Advanced Centrifugal Microfluidics: Timing, Aliquoting and Volume Reduction

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Abstract

Diagnostics and analytics can be fundamentally improved by automation, miniaturization and parallelization. A tool to enable automation, miniaturization and parallelization highly efficiently in compact and easy to use instruments is centrifugal microfluidics. With recent advances the field of centrifugal microfluidics has become more attractive than ever for the integration of complex laboratory workflows. This thesis introduces advanced centrifugal microfluidic unit operations and process chains for fluidic automation in three areas of centrifugal microfluidics:

- **Volume reduction**, allowing handling of volumes down to 40 nl within a new process chain for aliquoting and combination of three liquids in 20 different target ratios. The process chain is applied for preparation of protein samples in small-angle X-ray scattering (SAXS).
- Parallel **multi-liquid aliquoting**, which allows the parallel aliquoting of two liquids and subsequent pairwise combination of these aliquots in a single fluidic layer, and
- a new unit operation for **timing** of pumping and valving events, largely independent of the rotational frequency protocol.

The ability to handle nanoliter volumes is one of the key selling points of microfluidics as a field. However, most centrifugal microfluidic cartridges handle liquids in the microliter range. **Reduction of volumes** has the potential to both reduce the use of expensive sample material and increase the number of tests per cartridge. In **chapter 4**, this thesis presents a centrifugal microfluidic LabDisk for protein structure analysis via small-angle X-ray scattering on synchrotron beamlines. One LabDisk prepares 120 different measurement conditions, grouped into six dilution matrices. Each dilution matrix: (1) featuring the automatic generation of 20 different measurement conditions from three input liquids and (2) requiring only 2.5 µl of protein solution per dilution matrix, which corresponds to a tenfold reduction in sample volume in comparison to the state of the art. The total hands-on time for preparation of 120 different measurement conditions is less than 5 min. Read-out is performed on disk within the synchrotron beamline P12 at EMBL Hamburg (PETRA III, DESY). This work (1) demonstrates aliquoting of 40 nl aliquots for five different liquids typically used in SAXS and (2) confirms the fluidic performance of aliquoting, merging, mixing and read-out from SAXS experiments (2.7–4.4% CV of protein concentration). The LabDisk for SAXS is applied for basic analysis methods, such as the measurement of the radius of gyration, and
advanced analysis methods, such as the *ab initio* calculation of 3D models. Such experiments can be performed by a non-expert, since the only manual handling step for sample preparation remains the filling of the LabDisk for SAXS with regular pipettes. The new platform has the potential to introduce routine high-throughput SAXS screening of protein structures with minimal input volumes to the regular operation of synchrotron beamlines.

The second innovation presented focuses on the precise and highly reproducible metering and aliquoting of liquids, which is a pre-requisite for many analytical applications, e.g. the previously discussed analysis of protein structures. So far no solution has existed for assays that require simultaneous **aliquoting of multiple liquids** and the subsequent combination of aliquots in a single fluidic layer. In **chapter 5**, this work introduces 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, the unit operation is demonstrated for the simultaneous aliquoting of (1) a common assay reagent into 20x 5 μl aliquots and (2) five different sample liquids, each into four 5 μl aliquots. Subsequently, the reagent and sample aliquots are simultaneously transported and combined into 20 collection chambers. All coefficients of variation for metered volumes were between 0.4–1.0% for intra-run variation and 0.5–1.2% for inter-run variation. The aliquoting structure is compatible with 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.

The last innovation introduced focuses on the precise, and in large part rotational frequency protocol independent, **timing** of microfluidic operations. Timing of microfluidic operations is essential for the automation of complex laboratory workflows, in particular for the timed supply of samples and reagents. In **chapter 6**, this thesis presents a new unit operation for timed valving and pumping in centrifugal microfluidics. It is based on the temporary storage of pneumatic energy and the time-delayed sudden release of said energy. The timer is loaded at a relatively higher spinning frequency. The countdown is started by reducing to a relatively lower release frequency, at which the timer is released after a pre-defined delay time. Timing is demonstrated for (1) the sequential release of 4 liquids at times of (2.7 ± 0.2) s, (14.0 ± 0.5) s, (43.4 ± 1.0) s and (133.8 ± 2.3) s, (2) timed valving of typical assay reagents (contact angles 36–78°, viscosities 0.9–5.6 mPa·s) and (3) on-demand
valving of liquids from 4 inlet chambers in any user-defined sequence controlled by the spinning protocol. The microfluidic timer is compatible with all wetting properties and viscosities of common assay reagents and does neither require external means nor coatings.

In summary, three new centrifugal microfluidic operations are presented for the integration of complex laboratory workflows. The fluidic operations introduced aim to benefit the development of test carriers that are user-friendly, offer robust fluidics, can be fabricated using scalable fabrication technologies and do not require surface coatings or external means.
Zusammenfassung

Diagnostik und Analytik können durch erhöhte Automatisierung, Miniaturisierung und Parallelisierung fundamental verbessert werden. Die zentrifugale Mikrofluidik ist ein Werkzeug, welches erhöhte Automatisierung, Miniaturisierung und Parallelisierung mit Hilfe von einfach bedienbaren und kompakten Prozessiergeräten erlaubt. Jüngste Fortschritte haben die zentrifugale Mikrofluidik attraktiver denn je für die Automatisierung komplexer Laborabläufe gemacht. Die vorliegende Dissertation erweitert die zentrifugale Mikrofluidik um Einheitsoperationen und Prozessketten in drei Bereichen:


- **Paralleles Aliquotieren mehrerer Flüssigkeiten**, die neue Einheitsoperation erlaubt das parallele Aliquotieren von zwei Flüssigkeiten und die anschließende paarweise Kombination der Aliquots innerhalb einer einzigen fluidischen Lage, und

- eine neue Einheitsoperation zum **zeitgesteuerten Schalten und Pumpen**, wobei die zeitliche Steuerung großteils unabhängig von der Rotationsfrequenz der Kartusche erfolgt.


Die zuletzt präsentierte Innovation beschreibt die präzise und großteils frequenunabhängige **zeitliche Steuerung** von mikrofluidischen Operationen. Dies ist
essentiell für komplexe Laborabläufe, insbesondere für die sequentielle und zeitgenaue Zuführung von Reagenzien und Proben. In Kapitel 6 dieser Arbeit wird eine neue zentrifugalmikrofluidische Einheitsoperation zum zeitgesteuerten Pumpen und Schalten präsentiert. Die neue Operation basiert auf einer zeitlichen Speicherung von pneumatischer Energie und deren zeitverzögerter, plötzlicher Freisetzung. Der „Timer“ wird bei einer relativ höheren Drehfrequenz geladen. Durch die Reduktion der Drehfrequenz auf eine relativ niedrigere Drehfrequenz wird ein Countdown gestartet, bei dem der „Timer“ nach einer vordefinierten Zeit die gespeicherte Energie freisetzt. Die zeitliche Steuerung wird demonstriert für (1) die sequentielle Freisetzung von vier Flüssigkeiten bei Zeiten von (2.7 ± 0.2) s, (14.0 ± 0.5) s, (43.4 ± 1.0) s and (133.8 ± 2.3) s, (2) zeitgesteuertes Schalten von typischen Assayreagenzien (Kontaktdruck 36–78°, Viskositäten 0.9–5.6 mPa·s) und (3) „on-demand“ Schalten von Flüssigkeiten aus vier Einlasskammern in jeglicher nutzerdefinierten Reihenfolge, lediglich gesteuert durch das Frequenzprotokoll. Der „Timer“ ist kompatibel mit allen Benetzungseigenschaften und Viskositäten typischer Assayreagenzien und benötigt weder externe Aktuierungsmethoden noch Oberflächenbeschichtungen.

Zusammenfassend werden drei neue zentrifugalmikrofluidische Operationen präsentiert, welche der Integration komplexer Laborabläufe dienen. Die vorgestellten Operationen zielen darauf ab, die Entwicklung von mikrofluidischen Kartuschen mit nutzerfreundlichen und robusten Fluidiken zu unterstützen. Ein besonderes Augenmerk liegt darauf, dass die neuen Operationen kompatibel mit skalierbaren Fertigungsmethoden sind und weder Oberflächenbeschichtungen noch externe Aktuierungsmethoden benötigen.
List of Publications

First author publications in peer reviewed journals


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Publications at National and International Conferences


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## 7.2 Parallel aliquoting of multiple liquids

## 7.3 Timing of operations

## Glossary

## Abbreviations

## Physical measures

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1 Introduction

This chapter discusses the relevance and advantages of centrifugal microfluidics. The motivation for the work is illustrated and the main goals of the thesis are provided. Definitions of important terms are given in Table 1.1. The structure of this thesis is described at the end of this chapter.

1.1 Relevance and advantages of centrifugal-microfluidics

Automation, miniaturization and parallelization can improve the efficiency and reliability of analytics and diagnostics significantly:

- Automation allows more robust processes, eliminating the risk of human errors.
- Miniaturization reduces the consumption of samples and reagents, reducing costs and enabling more tests per sample, especially with rare and precious sample materials.
- Parallelization allows running multiple tests in parallel, increasing throughput and the information collected per sample.

A common solution for automation and parallelization in analytics are pipetting robots, but also microfluidics is often cited in this context, as it promises smaller reaction volumes and the automation of liquid handling without the bulkiness of robotic workstations [3–5]. Formerly a small niche of microfluidics, centrifugal microfluidics has become dramatically more popular within recent years [6–9]. Applications have been successfully demonstrated for many analytical and diagnostic applications, including nucleic acid analysis [10–14], immunoassays [15–18], clinical chemistry [19–22], cell analysis [23–27], food and soil analysis [28–33] and protein structure analysis [34, 35]. Major companies such as Abaxis [36], Roche [37], 3M [38], Panasonic and Samsung [39] have already commercialized centrifugal microfluidic products, and a number of smaller companies and startups are known to work on new innovative centrifugal microfluidic products (see Table 3.1 for more details). The reasons for centrifugal microfluidics being such an attractive technology for analytics and diagnostics are manifold:

In centrifugal microfluidics a single rotational motor can control complex microfluidic workflows, allowing simple and relatively cheap processing devices. For the integration of centrifugal microfluidic workflows, a large range of unit operations and process chains are
already available [9, 6]. These can be easily combined and integrated into monolithic cartridges, which can be produced using scalable, cost-efficient fabrication techniques like injection molding and thermoforming.

Other advantages of centrifugal microfluidics are related to the use of volume forces. Volume forces are long-range forces like gravity or centrifugal forces that act equally on every liquid element within a sufficiently small area. The opposite of volume forces are the short-range surface forces. Surface forces include capillary forces, cohesion forces and normal forces, e.g. the force from the piston of a syringe pump. Volume forces in centrifugal microfluidics can be adjusted by simply changing the rotational frequency to the needs of a specific process: Suspensions can be separated by sedimentation, e.g. for integrated blood plasma separation; bubbles can be removed from channels or chambers due to buoyancy, eliminating a typical problem in microfluidics. In centrifugal microfluidics a simple pipetting port can serve as the world-to-chip interface. This is possible since volume forces will act on the liquid no matter how it is situated within the inlet chamber. Similarly, simple to use and easy to fabricate world-to-chip interfaces have been an active field of research in other microfluidic platforms for more than a decade [40, 41]. Another advantage of centrifugal microfluidics is that liquid loss on channel walls due to surface forces can be minimized by simply increasing the rotational frequency, allowing centrifugal microfluidic cartridges to handle nanoliter volumes without any carrier liquid. The suitability for low input volumes becomes especially obvious when comparing this procedure to pressure-driven microfluidics, where large liquid volumes are lost in channels and tubings. Finally, centrifugal microfluidic cartridges can be implemented as closed monolithic disposables without any fluidic connections, such as tubes or pumps, reducing the risk of cross-contaminations. Furthermore, since no connections for tubes or pumps are necessary, one processing device can handle diverse cartridges with completely different fluidics, simply by changing the frequency protocol.
1.2 The need for advanced unit operations and process chains in centrifugal microfluidics

Table 1.1: Definitions of important terms as used in this thesis.

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tr>
<td>Microfluidic platform</td>
<td>According to Mark et al. [42] “a microfluidic platform comprises an easily combainable set of microfluidic unit operations that allows assay miniaturization within a consistent fabrication technology” and thereby facilitates the efficient integration of laboratory workflows and applications.</td>
</tr>
<tr>
<td>Centrifugal microfluidics</td>
<td>Centrifugal microfluidics is a sub-category of microfluidics, defined by its liquid actuation mechanism. In contrast to other microfluidic approaches, centrifugal microfluidics utilizes the rotation of a microfluidic cartridge as one important control parameter for liquid handling. Forces used for liquid manipulation unique to centrifugal microfluidics are the centrifugal force, Euler force and Coriolis force. One centrifugal-microfluidic cartridge can integrate a full laboratory workflow by integration of a sequence of microfluidic unit operations and process chains.</td>
</tr>
<tr>
<td>Microfluidic process chain</td>
<td>Strohmeier et al. [9] define process chains as “assemblies of fluidic unit operations and external means that represent laboratory workflows on a higher level of integration”, e.g. blood plasma separation or nucleic acid extraction.</td>
</tr>
<tr>
<td>Microfluidic unit operation</td>
<td>A microfluidic unit operation is an implementation of a basic fluidic functionality, such as valving, switching and metering [42]. Depending on the setting, the same functionality can be realized using different unit operations. For example depending on the application different types of valves may be appropriate.</td>
</tr>
</tbody>
</table>

1.2 The need for advanced unit operations and process chains in centrifugal microfluidics

Table 1.2 shows a comparison of different microfluidic platforms. Comparatively simple applications, like pregnancy tests, can be implemented in lateral flow tests. However, lateral flow tests fail when more complex workflows, such as precise metering, are required. On the other end of the spectrum, highly complex workflows with many parameters are best implemented using large-scale integration or segmented flow-based microfluidics. However, these platforms require expensive instruments, which are not suited for the point-of-care, and need to be operated by expert users. The strength of centrifugal microfluidics lies in the integration of complex workflows in relatively simple monolithic cartridges and processing devices that can be operated with minimal training, most often at the point of care.
In order to integrate such complex laboratory workflows, advanced centrifugal microfluidic unit operations and process chains are essential. While a large number of unit operations are readily available in centrifugal microfluidics, important drawbacks remain. This thesis aims to improve the integration of complex workflows in centrifugal microfluidics with three innovations:

1. **Volume reduction and increased integration**: The new process chain generates 20 different dilutions in the nanoliter range from three input liquids. The process chain is applied for preparation of protein dilutions for structural analysis via small-angle X-ray scattering.

