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Centrifugo-pneumatic multi-liquid aliquoting – parallel aliquoting and combination of multiple liquids in centrifugal microfluidics†

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The generation of mixtures with precisely metered volumes is essential for reproducible automation of laboratory workflows. Splitting a given liquid into well-defined metered sub-volumes, the so-called aliquoting, has been frequently demonstrated on centrifugal microfluidics. However, so far no solution exists for assays that require simultaneous aliquoting of multiple, different liquids and the subsequent pairwise combination of aliquots with full fluidic separation before combination. Here, we introduce the centrifugo-pneumatic multi-liquid aliquoting designed for parallel aliquoting and pairwise combination of multiple liquids. All pumping and aliquoting steps are based on a combination of centrifugal forces and pneumatic forces. The pneumatic forces are thereby provided intrinsically by centrifugal transport of the assay liquids into dead end chambers to compress the enclosed air. As an example, we demonstrate simultaneous aliquoting of 1.) a common assay reagent into twenty 5 μl aliquots and 2.) five different sample liquids, each into four aliquots of 5 μl. Subsequently, the reagent and sample aliquots are simultaneously transported and combined into twenty collection chambers. All coefficients of variation for metered volumes were between 0.4%–1.0% for intra-run variations and 0.5%–1.2% for inter-run variations. The aliquoting structure is compatible to common assay reagents with a wide range of liquid and material properties, demonstrated here for contact angles between 20° and 60°, densities between 789 and 1855 kg m^{-3} and viscosities between 0.89 and 4.1 mPa s. The centrifugo-pneumatic multi-liquid aliquoting is implemented as a passive fluidic structure into a single fluidic layer. Fabrication is compatible to scalable fabrication technologies such as injection molding or thermoforming and does not require any additional fabrication steps such as hydrophilic or hydrophobic coatings or integration of active valves.

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Introduction

Centrifugal microfluidics enables automation, miniaturization, and parallelization of laboratory workflows.[1–3] In order to reach reproducible performance in such assays, accurate metering of multiple liquid sub-volumes – so-called aliquoting – is essential. Aliquoting principles can be categorized in one-stage aliquoting and two-stage aliquoting. In one-stage aliquoting, the volume is defined by completely filling the read-out chamber. Excess liquid flows *via* an overflow to a waste region. The assay is then performed directly in the read-out chamber. One-stage aliquoting has been used for diverse areas such as digital PCR[4] or chemical analysis.[5] However, it is limited to processing of one liquid, only. If further processing of the metered liquid outside of the metering chamber, *e.g.* mixing with a second liquid is required, one-stage aliquoting is not suitable.

In two-stage aliquoting, the metering chamber is combined with a valve for transport of the liquid after metering. Besides ensuring a full separation of liquids, *e.g.* for geometric multiplex PCR,[6] two-stage aliquoting allows further liquid processing after metering. Typical valves for two-stage aliquoting are hydrophobic valves[7–9] or geometric valves.[10–12] Both types of valves depend strongly on the contact angle and surface tension of the metered liquid. Highly wetting liquids cannot be handled with these types of valves. Furthermore, geometric valves are additionally based on sharp edges, which require complex tooling for injection molding. For hydrophobic valves, localized surface modifications are introduced by an additional surface coating during

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manufacturing of the cartridge. Centrifugo-pneumatic aliquoting[13,14] is based on pneumatic counter-pressure from an enclosed air volume in the collection chamber. Since the counter pressure from the trapped air is independent of liquid properties, the centrifugo-pneumatic aliquoting can be used for liquids with a wide range of contact angles. Strohmeier *et al.* aliquoted two liquids into shared collection chambers by sequential processing of the two liquids within a centrifugo-pneumatic aliquoting structure. However, such sequential aliquoting is limited to the same aliquoted volumes and aliquoting pattern. Moreover, the second liquid comes into contact with residue from the first liquid, which can lead to undesired premature reactions outside of the collection chambers.[15]

For assays where liquids of different volumes need to be combined in one reaction, the centrifugo-pneumatic valves can be combined with dissolvable films.[16–18] Dissolvable films require a multi-layer cartridge by design and need additional fabrication steps for integration of the dissolvable films during fabrication. Aliquoting based on laser actuated ferro-wax valves[19,20] is also not limited with respect to contact angles or surface tensions. However, additional process steps are required for integration of the wax valves and the respective processing device needs to include a laser for melting of the ferro-wax valves.

So far, no aliquoting principle is available for aliquoting of multiple liquids in a single fluidic layer, which guarantees liquid separation till the collection chamber. A likely reason no such aliquoting principle is available is the traditional radially outwards transport of liquids in combination with fundamental geometric limitations of crossing channels (see ESI† Fig. S1). With new unit operations for centripedal pumping it now becomes possible to overcome such geometric limitations.[21–26] In this manuscript we introduce the centrifugo-pneumatic multi-liquid aliquoting. This aliquoting principle allows aliquoting in both the radially outwards and the radially inwards direction. The aliquoting overcomes previous limitations and allows for the first time a pairwise combination of aliquots with full fluidic separation before combination. It is based on pneumatic pumping,[27–30] which has recently been extended to centrifugo-dynamic inward pumping.[26] The fluidic principle relies only on pneumatic pressure and viscous dissipation and thus allows for aliquoting that is largely independent of surface tensions and contact angles. Since it does not require hydrophobic patches, capillary siphons or sharp edges, it can be easily fabricated with standard fabrication technologies, such as injection molding and micro-thermoforming.

