area) of mixture are gated into adjacent chamber by acceleration to 50 Hz. Steps (a) – (c) are repeated five times.

Acceleration to 50 Hz then gates the Fluorescein into the first dilution chamber. Fluorescein and PBS are mixed by alternating the spinning frequency between 15 Hz and 50 Hz for 10 times. Subsequently, rotation is stopped and the capillary siphon primes. From the dilution chamber, 15 µL or 7.5 µL respectively, are then transferred to the adjacent chamber by reacceleration to 50 Hz and mixed again as described above. The steps of transport and mixing are repeated five times (Figure 3, a-c) to generate five discrete dilutions with 30 µL volume each.

Finally, for quantification of fluorescence, 20 µL of each dilution are transferred to a microwell plate by pipetting which was the read out in a plate reader (Victor 1420 Multilable Counter, Perkin Elmer, Germany). Results were compared with manually pipetted reference dilution series and theoretical values for perfect dilution.

3. RESULTS AND DISCUSSION

For 1:3 and 1:5 dilution ratios respectively always one experiment was conducted on the lab centrifuge while, under identical conditions, three further experiments were conducted on a centrifugal test set-up which allowed real-time image analysis [5]. The current microfluidic layout enables for parallel generation of two separate dilution series. The generation of two dilution series in parallel required approximately 8 minutes vs. 10 minutes required for manual pipetting.

For visualization purposes, an additional 1:3 dilution series was generated by dilution of green ink in PBS *(Figure 4)*.

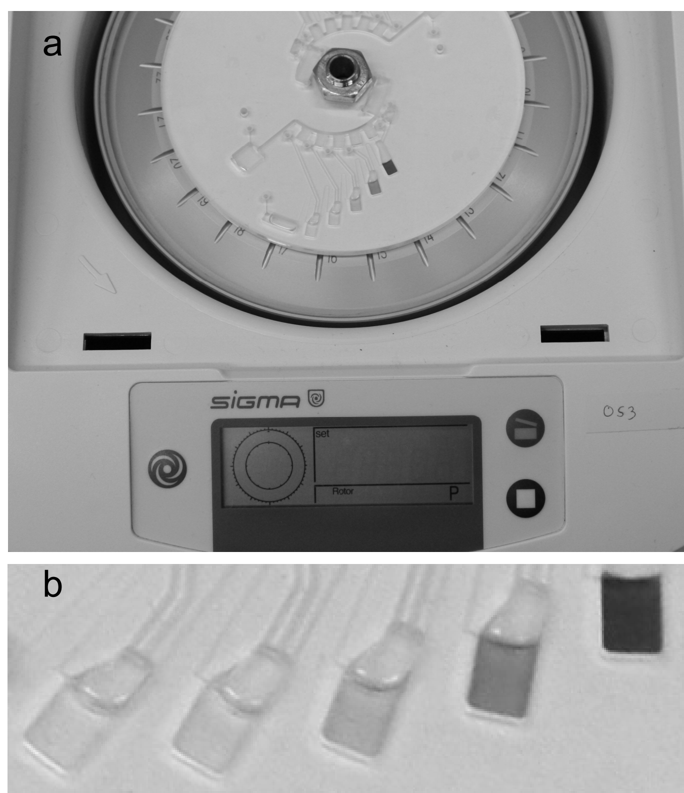

Figure 4: (a) Preparation of a 1:3 dilution series of green ink on disk mounted in a standard laboratory centrifuge; (b) Magnified image of the generated dilution series.

Since one dilution series comprises five discrete dilutions and eight on-disk experiments were performed, a total number of 40 single dilution steps were carried out. Out of that, only 2 failed due to bad prototyping what resulted in a failure of capillary priming of the corresponding siphon. Hence, the reliability of the fluidic process was 95%.

Mean values and coefficient of variations were calculated from the ratios of fluorescence signals *(Table 1)*. Initial concentrations of Fluorescein were 100 µM and 10 µM for 1:3 and 1:5 dilutions, respectively. For better comparison, fluorescence signals were normalized to an initial relative fluorescence of 100 % and subsequently compared with reference and theory *(Figure 5)*.

Table 1: Comparison of mean dilution ratios and coefficient of variation for 1:3 and 1:5 dilutions.

Dilution coeff.	**On-Disk**		**Reference**	
	Mean	CV [%]	Mean	CV [%]
1:3	3.02	14.2	2.85	16.5
1:5	4.06	15.8	4.89	6.2

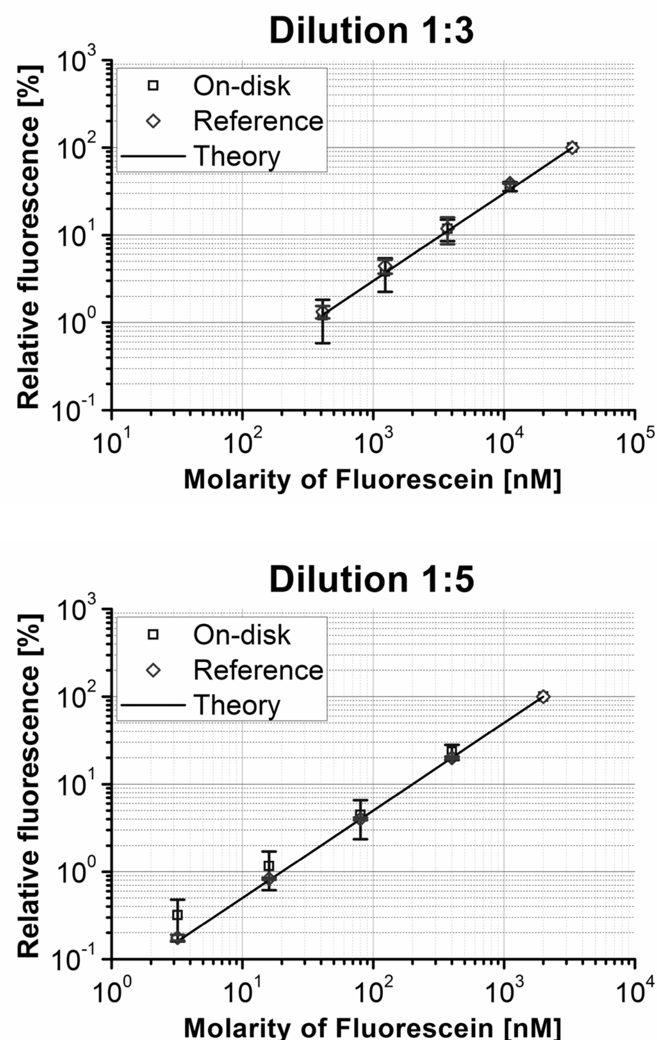

Figure 5: Normalized fluorescence for 1:3 diluted Fluorescein (100 µM) and 1:5 diluted Fluorescein (10 µM). Results from on-disk measurements (square) vs. manually pipetted reference (diamond) compared to theoretical values (black line).

4. CONCLUSION

We successfully demonstrated the fully integrated generation of dilution series on a disposable centrifugal microfluidic disk utilizing a standard laboratory centrifuge. For 1:3 dilutions, slightly better results than with manual pipetting were achieved. However, one has to keep in mind, that every failure in dilution series generation is propagated and thus effects the following results.

The precision of the system may be further improved by adjustments of the microfluidic designs. For example position and dimension of the capillary siphons and narrowing of the dilution chamber may lead to a more precise transfer of liquids. Time to result could be reduced by using the centrifuges RS 232 interface and suitable software for complete automation.

5. REFERENCES

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