2. **Multi-liquid aliquoting**: The new unit operation allows parallel aliquoting and the combination of multiple liquids for a wide range of liquid properties.

3. **Timing of microfluidic operations**: The new unit operation allows timed pumping and valving, largely independent of spinning frequencies, in passive centrifugal microfluidic cartridges.

In the following sections the specific relevance of these three innovations is explained.
Table 1.2: Comparison of different microfluidic platforms in terms of instrument, disposable and liquid handling. This table was originally published by Mark et al. [42]. The table was modified to include "low disposable costs per sample", "low reagent consumption per sample" and "world-to-chip interfacing suitable to PoC devices".

<table>
<thead>
<tr>
<th>Microfluidic platforms</th>
<th>Instrument</th>
<th>Disposable</th>
<th>Liquid handling</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Portability / wearability</td>
<td>Throughput: number of samples / assays per day</td>
<td>Low-cost instrument</td>
</tr>
<tr>
<td>Classical liquid handling (pipetting robots)</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Lateral flow test</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Linear actuated devices</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<tr>
<td>Pressure driven laminar flow</td>
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<td>+</td>
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<td>Microfluidic large scale integration</td>
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<tr>
<td>Segmented flow microfluidics</td>
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<td>Electrokinetics</td>
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<td>Electrowetting</td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Surface acoustic waves</td>
<td>+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Dedicated systems for massively parallel analysis</td>
<td>-</td>
<td>+</td>
<td>-</td>
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</table>

Note: + indicates good suitability, - indicates limited suitability, and 0 indicates no suitability.
1.3 The relevance of timing, aliquoting and volume reduction

1.3.1 Volume reduction

One motivation of microfluidics is the reduction of volumes, e.g. for precious sample material or expensive reagents. Research in microfluidics has so far mainly focused on high-throughput applications to reduce the sample volume per measured parameter. In comparison, the total sample volume is much harder to reduce. In pressure-driven microfluidics dead volumes in channels, tubings and reservoirs are required for fluidic function, but do not take part in the actual assay. Such dead volumes typically limit the minimal input volume to tens or hundreds of microliters. Since centrifugal microfluidics is based on volume forces, it does not require liquid volumes in tubings for liquid propulsion. Therefore centrifugal microfluidics is an excellent candidate for low-input volume applications. An example of such centrifugal microfluidic disks are the Gyrolab BioAffy CDs that are used for enzyme-linked immunosorbent assays (ELISA). The Gyrolab Bioaffy CDs are filled automatically by the Gyrolab workstation with down to 3.5 µl input volume for a duplex reaction of one sample [43]. Although many applications might benefit from such low input volumes, only a very limited number of applications have been presented for the volume range of tens to hundreds of nanoliters.

This thesis presents a new process chain that automatically generates a dilution matrix of 20 combinatorial dilutions from three input liquids. The liquid volumes of 2.5-3.5 µl can be pipetted directly into an inlet on the disk. The liquids are split into 40 nl aliquots, and 20 sets of six aliquots each are combined in different ratios. The process chain is presented for the application of small angle X-ray scattering, a method in structural biology that requires purified protein samples. Purified proteins are often expensive and hard to obtain in large volumes, and therefore protein structures are typically measured under a very limited set of conditions using SAXS. In comparison to state-of-the-art liquid handling in SAXS, the new process chain allows for a more than tenfold reduction in protein volumes and consequently more than ten times the experiments with the same amount of protein. The new process chain is described in chapter 4.
1.3 The relevance of timing, aliquoting and volume reduction

1.3.2 Multi-liquid aliquoting

Aliquoting is one of the most important unit operations in centrifugal microfluidics and an essential operation in many laboratory workflows, e.g. for the analysis of multiple parameters from one sample. When comparing centrifugal microfluidic platforms to the most important competing platform, linear actuated microfluidics, one major advantage of centrifugal microfluidics are the well characterized unit operations for aliquoting (see Table 1.2). This is also evident from the fact that aliquoting is a feature of almost all commercially available centrifugal microfluidic cartridges [16, 36, 37, 32].

However, current centrifugal microfluidic aliquoting principles are focused on aliquoting single liquids. If aliquoting of multiple liquids is available, then both liquids are aliquoted sequentially in the same structure and with the same aliquoting pattern [43] or the combination of liquid aliquots requires a second fluidic layer [44], which significantly increases the complexity during the fabrication of the cartridge. Many laboratory workflows depend on the metering and combination of multiple liquids, e.g. clinical chemistry assays, where one sample needs to be combined with multiple liquid reagents or PCR reactions, where one master-mix is combined with multiple samples. For the integration of such laboratory workflows a new type of unit operation is required, which is dedicated to the aliquoting and subsequent pairwise combination of multiple liquids.

One of the innovations described in this thesis is the centrifugo-pneumatic multi liquid aliquoting: An aliquoting principle specifically designed for the parallel aliquoting of multiple liquids and the efficient combination of these aliquots on a single fluidic layer. Especially noteworthy is the capability of the new aliquoting principle to aliquot into radially inward collection chambers from radially outwards metering chambers. The centrifugo-pneumatic multi-liquid aliquoting is described in detail in chapter 5.
1.3.3 Timing of microfluidic operations

Typically the timing of fluidic operations in centrifugal microfluidics is performed by the change of spinning frequencies. The parallel implementation of several different, independent processes on the same cartridge was difficult so far, because all areas of the cartridge experience the same spinning frequency.

To overcome this and similar limitations, different research groups have focused on actuation independent of rotation, using so-called external means. Unit operations based on external means include magnets in the processing device [45], lasers in the processing device and corresponding valves in the disk [46], actuation via pressurized air from an external pressure source [47, 48] or heating by the processing device [49]. While external means allow for actuation independent of rotation, they increase the complexity of the processing device.

In many cases for the integration of more complex processes it is sufficient, if a valving or pumping event does not happen directly with a change in rotational frequency, but instead is delayed in time. Ducrée’s group demonstrated such a time delay via the integration of dissolvable films [50]. Through a combination of dissolvable films and air venting it was possible to trigger the valving event of a second liquid with the completion of the valving event of a first liquid [51]. The principle was later extended on by inclusion of a paper strip. By placing dissolvable films at defined distances of the paper strip, the imbibition of the paper could be used for the timed valving of reagents. This allowed for 22 successive liquid handling steps in a single cartridge [52], which is, arguably, the most complex succession of valving events presented so far in a passive centrifugal microfluidic cartridge. The disadvantage of this concept is that the dissolvable films need to be integrated within an extra processing step and microfluidic cartridges require at least two fluidic layers.

Thus, for the integration of complex laboratory workflows, there is a need for a monolithic unit operation that requires no extra fabrication steps or external means and allows for the timing of microfluidic operations. This thesis presents the microfluidic timer, a unit operation that is based on the storage of pneumatic energy and the sudden release of said energy after a defined time period. In the release-on-demand mode the microfluidic timer is the first centrifugal microfluidic unit operation with no external means that facilitates a certain degree of on-demand programmability in centrifugal microfluidic cartridges. The microfluidic timer is described in detail in chapter 6.
1.4 Aim and structure of this thesis

In summary, this thesis presents advanced centrifugal microfluidic unit operations and process chains for the integration of complex laboratory workflows. Three areas in centrifugal microfluidics were identified that are particularly relevant to the integration of more complex workflows: volume reduction, aliquoting and timing. The corresponding investigations, designs, experimental results and discussions are presented in the following chapters:

1. Introduction sets the stage for the rest of the thesis by motivating the work;
2. Fundamentals explains the underlying physical principles employed within this work;
4. Volume reduction showcases a new process chain for low volume aliquoting and combination of three liquids. The chapter starts with a short state of the art for nanoliter handling in centrifugal microfluidics. The newly designed process chain is applied to and characterized in the context of sample preparation for small angle X-ray scattering. To better understand the context of small angle X-ray scattering, a brief introduction to SAXS is given;
5. Multi-liquid aliquoting the first part of the chapter summarizes the state of the art in aliquoting. The second part of the chapter demonstrates a new unit operation for the parallel aliquoting of multiple liquids on a single fluidic layer and the performance of this aliquoting technique for a wide range of liquids;
6. The chapter The microfluidic timer describes a new unit operation that can be employed for timed valving and pumping in centrifugal microfluidic cartridges. The use of the timer is demonstrated in a sequential release and release-on-demand mode;
7. Overall conclusion & outlook summarizes the work and gives an outlook of future developments;

Finally, the chapter Glossary provides definitions for terms used in this thesis, the chapter Appendix, gives supporting information and the chapter References, lists the references for the thesis.

Parts of chapters 4, 5 and 6 are published as journal publications. As with most papers in interdisciplinary fields, these journal publications were written by multiple authors. Therefore, where applicable, the work of co-authors in these publications is listed.
2 Fundamental forces

The following chapter will briefly describe the theoretical background required to design centrifugal microfluidic unit operations and process chains. This includes pseudo forces in the rotating reference frame and other relevant forces, such as viscous and capillary forces.

2.1 Pseudo forces in a rotating reference frame

Fundamental forces and corresponding pressures in centrifugal microfluidics can be categorized into pseudo forces that act due to Newton’s second law of motion in the rotating reference frame, and non-pseudo forces that are also present in non-rotating systems. The pseudo force densities present in centrifugal microfluidics are the centrifugal force density (eqn (2.1)), the Coriolis force density (eqn (2.2)) and the Euler force density (eqn (2.3)). The pseudo forces are illustrated in Figure 2.1 [9].

\[
f_{\text{centrifugal}} = -\rho \, \omega \times (\omega \times r)
\]

\[
f_{\text{Coriolis}} = -2 \, \rho \, \omega \times \frac{d}{dt} r
\]

\[
f_{\text{Euler}} = -\rho \, \frac{d}{dt} \omega \times r
\]

Here, \( \omega \) is the angular velocity, \( r \) is the position of the liquid element from the center of rotation and \( \rho \) is the density of the liquid element.

For the design process of centrifugal microfluidic cartridges, pressures are typically more useful than the corresponding forces. To calculate the centrifugal pressure, we can integrate the scalar centrifugal force density \( f_{\text{centrifugal}} \) over a liquid plug with an outer radius of \( r_2 \) and an inner radius of \( r_1 \). In the case that the radial vector \( r \) is orthogonal to the angular velocity vector \( \omega \), the scalar centrifugal force density \( f_{\text{centrifugal}} \) is given by [53]:

\[
f_{\text{centrifugal}} = \rho \, \omega^2 r
\]
2.1 Pseudo forces in a rotating reference frame

\[
p_{\text{cent}} = \frac{1}{2} \rho \omega^2 (r_2^2 - r_1^2)
\]

The Euler pressure can be calculated via the integration of the scalar Euler force density \( f_{\text{Euler}} \) over an isoradial liquid segment of length \( l_{\text{iso}} \) [53]:

\[
f_{\text{Euler}} = \rho \dot{\omega} r
\]

\[
p_{\text{Euler}} = \int_{0}^{l_{\text{iso}}} f_{\text{Euler}} \, dl = l_{\text{iso}} \rho \dot{\omega} r
\]

Induced pressures from the Coriolis force within chambers and channels are typically small and neglected. However, the Coriolis force influences the flow profile within channels, which can be used for mixing of liquids [54–56]. The deflection of liquid flow due to the Coriolis force can be used to switch liquid between different target reservoirs [57].

Figure 2.1: Fundamental forces in centrifugal microfluidics. A liquid column is displayed in blue. This figure was adapted from ref. [9].
2.2 Other forces relevant to centrifugal microfluidics

Besides the described pseudo forces, which are specific to centrifugal microfluidics, viscous forces (eqn (2.8)) and inertial forces (eqn (2.11)) are present in any fluidic system with a moving or accelerated fluid. The viscous pressure drop can be calculated from the relation between the hydraulic resistance $R$ and the flow rate $Q$ [58]:

$$p_{\text{visc}} = R \, Q$$  \hspace{1cm} 2.8

The hydraulic resistance depends on the geometry of the channel and the viscosity of the liquid. In a square channel of length $l$ with $w > h$ and for a liquid of dynamic viscosity $\eta$ the hydraulic resistance can be calculated as follows [58]:

$$R = \frac{\alpha \, \eta \, l}{w \, h^3}$$  \hspace{1cm} 2.9

$$\alpha = 12 \left[ 1 - \frac{192 \, h}{\pi^5 \, w} \, \tanh \left( \frac{\pi \, w}{2 \, h} \right) \right]^{-1}$$  \hspace{1cm} 2.10

The dimensionless number $\alpha$ depends only on the relation between height $h$ and width $w$ of the channel [58]. This resistance is based on the Hagen-Poiseuille equation and assumes laminar flow with a parabolic flow profile. In microfluidics laminar flow and a parabolic flow profile is typically present due to the low Reynolds numbers, the relation between inertial to viscous forces. However, as discussed before, in centrifugal-microfluidics the parabolic flow profile is deformed under rotation due to the Coriolis force [56]. This leads to an effective increase of the hydraulic resistance in centrifugal microfluidics, which has not been studied in detail and is generally ignored. In first simulations the effect was found to be small at low rotational frequencies and flow rates [59]. However, more studies are needed to better understand the effect.