The centrifugo-pneumatic multi-liquid aliquoting can be positioned on a wide range of radial positions. Especially noteworthy is that the aliquoting principle enables aliquoting from an outer array of metering chambers to an inner array of collection chambers using centrifugo-dynamic inward pumping.[20] The aliquoting of multiple liquids on a single structured side significantly reduces the complexity of fabrication in comparison to state-of-the-art aliquoting. Furthermore, the aliquoting allows for simultaneous combination of liquids in an array of collection chambers, which is especially useful for reactions with fast reaction kinetics. These distinct advantages are useful for many applications, *e.g.* nucleic acid analysis of multiple samples with a shared master mix or multiple colorimetric assays with a shared sample material. We demonstrate the combination of liquid aliquots in joint reservoirs between an inner and an outer aliquoting structure within only one single fluidic layer. The generation of 5 μl aliquots for two implementations of the aliquoting structure is characterized in detail. We investigate the system's robustness by evaluating the influence of over- and underfilling of the disk and the dependency on liquid properties using three significantly different liquids. Furthermore, we give design rules that can be used for the layout of the centrifugo-pneumatic multi-liquid aliquoting.

Aliquoting principle

The goal of the centrifugo-pneumatic multi-liquid aliquoting is the combination of one shared reagent with multiple samples in an array of collection chambers. The shared reagent is metered and split in an outer aliquoting structure. The samples are metered and split in inner aliquoting structures. Pairs of sample and reagent aliquots are combined in an array of collection chambers in between the aliquoting structures. Both aliquoting structures are implemented as two-stage aliquoting, where valving is based on pneumatic pumping. A flow chart of the aliquoting principle can be seen in Fig. 1. The principle of metering for an inner and an outer aliquoting structure and its implementation in centrifugal microfluidics is depicted in Fig. 2. It consists of a metering chamber with a pneumatic chamber connected radially inwards. The metering chamber is connected to an inlet channel and *via* a transfer channel to a collection chamber. After filling of the samples into the inlets, the disk is rotated. Due to the resulting centrifugal force, liquid is transported from the inlet to the metering chambers. As soon as liquid fills the metering chambers, the gas volume is entrapped and compressed by the liquid inflow (Fig. 2A) until the pneumatic overpressure is equal to the centrifugal pressure (Fig. 2B). When the metering chamber is completely filled, excess liquid is transported to the waste located within the pneumatic chamber. The metered volume is now precisely defined by the geometry of the metering chamber and the transfer channel. In the next step, the centrifugation frequency is quickly reduced. The entrapped gas in the compression chamber expands, pushing the metered sample out of the metering chamber. Since the fluidic resistance of the inlet channel is much higher than the fluidic resistance of the transfer channel, the majority of the metered sample is pushed through the transfer channel (Fig. 2C).[26] Even if the metering chamber is completely empty, the air in the compression chamber is still compressed by the sample volume remaining in the waste. This additional air volume pushes the residual sample

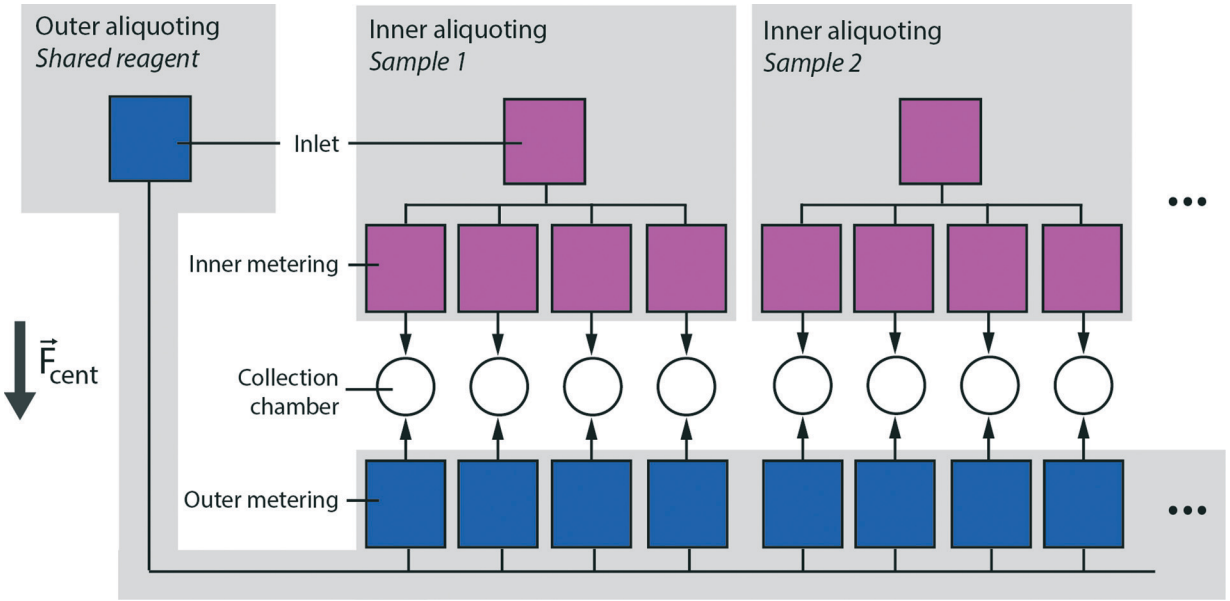

Fig. 1 The process chain of centrifugo-pneumatic multi-liquid aliquoting consists of the unit operations inner and outer aliquoting. Each aliquoting structure comprises an inlet and multiple metering chambers connected to the inlet. Each aliquot from the outer aliquoting is combined with a complimentary aliquot from the inner aliquoting within a collection chamber. The principle of the metering structures to be implemented in centrifugal microfluidics is depicted in Fig. 2.

material from the transfer channel into the collection chamber, enabling a transfer efficiency of close to 100% for the metered volume. After transfer, the metered sample is available in the collection chamber.