If liquid is accelerated, inertial pressures need to be taken into account. For a channel of length $l$ and a change of flow rate $\dot{Q}$ within the channel, the inertial pressure can be calculated from [53]:

$$p_{\text{inertial}} = - \frac{\rho \, l \, \dot{Q}}{w \, h}$$  \hspace{1cm} 2.11

Other pressures that are relevant to the design process in centrifugal microfluidics are capillary pressures (eqn (2.12)) and pneumatic pressures (eqn (2.13)). The capillary pressure
of a liquid meniscus in a square channel of width \( w \) and height \( h \) for a surface tension of \( \sigma \) can be calculated via [58]:

\[
p_{\text{cap}} = 2\sigma \cos \theta \left( \frac{1}{w} + \frac{1}{h} \right)
\]

The last pressure relevant to this thesis is the pneumatic pressure. In centrifugopneumatic unit operations air is compressed and decompressed in specially designed pneumatic chambers. The pneumatic overpressure of trapped air of volume \( V \) with an initial volume \( V_0 \) at atmospheric pressure \( p_0 \) can be calculated according to the ideal gas law:

\[
p_{\text{pneu}} = p_0 \left( \frac{V_0}{V} - 1 \right)
\]

Actuation principles that make use of other forces have been employed in centrifugal microfluidics, but are not directly relevant for this thesis. These forces include thermopneumatic actuation, magnetic actuation, electric actuation, actuation by external lasers, external air pressures, delamination of frangible seals and the use of dissolvable films.
3 State of the art in centrifugal microfluidics

This chapter gives a short overview of the relevant state of the art. The chapter starts with a short history of centrifugal microfluidics and then highlights recent trends in centrifugal microfluidics relevant to the integration of complex laboratory workflows. The chapter only discusses a small selection of recent advances in centrifugal microfluidics. For a more detailed description of such advances, the author recommends reviews on unit operations and applications in centrifugal microfluidics [23, 60, 8, 9], and microfluidic platforms in general [61, 42]. The specific state of the art of the respective research fields for volume reduction, multi-liquid aliquoting and the microfluidic timer are described in the corresponding chapters.

3.1 Short history of centrifugal microfluidics

The first papers in centrifugal microfluidics were published in the 1960s. These first developments focused on the centrifugal analyzer from N. Anderson at Oak Ridge National Labs [62]. Based on these developments, the Piccolo Xpress was introduced in the mid-1990s. The Piccolo Xpress is a blood analyzer in the field of clinical chemistry [36]. In the early 2000s Gyros and Tecan developed new applications, e.g. in the field of immunoassays, and generated a broad range of intellectual property. Many of the patents generated by these companies are still in force today [63–71]. In the last five to ten years, research in the field of centrifugal microfluidics has been more active than ever (see Figure 3.1). Global players like 3M, Roche and Samsung have entered the market with centrifugal microfluidic products, and numerous startups are aiming to introduce innovative products in the near future (see Table 3.1). While some research fields start to turn into products, many fields in analytics and diagnostics can still benefit from centrifugal microfluidic automation. It can be speculated that if some of the new products are successful, the growth of centrifugal microfluidics will continue. In the following I will discuss how recent trends in centrifugal microfluidics support the development of new products and what operations in centrifugal microfluidics can benefit from advanced unit operations and process chains.
3.1 Short history of centrifugal microfluidics

Figure 3.1: The history of centrifugal microfluidics as described in a recent review on advanced centrifugal microfluidics [9]. The annual number of publications relates to centrifugal microfluidics (source: Thomson Reuters ISI web of science; search term: "centrifug* AND (microfluid* OR analyzer* OR analyser)" in the category “topic”, accessed on March 15, 2015). Research between the 1960s and the early 1990s focused mainly on the centrifugal analyzer. The Abaxis PiccoloXPress was introduced in 1995. In the last 15 years the research field of centrifugal microfluidics has been steadily growing. Since 2009 major international companies have introduced first centrifugal microfluidic products, including 3M, Roche and Samsung.
Table 3.1: Embodiments of centrifugal microfluidic platforms that are either currently commercially available, in a precommercial phase announcing a release date in the near future, or showing promising developments. The table is an updated version from a recent review ref. [9].

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Provider (developer)</th>
<th>Identifier cartridge/name of system</th>
<th>Applications</th>
<th>Commercialization status</th>
</tr>
</thead>
<tbody>
<tr>
<td>[39]</td>
<td>Samsung</td>
<td>LABGEO IB10</td>
<td>Immunoassays</td>
<td>Commercially available</td>
</tr>
<tr>
<td>[38]</td>
<td>Focus Diagnostics (3M)</td>
<td>Universal Disc &amp; Direct Amplification Disk/Integrated Cycler</td>
<td>Nucleic acid analysis</td>
<td>Commercially available</td>
</tr>
<tr>
<td>[37]</td>
<td>Roche (Panasonic)</td>
<td>Cobas 101b</td>
<td>Blood parameter analysis (HbA1c and lipid panel)</td>
<td>Commercially available</td>
</tr>
<tr>
<td>[72]</td>
<td>Capital Bio</td>
<td>RTisochip</td>
<td>Nucleic acid analysis (respiratory tract infections)</td>
<td>Commercially available</td>
</tr>
<tr>
<td>[43]</td>
<td>Gyros AB</td>
<td>Gyrolab Bioaffy CD</td>
<td>Immunoassays</td>
<td>Commercially available</td>
</tr>
<tr>
<td>[32]</td>
<td>LaMotte</td>
<td>Water Link Spin Lab</td>
<td>Water analysis</td>
<td>Commercially available</td>
</tr>
<tr>
<td>[73]</td>
<td>Skyla</td>
<td>VB 1 Veterinary Clinical Chemistry Analyzer</td>
<td>Blood chemistry testing for veterinary applications</td>
<td>Commercially available</td>
</tr>
<tr>
<td>[74]</td>
<td>Biosurfit</td>
<td>Spinit</td>
<td>Immunoassays/blood parameter analysis</td>
<td>Commercially available</td>
</tr>
<tr>
<td>[75]</td>
<td>Radisens Diagnostics</td>
<td>Unknown</td>
<td>Immunoassay, clinical chemistry, and hematology assays</td>
<td>Precom</td>
</tr>
<tr>
<td>[76]</td>
<td>GenePOC-Diagnostics</td>
<td>Unknown</td>
<td>Nucleic acid analysis</td>
<td>Precom (planned 2016)</td>
</tr>
<tr>
<td>[77]</td>
<td>SpinChip Diagnostics</td>
<td>Unknown</td>
<td>Blood analysis</td>
<td>Development</td>
</tr>
<tr>
<td>[78]</td>
<td>Espira Inc.</td>
<td>Unknown</td>
<td>Nucleic acid analysis</td>
<td>Development</td>
</tr>
<tr>
<td>[79]</td>
<td>Hahn-Schickard</td>
<td>LabTube</td>
<td>Various applications</td>
<td>Development</td>
</tr>
<tr>
<td>[14]</td>
<td>Hahn-Schickard</td>
<td>LabDisk</td>
<td>Various applications</td>
<td>Development</td>
</tr>
<tr>
<td>[80]</td>
<td>SpinDiag</td>
<td>SpinDiag One</td>
<td>Nucleic acid analysis</td>
<td>Development</td>
</tr>
<tr>
<td>[81]</td>
<td>Sandia National Labs</td>
<td>Spin DX</td>
<td>Various applications</td>
<td>Development</td>
</tr>
<tr>
<td>[82]</td>
<td>BluSense Diagnostics</td>
<td>Blubox</td>
<td>Blood analysis</td>
<td>Development</td>
</tr>
</tbody>
</table>
3.2 Recent trends in centrifugal microfluidics

First applications in centrifugal microfluidics were limited to relatively simple operations such as radially outwards pumping by centrifugal force, siphon valves [62, 21], geometric and hydrophobic valves [83, 84] and one-stage aliquoting [85]. These unit operations formed the foundation of the centrifugal microfluidic platforms. Such unit operations were sufficient to integrate medium complexity workflows within a simple device, like the clinical chemistry tests within the Piccolo Xpress [36] and complex workflows, where some of the automation steps are supported by the processing device, like the BioAffy disks in the GyroLab workstation [16]. In recent years there has been a trend towards the integration of complex sample-to-answer workflows within simple processing devices that are suitable for point-of-care diagnostics [86–88]. These developments became possible due to several new types of advanced unit operations (Table 3.2), most of which can be categorized in one or several of three general trends:

1) External means
2) Functional materials
3) Centrifugo-pneumatic unit operations

External means are functionalities of the processing device other than rotation, including actuation based on temperature, magnetic or electric forces and pneumatics. Unit operations based on external means allow fluidic design with an additional degree of freedom, and therefore can allow complex workflows in relatively simple cartridges. Various different unit operations have already been presented using external means: Prof. Madou’s group demonstrated actuation via temperature changes [49, 89]. These unit operations are based on pneumatic chambers. By heating the enclosed air an overpressure in these pneumatic chambers is generated, which can be used to actuate liquid flow. Similar operations have been introduced by Keller et al. for thermopneumatic pumping and aliquoting in standard laboratory instruments [90, 91]. An advantage of centrifugo-thermopneumatic operations is that many processing devices will already include heating of the cartridge due to requirements of the biochemical reactions automated within the cartridge, e.g. for polymerase chain reaction (PCR). Samsung & Prof. Cho’s group presented ferrowax valves. The ferrowax valves are integrated as plugs to block a fluidic pathway. The blocked fluidic pathways can then be selectively opened by melting of the ferrowax valve using a laser diode [46]. Ferrowax valves proved to be a versatile tool for the integration of complex workflows, including immunoassays and water analysis [11, 17, 92, 87]. Other wax valves have been used to block fluidic pathways or venting, and can be actuated by global or
local heating, e.g. with a halogen lamp [93] or a hot air gun [94]. A unit operation that makes use of active cooling is the ice-valve. For the ice valve, liquid plugs are frozen using thermoelectric elements to block channels or air vents. The ice plugs were used to block evaporation during thermocycling for PCR [95]. Strohmeier et al. presented the centrifugal gas-phase transition magnetophoresis [45]. This method allows the transfer of magnetic particles between isoradially arranged chambers, by appropriately positioning the disk under a magnet in the processing device. The system was used by Hahn-Schickard and IMTEK to integrate several sample-to-answer assays, e.g. for the diagnosis of sepsis [14] or respiratory infections [86]. Prof. Salin’s group introduced the use of a stream of compressed air from an external pressure source. The compressed air can be focused on different air inlets on the disk. This external pressure was used to actuate liquid flow for valving, switching, pumping and mixing liquids in centrifugal microfluidic cartridges [96, 48, 47, 97]. A similar concept, introduced recently, uses external air pressure connected at eight electromechanically addressable pressure ports on the cartridge. The new system has been demonstrated for valving, switching, mixing and inward pumping [98]. While potentially allowing for very simple cartridges, a disadvantage of external air pressure is the high volume of air exchanged between the cartridge and the environment, which increases the risk of contaminations. In general a disadvantage of external means is the added complexity in the processing device. Furthermore, most unit operations based on external means also require integration of functional materials, e.g. the integration of ferrowax valves.

The second trend is this integration of functional materials for more complex unit operations. Functional materials in this context are materials, other than the cartridge substrate, that are specifically integrated to serve a fluidic function. Next to the already mentioned (ferro-)wax valves, the most prominent type of functional material is the dissolvable film valve by Ducreé’s group. Dissolvable films are polymer foils that dissolve when being brought in contact with water-based liquids. Fluidic actuation can be pre-programmed using dissolvable films that block channels for liquid flow or air venting. Dissolvable films have already been employed for valving, aliquoting, timing of operations and event triggered valving [50, 99, 51, 52]. Another functional material used for valving are latex films for centrifugo-pneumatic siphon valving at lower rotational frequencies [100]. The latex films were later used for valving as an intermediate layer and combined with thermopneumatic actuation. The latex film is deflected due to a thermopneumatic overpressure in a pneumatic chamber. The latex film then blocks liquid flow on a second fluidic layer of the cartridge, acting as a reversible temperature actuated valve [101]. An example for mixing by use of functional materials is the buoyancy driven bubble mixing. In this unit operation H₂O₂ is broken down into H₂O and O₂ using a catalyst integrated in the
cartridge. The generated gas is then routed through a liquid column, the liquid column is mixed by the flow induced from the rising gas bubbles [102]. In summary, if used properly, functional materials can allow for complex workflows using very simple frequency protocols. More importantly, since the frequency protocols are so simple, the processing devices can use a smaller motor, allowing for a smaller and more easily portable processing device that requires less energy. A shared disadvantage of functional materials is that they need to be integrated in an extra manufacturing step, which increases the cost and complexity of cartridge fabrication.

The third trend is the use of pneumatic forces via controlled compression and decompression of entrapped air within centrifugal microfluidic cartridges. The principle of pneumatic actuation was first published in a patent application by 3M company for mixing via reciprocation [103]. The principle of reciprocation mixing was later extended with a pneumatically actuated siphon valve for valving after mixing [104]. Pneumatic siphon valves were also used for cascading biochemical assays [22] and to integrate blood plasma separations [105, 22]. Other examples of centrifugo-pneumatic unit operations include under-pressure valves, and the thermopneumatic unit operations named earlier [106, 90, 91, 49]. In recent years Hahn-Schickard has mainly focused on centrifugo-pneumatic unit operations. The first unit operation using pneumatic forces within Hahn-Schickard was the centrifugo-pneumatic valve, introduced by Mark et al. The valve allows for handling highly wetting liquids and was later employed in centrifugo-pneumatic aliquoting [107, 108].

By engineering the fluidic resistances in conjunction with the centrifugo-pneumatic pressure within the centrifugal microfluidic cartridge, more complex pneumatic unit operations became possible. Examples include the centrifugo-pneumatic inward pumping [53], centrifugo-pneumatic handling of microparticles [109] and centrifugo-pneumatic switching [1]. The microfluidic timer [110] and the centrifugo-pneumatic multi-liquid aliquoting as presented in this thesis also fall in this category [111]. One major advantage of pneumatic unit operations is their robustness to variation in capillary pressure. Contact angles vary between liquids and can change with the age and storage conditions of the cartridge [112, 113]. Therefore, variation in capillary forces is a common error source in microfluidics. Centrifugo-pneumatic unit operations do not depend on capillary forces and can be well controlled at high rotational frequencies (>50 Hz). At such high rotational frequencies, pneumatic and centrifugal pressures are typically orders of magnitude greater than capillary pressures, and even extreme changes in contact angles can be tolerated. A second advantage of centrifugo-pneumatic operations is that their fluidic function can be well predicted using network simulations. Since 2012, the development of new unit
operations at Hahn-Schickard is supported by such network simulations [59]. The combination of robust centrifugo-pneumatic unit operations in conjunction with design by network-based simulations leads to a more efficient design process, which requires less design iterations to get to a robust microfluidic design. The biggest drawbacks of pneumatic unit operations are the need for more powerful motors and the required footprint on the disk for pneumatic chambers.

Lastly, some unit operations do not fall into any of these three categories of trends: Obvious examples include unit operations based on capillary forces, e.g. capillary siphon valves and geometric valves. More recent examples include the use of gravity based pumping, where gravity acts as an independent actuation parameter through the tilting of the processing device so that the axis of rotation is not parallel to the direction of gravity. According to a patent application by Panasonic, this actuation principle is likely in use in the Roche Cobas b 101 [114]. Another example is centrifugal microfluidic droplet generation via step emulsification, which has been used to perform digital recombinase polymerase amplification (RPA) and digital PCR [2, 115].

In summary the trend towards integration of ever more complex workflows in centrifugal microfluidics is supported by the development of new unit operations. Most of these unit operations can be categorized into one or more of three types of unit operations: Unit operations based on external means, unit operations based on functional materials and unit operations based on pneumatic forces. Some unit operations make use of several of those trends, e.g. unit operations using dissolvable films (functional materials) oftentimes also make use of pneumatic forces (centrifugo-pneumatic). In extreme cases all three trends are combined: wax valves (functional materials) have been used to block air vents (centrifugo-pneumatics) and can be actuated via an external heat source (external means). Table 3.2 shows what trends the described centrifugal microfluidic unit operations make use of, what laboratory workflows have been demonstrated and what the drawbacks and advantages are in terms of cartridge and device complexity.
Table 3.2: Selection of advanced centrifugal microfluidic unit operations and process chains. Unit operations and process chains are categorized into different trends in centrifugal microfluidics and rated in terms of cartridge complexity and device complexity. The described classes of unit operations are given as a set of examples selected by the author and do not represent all unit operations that allow the integration of complex workflows in centrifugal microfluidics.

**Characteristics of the unit operation: is used (✓), not used ( )**

Performance of the unit operation: good (+), average (o), poor (-)

<table>
<thead>
<tr>
<th>Reference</th>
<th>Actuation principle</th>
<th>Major trends in centrifugal microfluidics</th>
<th>Cartridge complexity</th>
<th>Device complexity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>External means</td>
<td>Functional materials</td>
<td>Centrifugo-pneumatic</td>
</tr>
<tr>
<td>[83]</td>
<td>Geometric valve</td>
<td>✓</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>[85]</td>
<td>Capillary siphon valve</td>
<td>✓</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>[116]</td>
<td>Shake-mode mixing</td>
<td></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>[49, 89–91]</td>
<td>Thermopneumatic valving / inward pumping</td>
<td>✓ ✓</td>
<td>✓</td>
<td>o</td>
</tr>
<tr>
<td>[46]</td>
<td>Ferrowax valves</td>
<td>✓ ✓</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>[93] [94]</td>
<td>Wax valves</td>
<td>✓ ✓ ✓</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>[95]</td>
<td>Ice valve</td>
<td>✓</td>
<td>o</td>
<td>+</td>
</tr>
<tr>
<td>[45]</td>
<td>Gas-phase transition magnetophoresis</td>
<td>✓ ✓</td>
<td>✓</td>
<td>+</td>
</tr>
<tr>
<td>[96, 48, 47, 97]</td>
<td>Pneumatic pumping (external stream of pressurized air)</td>
<td>✓</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>[98]</td>
<td>Pneumatic pumping (switchable pressure ports)</td>
<td>✓</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
### 3 State of the art in centrifugal microfluidics

<table>
<thead>
<tr>
<th>Reference</th>
<th>Actuation principle</th>
<th>Major trends in centrifugal microfluidics</th>
<th>Cartridge complexity</th>
<th>Device complexity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>External means</td>
<td>Functional materials</td>
<td>Centrifugo-pneumatic</td>
</tr>
<tr>
<td>[50, 99, 51, 52]</td>
<td>Dissolvable films</td>
<td>✓ ✓ o -</td>
<td>✓ o +</td>
<td>+ +</td>
</tr>
<tr>
<td>[100]</td>
<td>Latex film valve</td>
<td>✓ ✓ o +</td>
<td>- o o +</td>
<td>+</td>
</tr>
<tr>
<td>[101]</td>
<td>Reversible thermo-pneumatic valves (deflection of latex membrane)</td>
<td>✓ ✓ ✓ o -</td>
<td>✓ o +</td>
<td>+ -</td>
</tr>
<tr>
<td>[102]</td>
<td>Buoyancy driven bubble mixer</td>
<td>✓ - + o +</td>
<td>+ + +</td>
<td>+</td>
</tr>
<tr>
<td>[103]</td>
<td>Mixing by reciprocation (pneumatic pumping)</td>
<td>✓ o + + o o +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>[107, 108]</td>
<td>Centrifugo-pneumatic valve</td>
<td>✓ + + + o + +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>[22]</td>
<td>Centrifugo-pneumatic siphon valve</td>
<td>✓ - + + o o +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>[53]</td>
<td>Centrifugo-pneumatic inward pumping</td>
<td>✓ - + + o - +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>[109]</td>
<td>Centrifugo-pneumatic handling of microparticles</td>
<td>✓ - + + - - +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>[1]</td>
<td>Centrifugo-pneumatic switching</td>
<td>✓ - + + - - +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>[114]</td>
<td>Gravity assisted pumping</td>
<td>+ + + + o +</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>[2]</td>
<td>Centrifugal step emulsification</td>
<td>+ + + - + +</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

Page 22
3.3 Setups & fabrication of microfluidic cartridges

The fabrication technology and measurement setups as used in the individual experimental series are described briefly in the corresponding chapters. The development of stroboscopic setups and the detailed fabrication technologies of microfluidic cartridges were not the focus of this thesis. To learn more about the materials and methods used within this thesis, the author recommends the following literature on the stroboscopic setups [117], the fabrication of thermoformed disks [118, 119] and the fabrication of multi-layered thermoformed disks [120].
4 Volume reduction

This chapter addresses the handling of nanoliter volumes and the reduction of sample volumes via centrifugal microfluidics. A new process chain for the generation of combinatorial dilutions in the nanoliter range is presented in the context of protein structure analysis via small-angle X-ray scattering (SAXS).

First, a short review of the state of the art for the handling of nanoliter volumes in centrifugal microfluidics is given. Second, this chapter explains why SAXS is an important field for microfluidic automation, and discusses why centrifugal microfluidics is well-suited for the automation of sample preparation for SAXS. Finally, the new process chain for the generation of combinatorial dilutions in the nanoliter range is presented in the context of the LabDisk for SAXS.

4.1 State of the art in low input volume centrifugal microfluidics

At the moment, most applications and unit operations in centrifugal microfluidics use liquid volumes in the range of tens to hundreds of microliters. However, as discussed earlier in this work, one of the strengths of centrifugal microfluidics is its ability to also handle low volumes in the nanoliter range by use of the scalable volume forces in the rotating system (see chapter 1.1). Some laboratory workflows that benefit from low reagent or sample consumption have already been presented in centrifugal microfluidics:

Sundberg et al. presented a cartridge with one-stage aliquoting for digital PCR. A 40 µl input volume was split into 1000 aliquots of 33 nl each [121]. In one-stage aliquoting, liquids are metered directly in a receiving chamber. One-stage aliquoting is therefore limited to processing one liquid, only. In contrast, two-stage aliquoting seperates the metering chamber from the downstream fluidics via a valve, allowing fluidic processing of aliquots after metering. Gyros offers a commercially available cartridge for nanoliter scale ELISA, which includes two-stage aliquoting [43]. The disk includes the metering of a 20 nl portion of the sample, as well as the two-stage aliquoting of reagents in 96x 200 nl aliquots [122]. One experiment can be run from as little as 3.5 µl of sample volume, which is automatically injected into the cartridge by the GyroLab Workstation.
Lastly, multiple cartridges have been presented for protein crystallization. Li et al. introduced a disk for two-stage aliquoting and the combination of two liquids in 24 chambers using 31 nl of protein volume per aliquot [35, 8]. SpinX also presented a centrifugal microfluidic cartridge for protein crystallization. In this cartridge, valving was automated via the perforation of a central film using high-precision lasers in the processing device. The volume in each reaction chamber was 360 nl and up to two liquids were combined in each reaction chamber [123]. The smallest volume metered at Prof. Zengerle’s group within a centrifugal microfluidic cartridge was, to the best of my knowledge, the metering of 500 nl of blood by Steigert et al. for clinical chemistry applications [124]. Steinert et al. discussed the handling of volumes down to 1 nl. However, in this case liquid volumes were defined by an external PipeJet dispenser, which cannot be directly compared to on disk metering [34].

Table 4.1 shows a summary of nanoliter volume operations presented for centrifugal microfluidic cartridges. One point to note is that cartridges with low liquid volumes not only decrease the amount of reagents and samples used, but due to the smaller footprints of channels and chambers such cartridges can also integrate more tests per cartridge. However, for several reasons, only a limited number of small input volume applications are automated using centrifugal microfluidics.

First, rapid prototyping technologies in centrifugal microfluidics are typically based on micromilling or CO₂ laser cutting. These prototyping technologies are mainly suitable for structure sizes of hundreds of micrometers to several millimeters. To handle nanoliter volumes, microstructures in the size of tens of micrometers are required. However, the fabrication of such small features requires sophisticated machinery. The applications presented get around this by using either complex external means, like SpinX [123], or by relying on hydrophilic or hydrophobic surface coatings [35, 122], resulting in more complex and expensive cartridges and processing devices. Furthermore, since only few applications have been demonstrated for the nanoliter range, unit operations and process chains are not readily available. To integrate a new application with nanoliter volumes new unit operations typically have to be redeveloped from scratch. Therefore the platform benefits in centrifugal microfluidics are mostly limited to the microliter range. All of these points combined result in a much higher entry barrier to develop nanoliter scale centrifugal microfluidics in comparison to microliter scale centrifugal microfluidics.

To expand the capabilities of centrifugal microfluidics in the nanoliter volume range, a new process chain is presented for the generation of a dilution matrix with 20 different combinatorial dilutions in the nanoliter range from three input liquids. This novel process chain does not require surface modifications or a complex processing device. Furthermore,
prototyping and fabrication strategies for milling, thermoforming and sealing of structure sizes down to 32 µm × 20 µm were adapted specifically to the requirements of nanoliter scale centrifugal microfluidics.¹

¹ The newly developed prototyping techniques are not in the focus of this thesis, and all prototyping-related work was performed in close collaboration with the Hahn-Schickard prototyping, namely Benjamin Rutschinsky, Dominique Kosse and Dirk Buselmeier [120].
Table 4.1: Implementations of low sample and reagent volume microfluidic operations in centrifugal microfluidics.

<table>
<thead>
<tr>
<th>Author</th>
<th>Application</th>
<th>Unit operation / Process chain</th>
<th>Smallest handled liquid volume</th>
<th>Smallest input volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Emamzadah et al. [123]</td>
<td>Protein crystallization</td>
<td>Two-stage aliquoting (1)</td>
<td>&lt; 360 nl</td>
<td>N/A</td>
</tr>
<tr>
<td>(SpinX)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Li et al. [35]</td>
<td>Protein crystallization</td>
<td>Two-stage aliquoting</td>
<td>31 nl</td>
<td>N/A</td>
</tr>
<tr>
<td>Steigert et al. [124]</td>
<td>Clinical chemistry</td>
<td>Two-stage metering</td>
<td>500 nl</td>
<td>N/A</td>
</tr>
<tr>
<td>Sundberg et al. [121]</td>
<td>Digital PCR</td>
<td>One-stage aliquoting</td>
<td>33 nl</td>
<td>40 µl</td>
</tr>
<tr>
<td>Andersson et al. [122]</td>
<td>ELISA</td>
<td>Two-stage metering</td>
<td>20 nl (injection by processing device)</td>
<td>3.5 µl (2)</td>
</tr>
<tr>
<td>(Gyros)</td>
<td></td>
<td>Two-stage aliquoting</td>
<td>200 nl (injection by processing device)</td>
<td>N/A (2)</td>
</tr>
<tr>
<td>Schwemmer et al. [This Thesis]</td>
<td>Small-angle X-ray scattering</td>
<td>Two-stage aliquoting</td>
<td>40 nl</td>
<td>2.5 µl</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Combinatorial dilutions</td>
<td>240 nl</td>
<td>2.5 µl</td>
</tr>
</tbody>
</table>

(1) Handling using high-precision lasers in the processing device

(2) Volume injected by processing device
4.2 The relevance of microfluidic liquid handling to SAXS

Small angle X-ray scattering (SAXS) is a method in structural biology that uses 1D X-ray scattering patterns for the determination of 3D protein structures [125–127]. In a typical SAXS measurement two scattering profiles are collected. First, the scattering profile of proteins within a buffer solution is collected. From this scattering profile a background scattering profile of only the buffer, without any proteins, is subtracted. The resulting scattering profile contains only the contribution from the proteins to the scattering pattern (Figure 4.1). Using this subtracted background scattering profile, different types of analyses allow for the calculation of various parameters from the protein, including the radius of gyration, the molecular mass, the foldedness of the protein or even the 3D structure via *ab initio* modeling. The resolution of SAXS for 3D structures is on the order of 1 nm, which is much lower than the ångström resolution of protein crystallography [128]. However, in contrast to protein crystallography, SAXS can detect protein structures directly from solution, without the need for protein crystals, making SAXS an attractive complementary option if crystallization trials are unsuccessful. More importantly the measurement of the actual protein structure in solution provides insights into changes in protein structure under different environmental conditions, e.g. different salt or pH conditions. Such interactions are impossible to measure using crystallography.