An aliquoting structure can be realized by connecting one inlet with multiple of the described metering structures (see Fig. 3). In order to meter and transfer two liquids in a shared collection chamber, the aliquoting principle can be implemented as an inner and an outer aliquoting structure. For the outer aliquoting structure, the transfer channel connects the metering chamber to a collection chamber positioned radially inwards. For the inner aliquoting, this transfer channel is implemented as a siphon, connecting the metering chamber with the collection chamber positioned radially outwards. The implementation, as qualified in this manuscript, is shown in Fig. 3.

Fluidic design rules

The key design parameters of the centrifugo-pneumatic multi-liquid aliquoting are the volume of the metering chamber, the volume of the pneumatic chamber and the volumes and fluidic resistances of the inlet channel and transfer channel. The volume of the metering chamber and the geometries of the transfer and inlet channel define the aliquoted volume. The size of the pneumatic chamber defines the pressures during the metering and transfer phase. A figure illustrating the individual volumes can be found in the ESI† Fig. S2. The volume aliquoted into the collection chamber V_{ali} can be calculated by:

$$V_{ali} = V_{meter} + V_{out} - V_{bf} \quad (1)$$

where V_{meter} is the volume of the metering chamber, V_{out} is the liquid volume in the transfer channel during the metering phase and V_{bf} is the volume backflow towards the inlet during the transfer phase. For transfer at no rotation, the volume backflow can be calculated *via* the Hagen–Poiseuille law:

$$V_{bf} = V_{meter}\frac{R_{in}}{R_{out}} \quad (2)$$

R_{in} and R_{out} are the fluidic resistances of the inlet channel and transfer channel respectively. In most cases it is beneficial to minimize backflow to the inlet. Therefore, the fluidic resistance of the inlet channel R_{in} should be much higher than the fluidic resistance R_{out} of the transfer channel. For the layout of the outer aliquoting presented in this manuscript $R_{in} > 100\ R_{out}$, so more than 99% of the volume is transferred to the collection chamber.

Another critical parameter for centrifugo-pneumatic multi-liquid aliquoting is the size of the pneumatic chamber. In order to prevent any liquid transfer to the collection chamber during the metering step, the pneumatic overpressure in the pneumatic chamber during metering phase is balanced by the applied centrifugal pressure. The pneumatic overpressure in the pneumatic chamber during metering is:

$$p_{pneu} = p_0\frac{V_{meter} + V_{pneu}}{V_{meter} + V_{pneu} - \left(V_{input} - V_{in} - V_{out}\right)} - p_0 \quad (3)$$

where V_{pneu} is the volume of the pneumatic chamber and V_{input} is the liquid volume pipetted by the user into the