In recent years, better automation has made the collection and analysis of SAXS data accessible to users who are not experts in SAXS [129–132], making SAXS significantly more popular amongst users. However, current automation principles still require at least 5 µl of precious, high-purity protein solution per measurement. Therefore only a low number of environmental conditions are probed per protein in a typical experiment [133]. Microfluidic environments offer the potential to screen many different environmental conditions at a lower consumption of protein volume. However, current microfluidic sample environments in SAXS are limited to expert users, since they require the manual attachment of tubings and syringe-pumps [133, 134]. What is needed is a microfluidic environment that is both easy to use for non-experts and requires only minimal sample volumes. These requirements can be well addressed with a centrifugal microfluidic cartridge, since the requirements overlap with the strength of centrifugal microfluidics (see section 1.1), specifically the simple world-to-chip interface and the compatibility to low input volumes.
Figure 4.1: A collimated, monochromatic X-ray beam is scattered on a sample. The scattering vector $s$ is the change in momentum of an incident photon based on elastic scattering within the sample. The scattering profile is collected on a 2D detector. In a typical protein solution, proteins are not arranged in any specific direction, but orientated randomly. Therefore, the scattering pattern will be ring-shaped, and can be integrated to a 1-dimensional scattering profile dependent on $s$. In a typical small-angle X-ray (SAXS) experiment two 1D scattering profiles are collected, a first scattering profile from a protein solution and a second scattering profile from the buffer without any proteins. The difference of the two profiles is the scattering profile of only the proteins. Figure from ref. [130].
LabDisk for SAXS: A centrifugal microfluidic sample preparation platform for small-angle X-ray scattering


- The following section has identically been published as an original research paper in Lab on a Chip in 2016 (Volume: 16, Pages: 1161 - 1170). In contrast to the published manuscript, the numbering of sections, figures, tables and references was adapted to the overall numbering of this dissertation. Minor improvements due to additional proof reading were implemented. Part of the figures and formulas from the electronic supplementary material were included in the main text to improve readability. The abstract was adapted and included in the chapter abstract of this thesis.

Contributions to this publication:

- Frank Schwemmer: Literature search and analysis, designed the microfluidic cartridge, planned, performed and analyzed the fluidic experiments, coordinated the design of the processing device and positioner and wrote the manuscript, except for the section “SAXS experiments” and “Materials & methods” related to the SAXS experiments.

- Clement Blanchet, Alessandro Spilotros, Manfred Rössle: Performed the SAXS experiments and "Materials & methods” related to the SAXS experiments.

- Clement Blanchet, Alessandro Spilotros: Wrote the manuscript section on “SAXS experiments”.

- Clement Blanchet, Alessandro Spilotros, Manfred Rössle: Analyzed and interpreted the SAXS data.
4.2 The relevance of microfluidic liquid handling to SAXS

- Manfred Rössle, Daniel Mark: Conceived the idea of designing a LabDisk for SAXS.

- Dominique Kosse: Developed the prototyping and fabrication strategies for the LabDisk for SAXS, especially the process for the fabrication of 3-layer thermoformed disks.

- Haydyn Mertens, Melissa Graewert: Prepared the protein samples for the SAXS experiments.

- Clement Blanchet, Alessandro Spilotros, Dominique Kosse, Steffen Zehnle, Haydyn Mertens, Melissa Graewert, Manfred Rössle, Nils Paust, Dimitri Svergun, Felix von Stetten, Roland Zengerle, Daniel Mark: Scientific advice during experiments and manuscript preparation, proof-reading of the manuscript.
**Preface**

The following section discusses a new process chain for the generation of combinatorial dilutions of 240 nl volume each from three input liquids. The reduction of sample and reagent volumes is one of the central reasons microfluidics is an attractive field of research and, as discussed previously, centrifugal microfluidics is well-suited for handling low liquid volumes.

Section 4.1 describes available centrifugal microfluidic unit operations for the handling of nanoliter volumes. However, a process chain for aliquoting and subsequent combination of three different liquids in the nanoliter volume range has not been presented. For the application of small-angle X-ray scattering (SAXS) aliquoting and combination of at least three liquids, the protein sample, a buffer and a screening agent, is required to efficiently screen proteins for structural changes under different environmental conditions.

In the following we present the generation of a dilution matrix with 20 combinatorial dilutions from three input liquids at 2.5-3.5 µl input volume. The new process chain is applied for sample preparation in small-angle X-ray scattering. The described LabDisk for SAXS allows for a more than tenfold reduction in protein volume compared to robotic sample changers or other microfluidic sample environments for SAXS. Currently, the EMBL in Hamburg is developing algorithms for the automatic positioning of the LabDisk for SAXS in the BioSAXS beamline at EMBL Hamburg. As soon as these new algorithms are available, the described LabDisk for SAXS will serve as a new sample environment for standard users of the BioSAXS beamline. For this purpose, a total of 200 disks with 1200 dilution matrices are available at the EMBL in Hamburg.
4.3 Introduction

Small-angle X-ray scattering (SAXS) is an important technique in structural biology for the analysis of macromolecules in the range of 1-100 nm [135]. One of the unique advantages of SAXS is its ability to determine low-resolution structures of macromolecules and complex assemblies directly from protein solutions without the need for protein crystals. This makes SAXS an ideal method for the measurement of nm scale structural transitions triggered by a change in the environmental conditions, e.g. pH or salt concentration [133]. Within recent years, new methods in SAXS data analysis [129, 130] and the introduction of automated sampling robots [131, 132, 136, 137] have led to a rapid increase in the popularity of SAXS among structural biologists. However, while sampling robots have increased the ease of use and throughput, these robots still require 5 to 30 µl of protein volume per measurement (Table 4.2). Since quantities of high purity protein samples produced for structural studies are often limited, typically only a low number of environmental conditions can be probed per protein.

Microfluidics offers the potential to provide high-throughput systems that reduce sample volumes and time per measured condition. Different microfluidic devices have been presented for SAXS. Continuous flow devices, where liquids are mixed and analyzed in micro-channels have been used for the measurement of folding and unfolding kinetics via time-resolved SAXS [138–141]. In these methods the spatial distance after the micro-mixer corresponds to a time after mixing. By probing at different distances from the micro-mixer, kinetics could be resolved down to ~100 microseconds [142], in comparison to multiple milliseconds in stopped-flow measurements [143]. Furthermore, there are a number of customized microfluidic solutions for specific questions in SAXS, e.g. the formation of spider silk [144] or the alignment of anisotropic particles in microchannels [145]. Nevertheless, next to these specialized microfluidic solutions, some promising Lab-on-a-Chip devices have been presented that allow for high-throughput screening of protein structures under different environmental conditions with minimal protein volume.

Stehle et al. presented a droplet based microfluidic system for SAXS [134]. The system demonstrated the formation and analysis of gold nanoparticles. The volume of a single droplet was only ~0.5 nl and the data from 9000 droplets were averaged during one SAXS measurement. In the future the system can be integrated with well-studied droplet microfluidics operations, e.g. splitting, merging, micro-injection and mixing of droplets, to form more complex analysis systems. The bioXTAS chip, developed by Toft et al., allows for automated mixing of down to 36 µl of sample solution and read-out in a 200 nl X-ray
chamber [146]. This device was later substantially extended by Lafleur et al., who developed a Lab-on-a-Chip system for automated high-throughput sample preparation [133]. The Lab-on-a-Chip system contained multiple rotary valves for on demand mixing of screening agents. Protein concentrations could be continuously verified using UV absorbance measurements and protein consumption was reduced by an on chip sample reservoir. This allowed a full analysis cycle with 6 different measurement conditions to be measured with a protein volume of only 15 µl.

However, while all microfluidic systems available offer unique advantages for SAXS, current systems suffer from typical “world-to-chip” interfacing problems, e.g. all systems require manual attachment of tubes for syringe pumps. This makes the current Lab-on-a-Chip systems impractical for novice users and calls for more user-friendly solutions. Furthermore, with the exception of the new version of the bioXTAS chip, all microfluidic systems have large dead volumes of tens to hundreds of microliters.

Here, we present the centrifugal microfluidic LabDisk for SAXS. In contrast to pressure driven microfluidic approaches, centrifugal microfluidic systems can be filled with regular pipettes and intrinsically benefit from low dead volumes since no liquid is lost in tubings [9, 8]. This simple “world-to-chip” interface and low dead volumes have led to the adoption of centrifugal microfluidic platforms in diverse diagnostic [147, 122, 85] and analytical [87, 31] applications. Especially noteworthy are centrifugal microfluidic systems for protein crystallography [34, 35]. The LabDisk for SAXS is a new high-throughput tool for SAXS experiments at the P12 beamline at EMBL (PETRA III, DESY). The protein volume for a full dilution matrix of 20 measurement conditions (15 protein solutions, 5 buffers) is 2.5 µl. This volume corresponds to 170 nl per protein containing measurement condition, which is more than 10× less protein volume per measurement in comparison to current sample changers and other microfluidic systems (Table 4.2). The LabDisk for SAXS includes six dilution matrices, each offers automatic and precise aliquoting, merging and mixing of different combinations from three input liquids: the protein sample (2.5 µl), a screening agent (3 µl) and a buffer solution (3.5 µl).

We demonstrate that the LabDisk for SAXS can be used with a range of different liquids relevant for SAXS. SAXS data collected using the LabDisk for SAXS can be fitted against crystallographic structure and data quality is sufficient to calculate ab initio 3-dimensional structures for glucose isomerase. Finally, we show a first application of the presented LabDisk for SAXS, via demonstration of different intermolecular effects for glucose isomerase depending on salt and protein concentration. The LabDisk for SAXS has the
potential to introduce routine high-throughput screening to a wide SAXS community, reducing protein use and measurement time at synchrotron beamlines for all users.

**Table 4.2:** Comparison of different automation concepts for SAXS in comparison to the LabDisk for SAXS. The listed sample changers are part of the standard sample environment in the corresponding user dedicated beamlines listed in brackets. So far, no microfluidic system is in use as a standardized sample environment in user-dedicated beamlines.

<table>
<thead>
<tr>
<th>Author</th>
<th>Type</th>
<th>Minimum input volume</th>
<th>Automated generation of dilutions</th>
<th># of different measurement conditions containing protein</th>
<th>Volume of protein solution per measurement</th>
<th>Time per measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hura et al. [132]</td>
<td>Sample changer (Berkley)</td>
<td>N/A</td>
<td>No</td>
<td>1</td>
<td>12 µl</td>
<td>N/A</td>
</tr>
<tr>
<td>Round et al. [148]</td>
<td>Sample changer (EMBL)</td>
<td>20 µl</td>
<td>No</td>
<td>1</td>
<td>5-20 µl</td>
<td>60 s</td>
</tr>
<tr>
<td>David et al. [131]</td>
<td>Sample changer (Soleil)</td>
<td>N/A</td>
<td>No</td>
<td>1</td>
<td>6 µl</td>
<td>N/A</td>
</tr>
<tr>
<td>Nielsen et al. [136]</td>
<td>Sample changer (CHESS)</td>
<td>10 µl</td>
<td>No</td>
<td>1</td>
<td>10 µl&lt;sup&gt;a&lt;/sup&gt;</td>
<td>N/A</td>
</tr>
<tr>
<td>Stehle et al. [134]</td>
<td>Microfluidic system (Droplet based)</td>
<td>&gt; 50 µl&lt;sup&gt;b&lt;/sup&gt;</td>
<td>No</td>
<td>1</td>
<td>~ 9000*0.5 nl = 4.5 µl</td>
<td>900 s&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lafleur et al. [133]</td>
<td>Microfluidic system (Lab-on-a-Chip system)</td>
<td>15 µl</td>
<td>Yes</td>
<td>5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>2.5 µl&lt;sup&gt;d, e&lt;/sup&gt;</td>
<td>30 s</td>
</tr>
<tr>
<td>LabDisk for SAXS</td>
<td>Microfluidic system (Centrifugal microfluidic)</td>
<td>2.5 µl</td>
<td>Yes</td>
<td>15</td>
<td>170 nl&lt;sup&gt;d&lt;/sup&gt;</td>
<td>30-60 s&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Via manual loading 5 µl are possible; <sup>b</sup> Estimated minimal fill for syringe pumps and tubings; <sup>c</sup> Time for measurement of 9000 droplets; <sup>d</sup> Six different measurement conditions (five containing protein) for 15 µl input volume; <sup>e</sup> Due to dilution, some of the measurements are performed with lower protein concentration than the input stock solution; <sup>f</sup> Including the manual positioning of wells. Automated image recognition based positioning is estimated to reduce time per measurement to 3-5 s.
Figure 4.2: LabDisk for SAXS. Each of the six segments includes the aliquoting of the three input liquids, the combination and the mixing in different predefined concentrations. The mixtures then reside in the read-out chambers. Read-out can be performed on disk in a synchrotron beamline. The fluidic function of the segments is explained in Figure 4.5-9.

Figure 4.3: The processing device for the LabDisk for SAXS.
4.4 Operation of the LabDisk for SAXS

To collect data at a synchrotron beamline with the LabDisk for SAXS, three components are required: (1) the disposable LabDisk for SAXS, which includes all microfluidics for the generation of the dilution matrices and the read-out chambers (Figure 4.2), (2) the custom-built processing device, which is used to rotate the disk and thereby process the LabDisk for SAXS fluidically (Figure 4.3) and (3) a custom-built positioner, which is used to position the read-out chambers of the LabDisk for SAXS with the X-ray beam in the synchrotron beamline (Figure 4.4).

The LabDisk for SAXS is inserted into the processing device. Reagents are pipetted into the three inlets of each of the dilution matrices. One to six dilution matrices can be run in parallel. Each dilution matrix (Figure 4.5) is filled with 2.5 µl of protein solution, 3 µl of screening agent and 3.5 µl of buffer solution. After pipetting, the lid of the processing device is closed, and the processing device starts. The disk is initially rotated at 10 Hz and slowly accelerated to 30 Hz over a 90 s period. Liquid is transported from the inlet into the inlet channel. The liquid then fills individual aliquoting fingers that are connected via an isoradial feeding channel. The liquid flow from the aliquoting fingers into downstream fluidic elements is stopped by geometric valves [83, 86] (32 µm × 20 µm) located at the radially outer end of each of the aliquoting fingers (Figure 4.6A). Each aliquoting finger has a volume of 40 nl. After the aliquoting fingers are filled, excess liquid flows over a radial extension of the feeding channel into the waste. The radial extension of the feeding channel increases the hydrostatic pressure from the inlet to the waste and ensures that liquid from the feeding
channel is completely drained. When the feeding channel drains, the individual aliquoting fingers are fluidically separated, completing the metering of the aliquots (Figure 4.6B). After the metering is completed, the rotational frequency is increased to 150 Hz. Centrifugal pressure increases until the liquid bursts through the geometric valves. Liquid aliquots from the upper aliquoting row (protein sample) and the center aliquoting row (screening agent) are transported over the backside foil and combined with aliquots from the lower aliquoting row (buffer) (Figure 4.5). Each of the six dilution matrices on the disk generates 120 aliquots. Aliquots are combined in six aliquots each with different ratios of protein solution, screening agent and buffer aliquots, e.g. 3 protein solution aliquots, 1 screening agent aliquot and 2 buffer aliquots (see Figure 4.7 for all combinations). The aliquots are mixed by reciprocation using a pneumatic chamber (Figure 4.8) [104, 149]. The rotational frequency alternates between 10 Hz and 150 Hz, pumping the liquid between the read-out chamber and the pneumatic chamber until the liquid plugs are mixed completely. After 10 mixing cycles, the disk is stopped, the liquid is pumped into the read-out chambers and ready for analysis via SAXS. Each dilution matrix then contains 20 different measurement conditions. A full disk of six dilution matrices contains 120 different measurement conditions in 120 read-out chambers. Out of the 2.5 μl input volume 680 nl are analyzed in the actual SAXS measurement. The rest of the volume is included for pipetting tolerance, filling and draining of the feeding channel and as excess volume to ensure complete filling of the read-out chambers (see Table A4.1).

After the fluidic protocol is completed, the disk can be transferred from the processing device to the positioner within the P12 beamline (PETRA III, DESY). Each dilution matrix contains 20 read-out chambers. The alignment of the read-out chambers within the SAXS beamline is performed via a custom built 3-axes motor stage. The 3-axes stage contains a rotational motor, which is used to roughly align the read-out chambers with the beam. Two linear motors, accurate to 20 μm, are used for fine alignment of the measurement chamber with the X-ray beam. The alignment was performed by manually controlling the motors for this manuscript, but will be automated in the future. The depth of the measured liquid column in the read-out chamber is 860 μm, the minimum diameter of the read-out chamber is 348 μm. The size of the X-ray beam was 50 μm × 50 μm. The alignment is supported by an in-axis camera setup. For identification each read-out chamber is individually numbered via a bitcode next to the read-out chamber. Data is automatically collected. After all 120 measurements are completed the disposable disk can be discarded and exchanged with the next disk.
Figure 4.5: One dilution matrix of the LabDisk for SAXS. The left side shows a top view of the dilution matrix, the right side shows a cross-section. Red circles indicate through holes in the center foil. After metering the three input liquids, the rotational frequency is increased. Aliquots from the sample and screening agent are transferred through holes in the center foil (red circles), transported over the backside fluidic layer and combined with the buffer aliquots in the read-out and mixing section. Aliquots are combined in 20 sets of six aliquots each, comprising different combinations of sample, screening and buffer aliquots. Arrows indicate liquid flow. Side view is not to scale. Aliquoting is described in Figure 4.6. The mixing is described in Figure 4.8. The different target concentrations, as generated in one dilution matrix, are shown in Figure 4.7.
Figure 4.6: Aliquoting principle. During filling the rotational frequency is slowly ramped up from 10 Hz to 30 Hz. When liquid from the inlet fills the inlet channel, the aliquoting fingers having 40 nl volume each are sequentially filled via the feeding channel and excess liquid is transported to the waste (A). All extra liquid above the metering fingers is transported to the waste due to the radial extension of the feeding channel (B). By ramping up the rotational frequency to 150 Hz a certain number of the 40 nl aliquots is merged to create multiples of 40 nl volumes and transferred to the mixing and read-out chambers (C). The aliquoting structures for protein solution, screening agent and buffer aliquoting contain 33 aliquots, 36 aliquots and 51 aliquots, respectively. Combination of aliquots is explained in Figure 4.5, mixing is explained in Figure 4.8.
Figure 4.7: Dilutions as generated in the dilution matrix of the LabDisk for SAXS. Dilutions are plotted in terms of input concentration of protein solution and screening agent. In each read-out chamber sets of six aliquots for protein solution (blue), screening agent (violet) and buffer (green) are combined. Numbers indicate the number of aliquots combined for each measurement condition.

Figure 4.8: Mixing via reciprocation. Aliquots are combined in the read-out chamber (A). By the alternation of rotational frequencies between high and low frequencies, the liquid is pushed back and forth between the read-out chamber and the pneumatic chamber (B). After 10 cycles mixing is completed and the dilution matrix can be transferred to the beamline for read-out (C).
4.5 Materials & methods

4.5.1 Prototyping & fabrication

A foundry service fabricated the LabDisks via a three-layer thermoforming process [150]. The process for the fabrication of the LabDisk for SAXS was described in detail by Kosse et al. [120], here in short.

LabDisks consist of three thermally bonded layers, a frontside foil containing most of the microfluidic structures, a backside foil connecting the radially inner aliquoting structures with the radially outer mixing chambers, and a center foil (160 μm, Topas COC 8007 and Topas COC 6013 co-extruded compound foil) with drilled holes connecting the frontside and backside fluidic layer. Microfluidic structures in the frontside and backside fluidic layers were designed using the computer-aided design software SolidWorks 2011 (Dassault Systèmes SOLIDWORKS Corp., France) and micro-milled using a KERN Evo (KERN Microtechnik GmbH, Germany) into a PMMA master (Plexiglas, Evonik, Germany). A negative of the microfluidic structures was replicated in PDMS (Elastosil RT-607, Wacker Chemie). The frontside and backside foils consist of custom-made 120 μm thick co-extruded Topas COC 8007 and Topas COC 6013 compound foils. Microstructures were replicated via thermoforming on a custom-built hot-press. The thickness of the X-ray viewing windows after thermoforming was ~70 μm for the frontside and ~115 μm for the backside foil. Frontside, center and backside foil were aligned and bonded by means of thermal bonding, using pressurized air in a hot press.

4.5.2 Fluidic processing & design

Stroboscopic images were taken using a LabDisk-Player modified with a stroboscopic light and camera to monitor microfluidic operation under rotation. SAXS Experiments were performed using a custom-built processing device. It allows for rotational frequencies of up to 200 Hz and facilitates acceleration and deceleration of up to 30 Hz s⁻¹ (Figure 4.3).
4.5.3 **SAXS data collection**

SAXS data were collected on the beamline P12 of EMBL, at the PETRA III storage ring (DESY, Hamburg) [143]. The disk was mounted in an about 3 cm wide air gap between the vacuum tube to the synchrotron and the vacuum tube to the detector. The disk was positioned on a custom-built 3-axes motor stage. The 3-axes stage contains a rotational motor, which roughly positions the read-out chambers in the X-ray beam path. Two linear motors are used for fine alignment. A custom-built on-axis camera provides images of the disk, facilitating the alignment of the chambers with the X-ray beam whose position on the image is known. A picture of the LabDisk for SAXS set up in the beamline (PETRA III, DESY) is depicted in Figure 4.4.

The X-ray beam (energy 10 keV) was collimated to an effective beam size of about 50 μm × 50 μm at sample position, yielding a flux of $5 \times 10^{11}$ photons per second. Data were collected on a Pilatus 2 M detector (Dectris, Villingen), with a sample-to-detector distance of 3.1 m covering a range of momentum transfer $0.02 \text{ nm}^{-1} < s < 4.8 \text{ nm}^{-1}$ ($s = 4\pi \sin \theta / \lambda$, where $2\theta$ is the scattering angle, and $\lambda = 0.12$ nm is the X-ray wavelength). On each read-out chamber, 20 frames of 50 ms exposure time were collected, radially averaged and normalized to the transmitted beam intensity. Individual frames were manually inspected and compared to identify radiation damage. Frames exhibiting significant differences in intensity were discarded.

The “batch-mode” measurements were performed using an in-vacuum flow through cell coupled to an autosampler robot [143, 148]. Twenty frames of 50 ms were collected and analyzed using the SAXS data analysis pipeline [151].

4.5.4 **Data processing**

Data were processed using programs from the ATSAS package [152]. The buffer scattering signal was subtracted from that of the sample to isolate the scattering signal of the solute. The radius of gyration, $R_g$, of each sample was computed using the Guinier approximation [153] on a restricted range ($0.21 \text{ nm}^{-1} < s < 0.4 \text{ nm}^{-1}$). The *ab initio* bead model of glucose isomerase was reconstructed from the experimental data using the program DAMMIN [154]. Predicted SAXS profiles of glucose isomerase were computed from the atomic structure (PDB ID. 1OAD) using Crysol [155] and used to compute signal to noise ratio.
4.6 Results

To test the compatibility of different liquids with the LabDisk for SAXS, the disk was filled with liquids typical for SAXS experiments and aliquoting was observed using a stroboscopic setup. Metered volumes were quantified from the filling height in the aliquoting finger directly before transfer to the mixing chambers. The variation in metered volume as determined from filling height was 0.2–1.0% CV (Figure 4.9). Inaccuracies in aliquoting originate mainly from the first and last aliquoting fingers. Liquid in the first and last aliquoting fingers slowly wicks out of the fingers and into the waste (Figure A4.1), leading to underfilling in these metering fingers. The metering works as expected for the tested liquids. It has to be noted that the given volumes in Figure 4.9 are not the same as the volumes aliquoted into the read-out chambers, since this evaluation does not take into account possible variations in aliquoting finger depths due to fabrication tolerances and neglects liquid lost on channel walls due to capillary forces during transfer. Some liquid loss due to capillary forces can commonly be expected in centrifugal microfluidics. To minimize the liquid loss on channel walls, the rotational frequency during transfer was 150 Hz, which is notably higher than the rotational frequencies typically used in centrifugal microfluidics.

For the SAXS experiments, only the protein and screening agent concentrations in the read-out chambers matter, the absolute volumes are irrelevant. To measure the actual variation of protein concentration in the read-out chamber, we quantified the relative protein concentrations in the read-out chamber from the SAXS scattering data for the dilution matrix with glucose isomerase. Relative protein concentrations were quantified by measuring the actual scaling factors vs. expected scaling factors in the SAXS scattering pattern. The variation of measured protein concentration at the three different diluted target concentrations was 2.7–4.4% CV. This variation includes variations in aliquoting, combination and mixing. Due to the geometric valves in the LabDisk for SAXS, liquids with very low advancing contact angles on the COC surface (<45°) cannot be used in the LabDisk for SAXS, i.e. liquids with high concentrations of surfactants. For such low advancing contact angles, there is cornerflow in accordance with the Concus–Finn condition [156, 157]. This leads to unpredictable filling of channels and in extreme cases even “bursting” of geometric valves before the rotation of the disk is started. Consequently, the aliquoting fails. Another limiting factor is viscosity. Increasing the viscosity of input liquids will reduce the flow rate in the cartridge. At some point, the liquid flow rate will be so low that aliquoting would not be finished before the rotational frequency is increased to 150 Hz. Liquid remaining in the inlet would then be transferred to the first read-out chamber. The liquid with the highest viscosity tested was water with 10% glycerol, with a viscosity of 1.31 mPa s at 20 °C. If liquids of
higher viscosities need to be handled, the frequency protocol can be adapted to include longer holding times. In general, failure of the fluidics is clearly visible from uneven filling of the read-out chambers and lack of liquid in one or more of the three waste chambers.

4.7 SAXS experiments

We used the LabDisk for SAXS to collect SAXS data on glucose isomerase (GI) at different protein and NaCl concentrations. The dilution matrix was prepared using a stock GI solution of 11 mg ml\(^{-1}\), the dilution buffer and a screening buffer containing 500 mM NaCl. The 20 conditions generated with the dilution matrix are reported in Table 4.3.