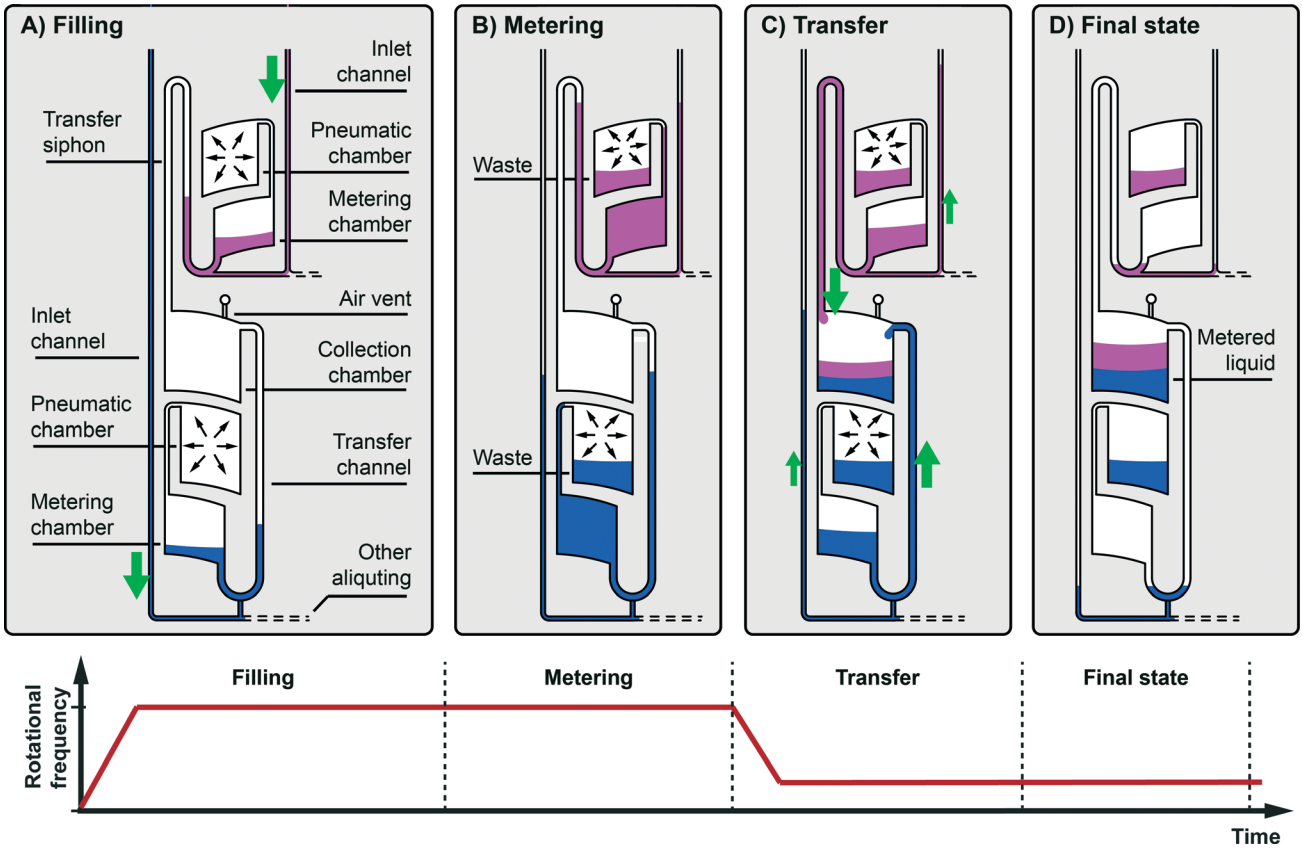

Fig. 2 Illustration of the metering principle. (A) The metering chambers are filled at high rotational frequency. The incoming liquid entraps the gas volume in the metering chamber and the pneumatic chamber. (B) When the metering chamber is completely filled, excess liquid flows into the pneumatic chamber and is trapped in a waste region. The liquid flow stops when equilibrium between centrifugal pressure and pneumatic pressure is reached. (C) After metering is completed, the rotational frequency is quickly decreased. The pneumatic pressure pushes the metered liquid out of the metering chamber, through the transfer channel and the inlet channel. Due to the much higher fluidic resistance of the inlet channel, most of the metered liquid is transported through the transfer channel into the collection chamber. (D) After the metering chambers are emptied, the transfer is completed and the metered liquid is available in the collection chamber.

inlet per aliquot. The liquid volume remaining in the inlet channel is V_{in} and p_0 is the ambient pressure. The maximal centrifugal pressure before overflow into the collection chamber is given by:

$$p_{cent} = 2\pi^2 \rho f_{max}{}^2 \left(r_{of}{}^2 - r_{out}{}^2\right) \quad (4)$$

where f_{max} is the maximal rotational frequency supported by the processing device, r_{of} is the position of the meniscus at the connection of the metering chamber to the pneumatic chamber and the r_{out} is the radial most inwards position of the transfer channel. The density of the liquid to be aliquoted is ρ. In a steady state, the pneumatic pressure of eqn (3) is equal to the centrifugal pressure of eqn (4). From this equation, the minimum volume of the pneumatic chamber with no liquid overflow to the collection chamber can be derived:

$$V_{pneu} = V_{input} - V_{in} - V_{out} - V_{meter} + \frac{p_0(V_{input} - V_{in} - V_{out})}{p_{cent}} \quad (5)$$

As shown previously, to ensure high pump efficiencies, the upper boundary for the combined size of the pneumatic chamber V_{pneu} and the metering chamber V_{meter} is four times the volume of the input liquid V_{input}.[26]

Materials & methods

The microfluidic disk was designed using SolidWorks (Dassault Systèmes SOLIDWORKS Corp.). Fabrication of 7 prototype disks was done by the Hahn-Schickard Lab-on-a-Chip Design and Foundry Service (www.hahn-schickard.de/fertigung/lab-on-a-chip-design-foundry-service/). The design was milled in a PMMA substrate (Plexiglas, Evonik, Germany) using a micro-precision mill (KERN Evo, KERN Microtechnik GmbH). From the PMMA master a PDMS-replicate was cast. This PDMS-replicate was then used for hot-embossing of the microfluidic disks in polystyrene by a custom built hot press (WICKERT Maschinenbau GmbH). The polystyrene disks were sealed *via* lamination using a pressure sensitive adhesive foil (# 900320, HJ Bioanalytik, Germany). Five of the disks were used twice. Between experiments the sealing was removed, the disks were cleaned with isopropanol, dryed with compressed air and resealed. Contact angles were measured using a contact angle meter (OCA 15+, DataPhysics Instruments GmbH). For the

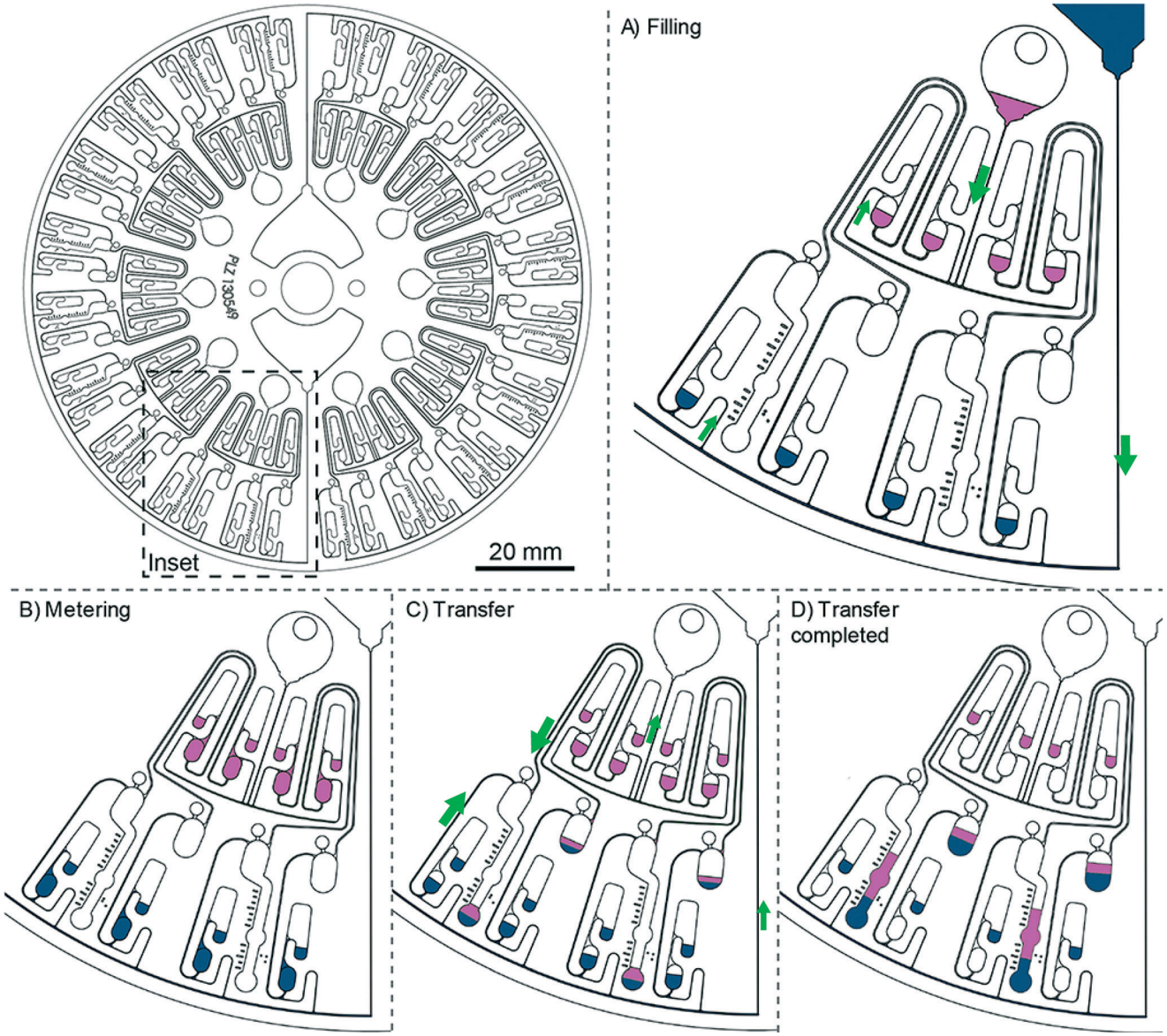

Fig. 3 Microfluidic disk with 10 inner aliquoting structures and two outer aliquoting structures. The microfluidic disk (upper left) includes two outer aliquoting structures with twenty aliquots each and ten inner aliquoting structures with four aliquots each. The fluidic operation of the inner and outer aliquoting is described step by step for the marked inset. Labels A)–D) describe the fluidic state of the inner and outer aliquoting as detailed in the caption of Fig. 2. Green arrows indicate liquid flow at selected locations. A detailed description of the implemented channels and chambers is given in ESI† Fig. S3. An experimental run of the implemented design using colored dye can be seen in Fig. 4.

experiments with aliquoted oil, Fluorinert Electronic Liquid FC-40 was used (3M Company).

Disks were processed in a prototype LabDisk Player (Qiagen Lake Constance GmbH, Germany), which was modified to include a stroboscopic setup[31] for monitoring of the microfluidic disk under rotation (BioFluidix GmbH). All reported aliquots were quantified using a custom programmed Matlab (MathWorks GmbH) based image recognition algorithm. To use this algorithm, every second collection chamber was equipped with an additional chamber designed for precise quantification of the aliquoted liquid volume (see Fig. 5). By detecting the two circular structures and the liquid meniscus the program quantifies volumes with a precision of approximately ±20 nl. A similar concept was recently reported in detail by Kazarine *et al.*[32]

Experiments

The implementation of the aliquoting described in this manuscript is depicted in Fig. 3. The liquid properties of the sample liquids chosen for characterization of aliquoting can be seen in Table 1. The target aliquoting volume is 5 μl. Each inlet of the inner aliquoting structure is filled with 25 μl for generation of 4 aliquots. The outer aliquoting is filled with 146 μl for generation of 20 aliquots. After filling, the disk is rotated at 90 Hz with an acceleration of 5 Hz s^{-1}. At this high

Table 1 Liquid properties of the aliquoted liquids

Liquid	Viscosity at 25 °C in mPa s	Density in kg m^{-3}	Advancing contact angle	Receding contact angle
Di water (0.1% Tween 20 v/v)	0.89	1000	62.5° ± 4.8°	12.0° ± 3.2°
Ethanol	1.1	789	42.7° ± 7.1°	7.1° ± 1.5°
Fluorinert FC-40	4.1	1855	19.0° ± 1.0°	0°