SAXS data were collected for the 20 different conditions, investigating protein dilution and the effect of NaCl concentration on intermolecular interactions. The radially averaged curves collected on the different read-out chambers of the disk were consistent with each other and had the same background except for one chamber that exhibited a very intense signal at a low angle (<0.4 nm\(^{-1}\)). The 2D images collected on this read-out chamber show a strong parasitic signal at low angle suggesting that the chamber was not properly aligned with the beam. This data set was not considered in the following. For the consistent read-out chambers, scattering data were collected from protein and buffer solutions and difference profiles generated for analysis. The scattering curves corresponding to the dilution matrix of GI without additional NaCl (where the differences between the curves are the largest) are depicted in Figure 4.10A.

![Figure 4.9](image.png)

**Figure 4.9:** Aliquoting of liquids relevant for SAXS (51 measurements each). Error bars correspond to one standard deviation. *Metered volume was quantified via the filling heights in the aliquoting fingers. Variations in volume of the aliquoting fingers due to fabrication tolerances and liquid loss during transport to the read-out chamber were not taken into account.
4.7.1 Comparison of the SAXS data collected on the LabDisk for SAXS with data from the sample changer

The data collected with the LabDisk for SAXS were first compared to data collected on the same sample with the standard autosampler coupled capillary setup for solution SAXS of the P12 beamline. Curves collected on GI at 5.5 mg ml$^{-1}$ (no salt) with the two setups are shown in Figure 4.10B. The data collected on the disk is noisier than the one collected on the sample changer: the signal-to-noise-ratio of the data collected on the disk is less than half of the one collected on the sample changer. But the two curves are in good agreement ($\chi^2 = 1.4$, see eqn (A4.1)).

(i) In the sample changer operation, the capillary in which the sample is loaded is in vacuum. The LabDisk for SAXS is operated in a 3 cm wide air gap. In addition to the scattering of air around the disk, two vacuum windows were added on the beamline to break the vacuum path (detector flight tube window: Kapton, 30 μm; on-axis camera window: polycarbonate, 125 μm). Although these windows have been chosen because of their low scattering property, they do contribute to the experimental background.

(ii) The total SAXS signal is lower because the path length of the X-ray in the capillary is reduced: the path length for the read-out chamber is 860 μm, whereas the path length for the sample changer capillary is 1.7 mm. For the photon energy used in these experiments (10 keV), the optimal path length would be 1.88 mm [158], i.e. the path length is closer to optimal in the sample changer capillary than in the disk. Furthermore, for the presented experiments, the beam was cut down to 50 μm × 50 μm to have the full beam well centered in the read-out chamber, which reduced the intensity of the incoming beam by approximately a factor of 10.

Even though the signal-to-noise ratio is reduced, the resulting SAXS profiles collected with the LabDisk for SAXS can be readily used for advanced modeling methods such as envelope determination. Figure 4.10C shows an *ab initio* bead model reconstructed from the LabDisk for SAXS data (5.5 mg ml$^{-1}$, 250 mM NaCl) using a P222 symmetry overlaid with the atomic structure of the GI tetramer (PDB ID. 1OAD). The SAXS model and high-resolution structure overlap well, showing that despite the increased noise, the disk data can be used to determine an accurate solute envelope *ab initio*. A comparison of the predicted scattering profile from the atomic structure and the experimental data is shown in Figure 4.10C,
demonstrating the very good fit of the atomic structure of tetrameric GI to the LabDisk for SAXS data ($\chi^2=0.75$, eqn (A4.1)).

4.7.2 Dilution matrix for structural screening of protein structures

The LabDisk for SAXS provides effective means of screening multiple conditions with a minimal amount of both protein sample and buffer solutions. As a first case study, the impact of increased salt concentration on the repulsive intermolecular interaction between GI tetramers in solution was investigated. From the difference profiles obtained using the disk, the $R_g$ was computed for each different condition (protein and salt concentration) plotted in Figure 4.11A.
Figure 4.10: SAXS data for glucose isomerase. SAXS curves of glucose isomerase collected on the LabDisk for SAXS without salt at different concentration (black: 1.8 mg ml\(^{-1}\), red: 3.7 mg ml\(^{-1}\), green: 5.5 mg ml\(^{-1}\), violet: 11 mg ml\(^{-1}\)) (A). Comparison of the SAXS curves of GI collected with the SAXS disk (black) and with the sample changer (red) (GI: 5.5 mg ml\(^{-1}\), no salt) (B). Curve collected on the LabDisk for SAXS (black) (GI: 5.5 mg ml\(^{-1}\), 250 mM NaCl) used to compute \textit{ab initio} the envelope shown in purple. The \textit{ab initio} model overlaps with the atomic structure used to compute the theoretical scattering pattern (red) (C).
A clear decrease of the computed $R_g$ is observed when the protein concentration increases: from 3.3 nm at 1.8 mg ml$^{-1}$ to 2.4 nm at 11 mg ml$^{-1}$. Such behavior is characteristic of systems showing strong intermolecular repulsion. This observed decrease in $R_g$ with protein concentration is significantly reduced in the presence of salt. At a GI concentration of 5.5 mg ml$^{-1}$ the apparent $R_g$ increases from 3.1 to 3.3 nm as the salt concentration is increased to 250 mM (Figure 4.11B), in agreement with the $R_g$ observed for dilute GI (<5.5 mg ml$^{-1}$) in the absence of salt. At lower protein concentrations no large variation of the $R_g$ is seen upon salt addition, presumably due to the effective protein concentration being below the critical value required for significant intermolecular repulsion to be observed.

The change in the apparent $R_g$ of GI tetramers determined from the LabDisk for SAXS data is clearly due to a partial ordering of the protein molecules in solution, characteristic of repulsive intermolecular interaction. Interactions between proteins in solution are readily seen by SAXS, notably in the low angle region ($s < 0.1$ nm$^{-1}$, where the observed scattering profile deviates from that of the form factor (the curve of an ideal solution at infinite dilution). Repulsion between the solutes results in a decrease in the SAXS signal at small angle. We applied a Guinier approximation to detect changes at low momentum transfers. From the resulting change in $R_g$, interactions between the solutes can be deduced, e.g. a decrease in the apparent $R_g$ corresponds to a stronger repulsion between the solutes.

As glucose isomerase has an overall negative surface charge at neutral pH (pI $\sim 3$), the repulsive interaction observed here can be attributed to electrostatic repulsion between the charged GI tetramers in solution. These repulsions are modulated by the addition of salts, which screen the charges of the protein:

- When the protein concentration increases (i.e. distance between solutes decreases), the interaction between the solutes increases, resulting in the decrease of the apparent $R_g$.

- At intermediate protein concentration (5.5 mg ml$^{-1}$), the repulsions are still present and affect the $R_g$. When the salt concentration increases, the charge of the solute is screened, there is less repulsion between the solutes and the apparent $R_g$ increases.

- For a lower concentration, the distances between the proteins become too large to see the interaction and no clear effect of the salt is observed.
Table 4.3: Concentration of protein and NaCl in the different read-out chambers as used in the dilution matrix for glucose isomerase. Read-out chamber are numbered from right to left.

<table>
<thead>
<tr>
<th>Read-out chamber</th>
<th>Protein concentration (mg ml(^{-1}))</th>
<th>NaCl concentration (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11.0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0.0</td>
<td>250</td>
</tr>
<tr>
<td>3</td>
<td>1.8</td>
<td>333</td>
</tr>
<tr>
<td>4</td>
<td>3.7</td>
<td>250</td>
</tr>
<tr>
<td>5</td>
<td>5.5</td>
<td>83</td>
</tr>
<tr>
<td>6</td>
<td>0.0</td>
<td>83</td>
</tr>
<tr>
<td>7</td>
<td>1.8</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>3.7</td>
<td>167</td>
</tr>
<tr>
<td>9</td>
<td>5.5</td>
<td>250</td>
</tr>
<tr>
<td>10</td>
<td>1.8</td>
<td>83</td>
</tr>
<tr>
<td>11</td>
<td>3.7</td>
<td>0</td>
</tr>
<tr>
<td>12</td>
<td>0.0</td>
<td>333</td>
</tr>
<tr>
<td>13</td>
<td>5.5</td>
<td>0</td>
</tr>
<tr>
<td>14</td>
<td>0.0</td>
<td>167</td>
</tr>
<tr>
<td>15</td>
<td>1.8</td>
<td>250</td>
</tr>
<tr>
<td>16</td>
<td>3.7</td>
<td>83</td>
</tr>
<tr>
<td>17</td>
<td>5.5</td>
<td>167</td>
</tr>
<tr>
<td>18</td>
<td>1.8</td>
<td>167</td>
</tr>
<tr>
<td>19</td>
<td>0.0</td>
<td>0</td>
</tr>
<tr>
<td>20</td>
<td>3.7</td>
<td>333</td>
</tr>
</tbody>
</table>
Figure 4.11: Apparent $R_g$ or glucose isomerase from one dilution matrix of the LabDisk for SAXS. The liquid volume in each read-out chamber was 123 nl. Apparent $R_g$ under varying NaCl concentration (black: 0 mM, red: 83 mM, green: 167 mM, yellow: 250 mM, blue: 333 mM) plotted in function of the protein concentration (A). Apparent $R_g$ computed for protein concentration of 5.5 mg ml$^{-1}$ in function of the salt concentration. Error bars are estimated as 0.1 nm. Results measured with the standard sample changer are given in light grey (B).
4.8 Summary & conclusion

We have presented a SAXS sample preparation platform. The LabDisk for SAXS contains six dilution matrices on one disk. Each dilution matrix automatically generates 20 different measurement conditions, from only 2.5 μl of protein solution. We have showed high performance aliquoting, combination, mixing and read-out with less than 5% CV in protein concentration. We demonstrated aliquoting for a range of liquids with typical contact angles and viscosities on the LabDisk for SAXS. Liquid properties compatible to the LabDisk for SAXS are mainly limited due to the aliquoting principle, which is based on geometric valves. A redesigned LabDisk for SAXS could make use of newer aliquoting principles, which are compatible to a wider range of liquids, e.g. the recently presented centrifugo-pneumatic multi-liquid aliquoting, which is based on pneumatic valves, and was demonstrated to offer robust aliquoting for contact angles down to 0° and viscosities up to 4.1 mPa s [11]. The presented LabDisk for SAXS is ideal for fast screening with a limited amount of sample volume to find the optimal sample conditions before eventually measuring in a low background dedicated setup that provides data with less noise but requires larger volumes of protein samples. Although the SAXS data collected with the LabDisk for SAXS are noisier than data collected with the in-vacuum flow cell of the sample changer, they can be used for basic and even advanced data analysis methods. Only 2.5 μl of protein stock solution was required for the whole dilution matrix. In comparison, the same experiments using the conventional SAXS sample changer at the BioSAXS beamline P12 would require 110 μl of protein sample volume (assuming 20 μl total volume per measurement condition). The new screening possibilities were demonstrated to monitor repulsive interactions of GI tetramers in function of both protein and NaCl concentration.

For the future, we plan to optimize the integration of the LabDisk for SAXS platform in the BioSAXS beamline P12. This includes automated positioning, assisted by image recognition, which will reduce the time per measurement to 3–5 s. Furthermore, we have fabricated 200 LabDisks for SAXS with a total of 1200 dilution matrices. These disks will be made available to regular users of the P12 beamline (PETRA III, DESY).
5 Multi-liquid aliquoting

This chapter addresses aliquoting in centrifugal microfluidics and specifically describes a new unit operation for the parallel two-stage aliquoting and combination of multiple liquids on a single fluidic layer. The first section is an excerpt from the review paper: “Centrifugal microfluidic platforms: advanced unit operations and applications”. The author of this thesis was responsible for literature search and for writing this section. The co-authors supported the work on the section via proof-reading and scientific advice. In the second section the new unit operation for centrifugo-pneumatic multi-liquid aliquoting is presented.

5.1 State of the art in centrifugal microfluidic aliquoting

Most microfluidically integrated applications require precise input volumes of liquids in order to obtain quantitatively reproducible results. Consequently, unit operations for the metering of liquid volumes are widely employed. Splitting an input liquid volume into multiple defined sub-volumes is referred to as aliquoting, which mostly involves multiple parallel metering steps. Aliquoting itself was subcategorized by Mark et al. into one-stage and two-stage aliquoting (Figure 5.1). The latter refers to a microfluidic aliquoting process in which single aliquots are transferred into fluidically separated chambers after metering [108]. The embodiments of centrifugal microfluidic unit operations for metering and aliquoting are listed in Table 5.1. In the simplest case, a metering structure consists of a connection channel to an inlet, a metering chamber with a defined volume and an overflow to a waste chamber for excess volume (Figure 5.1). The metering can be combined with valves at the radially outer end of the metering chamber to allow for further fluidic processing. The demonstrated valves include hydrophobic [122], capillary siphon [124] and centrifugo-pneumatic valves [107]. The metering accuracy is mainly affected by the variation of the cavity size within the fabrication tolerances [108] and the wicking effects at liquid interfaces due to capillary forces [124]. Capillary forces (eqn (2.12)) can be counteracted by centrifugal forces (eqn (2.5)), which leads to a high metering accuracy in centrifugal microfluidics even at nanoliter volumes. In single-stage aliquoting, fluid volumes are metered directly into the receiving chamber. Thus, the aliquoting process simply involves the transport of the liquid from an inlet into multiple receiving chambers, while the excess is gated into an overflow. As mentioned by Mark et al., single stage aliquoting bears the
problem that adjacent aliquots may still be connected by a liquid film. For example, if reagents are pre-stored within the receiving chambers, this fluidic connection can lead to cross-contamination [108] (Figure 5.1b). To avoid cross-contamination during digital PCR, Sundberg et al. used a mineral oil to fill the microfluidic channel and separate the aliquoted volumes after the aliquoting process [121].