rotational frequency, the sample liquid is transported to the array of metering chambers. After 30 s, the inlet has run empty and the sample is distributed in the metering chambers and the respective waste regions. The rotational frequency is then reduced to 15 Hz (in case of 0.1% Tween 20) for 1 s with 15 Hz s^{-1} or to 10 Hz (in case of ethanol and FC-40) for 10 s with 15 Hz s^{-1}. During this low centrifugation step, the metered liquids are pumped to the collection chambers. For the liquids with higher viscosities, the centrifugation speed was decreased to increase the pump rate. Additionally, the time at low centrifugation was increased to allow for a complete transport of the metered liquid. Detailed frequency profiles for both low and high viscosity liquids can be found in ESI† Fig. S4. After transfer of the metered liquid, the rotational frequency is increased to 90 Hz for 10 s to remove liquid plugs from all transfer channels. The metered liquid volume in the collection chambers is now available for further processing. For read-out of the aliquoted volumes, we chose a rotational frequency of 50 Hz. This ensures a flat meniscus for a precise measurement of volume in the quantification structure (Fig. 5). We quantified the performance of inner and outer aliquoting in 7 prototype disks. For each disk, half of the disk was tested for inner aliquoting and the other half of the disk was tested for outer aliquoting. Photographs from an experiment with dyed sample are shown in Fig. 4.

Results & discussion

According to ISO 8655-5:2002, the maximum permissible random error for a calibrated pipette at 5 μl is 1.5%. The maximum permissible systematic error is 2.5%. To be useful for automation of laboratory assays, the variations of the centrifugo-pneumatic multi-liquid aliquoting should be within these boundaries. To quantify how robust the centrifugo-pneumatic multi-liquid aliquoting works under varying conditions, we tested both, the inner and outer aliquoting for different liquids. The inner and outer aliquoting were tested for viscosities between 0.89–4.1 mPa s, advancing contact angles between 20–60° and densities between 789–1855 kg m^{-3} (see Table 1).

We quantify variations as overall variations, intra-run variations and inter-run variations. The overall variation is the coefficient of variation of all measurements of either the inner or outer aliquoting for a given liquid and input volume. For the quantification of variations between different runs it is useful to look at the inter-run CV, which is the coefficient of variation between the mean of individual runs. Variations within individual runs can be quantified by intra-run CVs, which is the coefficient of variation within one individual run. All underlying formulas are given in the ESI.† We define one run as the generation of 20 aliquots. Thus one run for outer aliquoting consists of 20 aliquots from one outer aliquoting structure. One run for inner aliquoting consists of 20 aliquots from 5 inner aliquoting structures, corresponding to one outer aliquoting structure.

The overall variation for inner aliquoting (outer aliquoting) was found to be between 0.5% and 1.1% (0.9% and 1.4%) for the tested liquids. The mean of the aliquoted volumes was increased by 1.2% to 2.5% (1.5% to 2.0%) from the targeted 5 μl for the inner aliquoting (outer aliquoting). This means both the inner aliquoting and the outer aliquoting structure satisfy the ISO 8655-5:2002 standard for pipettes in the same volume range for all tested liquids. Detailed results for all conditions, including the intra-run and inter-run variations, can be found in Table 2.

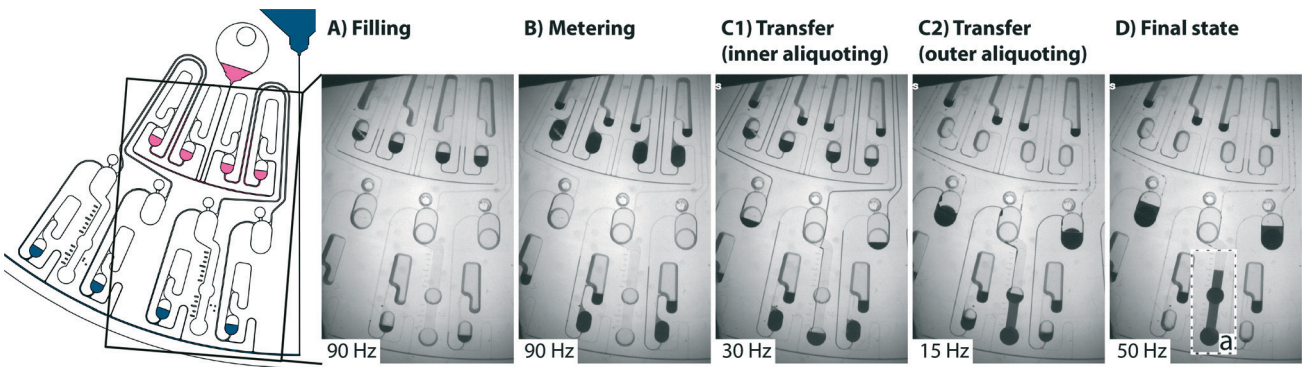

Fig. 4 Experiment showing principle of centrifugo-pneumatic multi-liquid aliquoting with stained sample material. Labels A)–D) describe the fluidic state of the inner and outer aliquoting as detailed in the caption of Fig. 2. Every second collection chamber is combined with a quantification structure (a) for measurement of aliquoted volumes. The quantification structure is described in detail in Fig. 5.

Table 2 Aliquoting variations for the centrifugo-pneumatic multi-liquid aliquoting. The aliquoting is categorized into inner and outer aliquoting for three tested liquids. The overall CV is defined as the standard deviation of all aliquot volumes with either the inner or outer aliquoting for a given liquid. The inter-run variation is the variation between the mean of runs. The intra-run variation is the coefficient of variation for an individual run.[34] The given intra-run CVs are the mean of the individual intra-run CVs for a given set of runs. One run is the generation of 20 aliquots, 10 of which are quantified *via* the attached quantification structures. Thus one run for outer aliquoting consists of aliquots from one outer aliquoting structure. One run for inner aliquoting consists of aliquots from 5 inner aliquoting structures

		Input volume	Mean volume in μl	Systematic error	Overall CV	Intra-run CV	Inter-run CV
Inner aliquoting	0.1% Tween 20	Default	5.08	1.6%	0.6%	0.6%	1.1%
		+10%	5.09	1.9%	0.5%	0.5%	—
		−10%	5.06	1.1%	0.3%	0.3%	—
	Ethanol	Default	5.06	1.2%	1.1%	1.0%	1.2%
	FC-40	Default	5.13	2.5%	0.5%	0.4%	0.7%
Outer aliquoting	0.1% Tween 20	Default	5.07	1.5%	1.4%	0.9%	1.1%
		+10%	5.09	1.8%	0.6%	0.6%	—
		−10%	5.11	2.1%	0.5%	0.5%	—
	Ethanol	Default	5.08	1.6%	0.9%	0.6%	0.9%
	FC-40	Default	5.10	2.0%	1.0%	0.8%	0.5%

Since a user can never fill the inlet with 100% accuracy, an aliquoting structure needs to tolerate varying input volumes. The maximum permissible variation for a calibrated pipette is 1% of systematic deviation and 0.5% for random variations in the range of 20–200 μl (ISO 8655-5:2002). In order for a microfluidic aliquoting structure to be robust, it should also tolerate additional variations for more complex liquid samples or mishandling by the operator. As a worst case scenario, we tested the aliquoting structures for variation of input volumes by ±10%. The mean aliquoted volume did not change within errors for the inner aliquoting 5.09 ± 0.03 μl (underfilling) and 5.06 ± 0.01 μl (overfilling) and for outer aliquoting 5.09 ± 0.03 μl (underfilling) and 5.11 ± 0.02 μl (overfilling). Furthermore, the coefficient of variation for aliquoted volumes did not increase for over & underfilling (see Table 2). Thus, the aliquoting principle tolerates variation of input volumes of at least ±10%. We determined the maximum permissible variation in input volume *via* a network simulation based approach.[26,33] For inner aliquoting the maximum permissible variation is 20% for underfilling and 40% for overfilling. For outer aliquoting the maximum permissible variation is 25% for both underfilling and overfilling. In the presented design the maximum volume is limited by the size of the waste reservoirs. For inner aliquoting the minimum volume is limited by the 5 μl target volume. For outer aliquoting some liquid volume is required in the waste chamber in order to push all liquid out of the transfer channel during aliquoting. The outer aliquoting fails if the input volume is less than 5.5 μl per aliquot.

A total of 238 aliquots were tested in 24 runs. Out of 238 aliquots, 237 aliquoted volumes were found to be between 4.75–5.25 μl. One aliquot failed (Fig. 6, run no. 14) because of particles associated with the prototyping environment that blocked one channel. One inner aliquoting structure was excluded in an optical quality control prior to experiments. The corresponding run (Fig. 5, run no. 9) shows results of the 8 remaining aliquots. Fabrication with high precision steel tools, automated sealing and clean room conditions is expected to further improve performance of the centrifugo-pneumatic multi-liquid aliquoting (as observed by Mark *et al.*[14] when comparing aliquoting CVs for prototyping and injection molding).

Summary & conclusion

We introduced the centrifugo-pneumatic multi-liquid aliquoting, a new process chain for metering and aliquoting of multiple liquids in centrifugal microfluidics. The aliquoting principle is solely based on pneumatic pressures, centrifugal pressures and viscous dissipation. We implemented the aliquoting in a new configuration, where one aliquoting structure is positioned on an outer disk radius and a second aliquoting structure is positioned on an inner

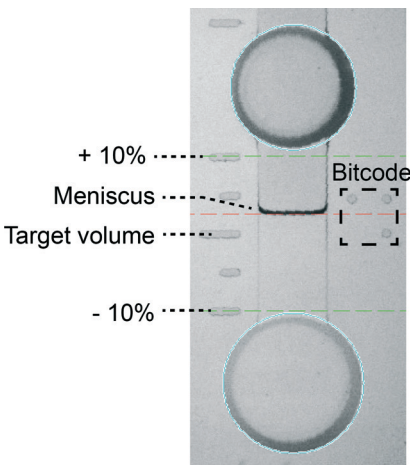

Fig. 5 Quantification structure for metering of individual aliquots. The structure consists of an upper and lower circular chamber, connected by a channel. The lower chamber has a volume of 4.5 μl and the channel has a total volume of 1 μl. For a filling volume of 5 μl the meniscus is positioned at the center between the circular chambers. The circular chambers and the position of the meniscus (red line) are detected by an automated Matlab algorithm. The aliquoted volume can then be calculated from the meniscus position within the channel with a precision of 20 nl. Each aliquot is identified *via* an individual bitcode number.

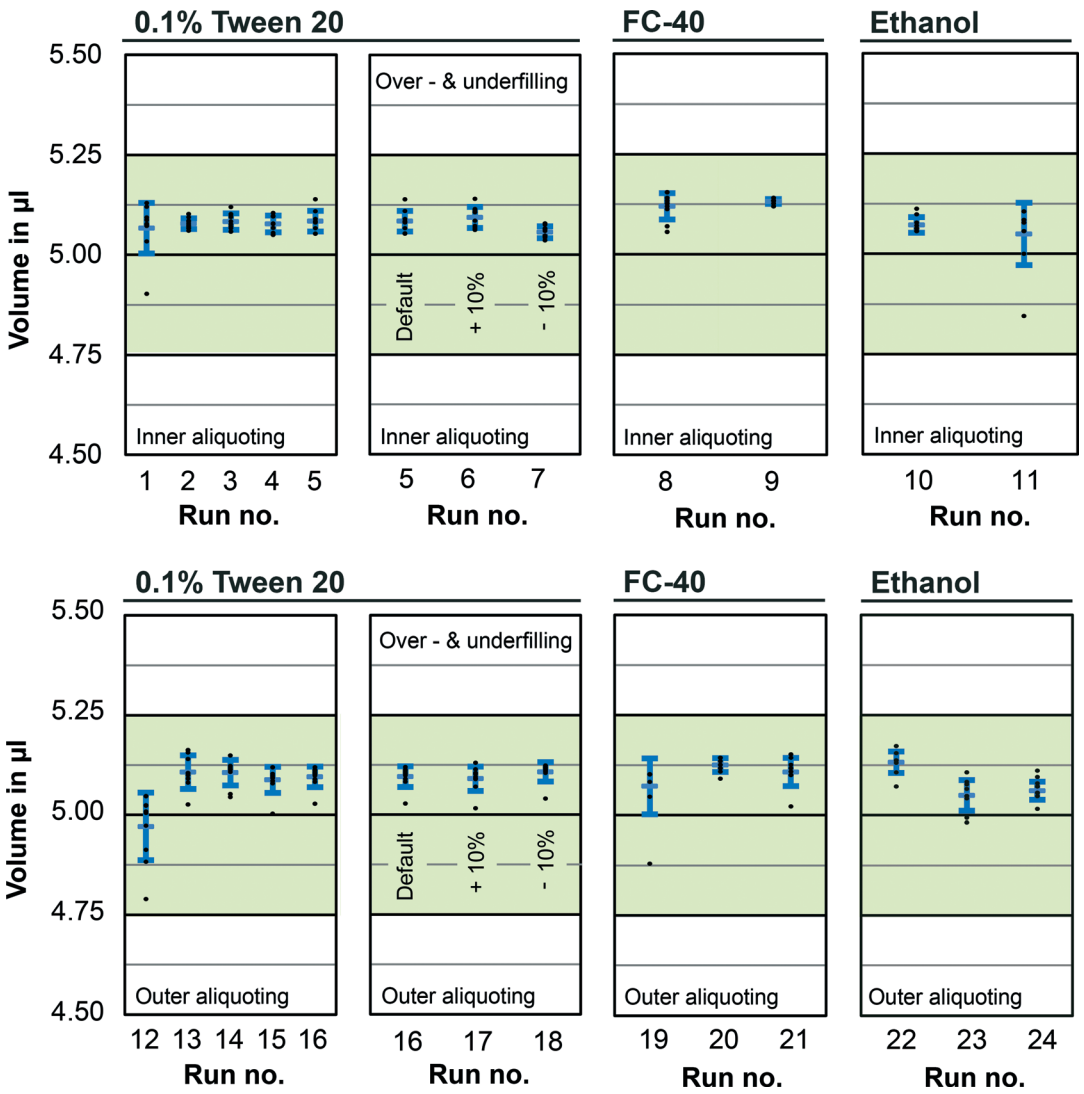

Fig. 6 Performance of centrifugo-pneumatic multi-liquid aliquoting for the inner and outer aliquoting structures with three different liquids. One run corresponds to 20 aliquots. Overall variation, inter-run variation and intra-run variation are given in Table 2. Inner aliquoting (upper row) and outer aliquoting (lower row) was performed for three different liquids (see Table 1) of varying contact angles, densities and viscosities. Overfilling by +10% volume and underfilling by −10% volume confirms robustness of the aliquoting against different input volumes. Error bars represent one standard deviation. Individual aliquots are represented by black dots. One aliquot of run no. 14 (Tween 20, outer aliquoting) was excluded from evaluation. The filling channel of this aliquot was completely clogged due to an error during fabrication.

disk radius. 20 aliquots from one outer aliquoting structure are combined with 20 aliquots from five inner aliquoting structures in an array of collection chambers in-between the aliquoting structures.

The centrifugo-pneumatic multi-liquid aliquoting is compatible to standard fabrication technologies and does not require sharp corners or surface modifications. Furthermore, we showed that the aliquoting principle is compatible to a variety of liquids and robust against changes in density and viscosity by more than a factor of two and four, respectively. The aliquoting was demonstrated for advancing contact angles between 20–60°. Such highly wetting liquids are very challenging to aliquot and cannot be processed with most of the available aliquoting principles. Lastly, the presented structures tolerate variations of input volumes by at least ±10%. For all tested liquids and volumes, the aliquoting meets the requirements in both accuracy and precision for calibrated pipettes in the same volume range as defined by ISO 8655-5:2002.

In the future, the aliquoting principle can be easily combined with microfluidic timers for timed sequential addition of metered reagents.[33] Reagent pre-storage can be included by use of miniature-stick-packs,[35] which can be introduced directly in the inlets to further reduce the number of required pipetting steps. Together with centrifugal step-emulsification the aliquoting could be used to combine multiple RPA master-mixes with a shared Mg^{2+} solution, prior to droplet generation for digital droplet RPA.[36] We expect the new aliquoting to be useful for automation of applications, where one sample needs to be combined with multiple liquid

reagents, or one liquid reagent needs to be combined with multiple samples. Possible applications range from PCR reactions of multiple samples and a shared master-mix, to multiparameter analysis of a single sample with different liquid reagents.

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