Two-stage aliquoting allows for full fluidic separation between adjacent aliquots, and therefore is usually applied when cross-contamination is an issue [13], or when further fluidic processing of the individual aliquots is required. Two-stage aliquoting combines the parallel metering of one-step aliquoting with normally closed valves at the radial outer side of each metering finger. After metering, the single aliquots can pass the valve and be used for further fluidic processing [122, 87].

**Figure 5.1:** Centrifugal microfluidic unit operations for metering and aliquoting. (a) Basic principle of metering. A liquid fills a metering chamber with a defined volume. The excess is gated into a waste chamber. The metered volume can subsequently be transferred into the microfluidic network via suitable valves. (b) Different aliquoting concepts. Part b of the figure is from Mark et al. [108].
Table 5.1: Centrifugal microfluidic unit operations for metering and aliquoting.

<table>
<thead>
<tr>
<th>Ref.</th>
<th>Integrated valve type</th>
<th>Aliquoted volume</th>
<th>CV (%)</th>
<th>Number of parallel aliquots</th>
</tr>
</thead>
<tbody>
<tr>
<td>Schembri C. T. et al. [85]</td>
<td>No valve</td>
<td>Not reported</td>
<td>&lt;2</td>
<td>4 or 21</td>
</tr>
<tr>
<td>Sundberg S. O. et al. [121]</td>
<td>No valve</td>
<td>33 nl</td>
<td>16</td>
<td>1000</td>
</tr>
<tr>
<td>Andersson P. et al. [122]</td>
<td>Hydrophobic valve</td>
<td>200 nl</td>
<td>0.75</td>
<td>112</td>
</tr>
<tr>
<td>Andersson P. et al. [122]</td>
<td>Hydrophobic valve</td>
<td>20 nl</td>
<td>1.90</td>
<td>1</td>
</tr>
<tr>
<td>Mark D. et al. [108]</td>
<td>Centrifugo-pneumatic valve</td>
<td>6–10 μl</td>
<td>2.2–3.6</td>
<td>8 or 16</td>
</tr>
<tr>
<td>Steigert J. et al. [124]</td>
<td>Capillary siphon</td>
<td>500 nl</td>
<td>&lt;5</td>
<td>1</td>
</tr>
<tr>
<td>Schwemmer F. et al. [44]</td>
<td>Capillary valve</td>
<td>40 nl</td>
<td>1–5.5</td>
<td>120</td>
</tr>
<tr>
<td>Li G. et al. [35]</td>
<td>Capillary valve</td>
<td>31 nl</td>
<td>2.80</td>
<td>24</td>
</tr>
<tr>
<td>Hwang H. et al. [87]</td>
<td>Ferrowax-based microvalves</td>
<td>100 μl</td>
<td>Not reported</td>
<td>5</td>
</tr>
</tbody>
</table>
Centrifugo-pneumatic multi-liquid aliquoting – parallel aliquoting and combination of multiple liquids in centrifugal microfluidics

Frank Schwemmer, Tobias Hutzenlaub, Dirk Buselmeier, Nils Paust, Felix von Stetten, Daniel Mark, Roland Zengerle, Dominique Kosse

- The following sections have identically been published as an original research paper in Lab on a Chip in 2015 (Volume: 15, Pages: 3250 - 3258). In contrast to the published manuscript, the numbering of sections, figures, tables and references was adapted to the overall numbering of this dissertation. Figures and formulas from the electronic supplementary material were included in the main text to improve readability. The introduction section was shortened to reduce redundancies with section 5.1. The abstract was adapted and included in the abstract section of this thesis.

Contributions to this publication:

- Frank Schwemmer: Literature search and analysis, conceived the idea for the aliquoting principle, designed the microfluidic integration, planned and performed all microfluidic experiments, programmed the image recognition for the analysis of aliquoted volumes, analyzed the experiments and wrote the manuscript.

- Tobias Hutzenlaub: Set up the network simulations, performed the network simulations and conducted the contact angle measurements.

- Dirk Buselmeier, Dominique Kosse: Developed the hot-embossing processes for the fabrication of disks.

- Dirk Buselmeier, Nils Paust, Felix von Stetten, Daniel Mark, Roland Zengerle, Dominique Kosse: Scientific advice during experiments and manuscript preparation, proof-reading of the manuscript.
Preface

The following sections describe a new centrifugo-pneumatic unit operation for the parallel two-stage aliquoting of multiple liquids on a single fluidic layer. One drawback of the LabDisk for SAXS presented in the previous chapter is the multi-layered fluidics and the resulting high fabrication cost per dilution matrix. This cost limits the use of the LabDisk for SAXS to the high-cost application at synchrotron beamlines. At a lower cost per disk, the LabDisk for SAXS would be relevant for the larger market segment of automation for X-ray sources in regular laboratories. However, so far there are no unit operations dedicated to aliquoting of multiple liquids in a single fluidic layer in centrifugal microfluidics.

Therefore one goal of this thesis was to develop a new unit operation for aliquoting multiple liquids on a single fluidic layer. This is challenging since liquids in centrifugal microfluidics are typically moved radially outwards, which leads to crossing channels if multiple aliquoting structures are used in parallel. The new unit operation should therefore (1) allow aliquoting in both the radially outwards and the radially inwards direction, be compatible to (2) a similar or greater variety of liquid properties as the centrifugo-pneumatic aliquoting, (3) facilitate scalable fabrication via both thermoforming and injection molding and (4) not be dependent on external means or surface coatings.

The developed multi-liquid aliquoting is now used in various projects, including the development of a LabDisk for the detection of botulinum neurotoxin (ZIM project “BoNT Disk”, project number KF2146328MD3), a LabTube for the point-of-care detection of Group B Streptococcus (BMBF project “Kreißsaal-Lab”, project number 031A214C) and is a key feature in several industry projects.
5.2 Introduction

As stated before, centrifugal microfluidics enables the automation, miniaturization and parallelization of laboratory workflows [8, 42, 9]. In order to attain reproducible performances in such assays, the accurate metering of multiple liquid sub-volumes – the so-called aliquoting – is essential. As described above, aliquoting principles can be categorized in one-stage aliquoting and two-stage aliquoting. One-stage aliquoting has been used for diverse areas such as digital PCR [121] or chemical analysis [85]. However, it is limited to the 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 the transport of the liquid after metering. Besides ensuring a full separation of liquids, e.g. for geometric multiplex PCR [119], two-stage aliquoting allows further liquid processing after metering. Typical valves for two-stage aliquoting are hydrophobic valves [159, 122, 160] or geometric valves [83, 161, 44]. 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 a complex tooling for injection molding. For hydrophobic valves, localized surface modifications are introduced by an additional surface coating during the manufacturing of the cartridge. Centrifugo-pneumatic aliquoting [108, 107] 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, 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 residues of the first liquid, which can lead to undesired premature reactions outside of the collection chambers [162].

For assays where liquids of different volumes need to be combined in one reaction, the centrifugo-pneumatic valves can be combined with dissolvable films [51, 50, 99]. Dissolvable films require a multi-layer cartridge by design and need additional fabrication steps for the integration of the dissolvable films during fabrication. Aliquoting based on laser actuated ferrowax valves [163, 87] is also not limited with respect to contact angles or surface tensions. However, additional process steps are required for the integration of the wax
valves, and the respective processing device needs to include a laser for melting the ferrowax valves.

So far, no aliquoting principle is available for the aliquoting of multiple liquids in a single fluidic layer, which guarantees liquid separation till the collection chamber. A probable reason for no such aliquoting principle being available is the traditional radially outwards transport of liquids in combination with fundamental geometric limitations of crossing channels (see Figure 5.2). With new unit operations for pneumatic pumping it now becomes possible to overcome such geometric limitations [164, 165, 53, 94, 49, 48]. In this manuscript we introduce centrifugo-pneumatic multi-liquid aliquoting. This aliquoting principle allows aliquoting in both the radially outwards and the radially inwards direction. Centrifugo-pneumatic multi-liquid 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 [22, 166, 104, 100], which has recently been extended to centrifugo-dynamic inward pumping [53]. 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.

Figure 5.2: This figure illustrates the limitations of aliquoting and combining multiple liquids in a single layer for the case that liquid transport is restricted to radially outwards transport. More explicitly, if liquid transport is restricted to outwards transport, pairwise combination in more than two collection chambers from two aliquoting structures becomes impossible within one fluidic layer and without premature contact of the liquids outside of the collection chambers.
Centrifugo-pneumatic multi-liquid aliquoting can be positioned on a wide range of radial positions. Especially noteworthy is the fact 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 [53]. 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, this form of aliquoting allows for a 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. the 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 provide design rules that can be used for the layout of centrifugo-pneumatic multi-liquid aliquoting.

5.3 Aliquoting principle

The goal of 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 Figure 5.3. The principle of metering for an inner and an outer aliquoting structure and its implementation in centrifugal microfluidics is depicted in Figure 5.4. It consists of a metering chamber connected with a pneumatic chamber 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 (Figure 5.4A) until the pneumatic overpressure is equal to the centrifugal pressure (Figure 5.4B). 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 (Figure 5.4C) [53]. 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 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.

\[ \text{Figure 5.3: The process chain of centrifugo-pneumatic multi-liquid aliquoting consists of the unit operations inner and outer aliquoting. Each aliquoting structure comprises an inlet and multiple metering chambers connected to the inlet. Each aliquot from the outer aliquoting is combined with a complimentary aliquot from the inner aliquoting within a collection chamber. The principle of the metering structures to be implemented in centrifugal microfluidics is depicted in Figure 5.4.} \]
Figure 5.4: 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 the 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.

An aliquoting structure can be realized by connecting one inlet with multiple of the described metering structures (see Figure 5.3). 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 Figure 5.5.
Figure 5.5: Microfluidic disk with 10 inner aliquoting structures and two outer aliquoting structures. The microfluidic disk (upper left) includes two outer aliquoting structures with 20 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 Figure 5.4. Green arrows indicate liquid flow at selected locations. A detailed description of the implemented channels and chambers is given in Figure 5.7. An experimental run of the implemented design using colored dye can be seen in Figure 5.8.
5.4 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. Figure 5.6 illustrates the individual volumes. The volume aliquoted into the collection chamber \( V_{\text{ali}} \) can be calculated by:

\[
V_{\text{ali}} = V_{\text{meter}} + V_{\text{out}} - V_{\text{bf}}
\]

where \( V_{\text{meter}} \) is the volume of the metering chamber, \( V_{\text{out}} \) is the liquid volume in the transfer channel during the metering phase and \( V_{\text{bf}} \) 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:

\[
V_{\text{bf}} = V_{\text{meter}} \frac{R_{\text{in}}}{R_{\text{out}}}
\]

\( R_{\text{in}} \) and \( R_{\text{out}} \) 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 \( R_{\text{in}} \) should be much higher than the fluidic resistance \( R_{\text{out}} \) of the transfer channel. For the layout of the outer aliquoting presented in this manuscript, it can be stated that \( R_{\text{in}} > 100 R_{\text{out}} \), 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 transfer of liquid to the collection chamber during the metering step, the pneumatic overpressure in the pneumatic chamber during the metering phase is balanced by the applied centrifugal pressure. The pneumatic overpressure in the pneumatic chamber during metering is:

\[ p_{\text{pneu}} = p_0 \frac{V_{\text{meter}} + V_{\text{pneu}}}{V_{\text{pneu}} + V_{\text{meter}}} - (V_{\text{input}} - V_{\text{in}} - V_{\text{out}}) - p_0 \]  

where \( V_{\text{pneu}} \) is the volume of the pneumatic chamber and \( V_{\text{input}} \) is the liquid volume pipetted by the user into the inlet per aliquot. The liquid volume remaining in the inlet channel is \( V_{\text{in}} \), and \( p_0 \) is the ambient pressure. The maximal centrifugal pressure before overflow into the collection chamber is given by:

\[ p_{\text{cent}} = 2 \pi^2 \rho f_{\text{max}}^2 (r_{\text{of}}^2 - r_{\text{out}}^2) \]  

where \( f_{\text{max}} \) is the maximal rotational frequency supported by the processing device, \( r_{\text{of}} \) is the position of the meniscus at the connection of the metering chamber to the pneumatic chamber and the \( r_{\text{out}} \) is the radial most inwards position of the transfer channel. The density of the liquid to be aliquoted is \( \rho \). In a steady state, the pneumatic pressure of eqn (5.3) is equal to the centrifugal pressure of eqn (5.4). From this equation, the minimum volume of the pneumatic chamber with no liquid overflow to the collection chamber can be derived:
\[ V_{\text{pneu}} = V_{\text{input}} - V_{\text{in}} - V_{\text{out}} - V_{\text{meter}} + \frac{p_0(V_{\text{input}} - V_{\text{in}} - V_{\text{out}})}{p_{\text{cent}}} \]

As shown previously, to ensure high pump efficiencies, the upper boundary for the combined size of the pneumatic chamber \( V_{\text{pneu}} \) and the metering chamber \( V_{\text{meter}} \) is four times the volume of the input liquid \( V_{\text{input}} \) [53].

### 5.5 Materials & methods

The microfluidic disk was designed using SolidWorks (Dassault Systèmes SOLIDWORKS Corp.). The 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, dried with compressed air and resealed. Contact angles were measured using a contact angle meter (OCA 15+, DataPhysics Instruments GmbH). For the experiments with aliquoted oil, Fluorinert Electronic Liquid FC-40 was used (3M Company).

The disks were processed in a prototype LabDisk Player (Qiagen Lake Constance GmbH, Germany), which was modified to include a stroboscopic setup [117] for monitoring 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 the precise quantification of the aliquoted liquid volume (see Figure 5.9). 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. [167].
Figure 5.7: Implemented microfluidic design including labels for channels, chambers and other features relevant to the microfluidic design.

Figure 5.8: Experiment showing the 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 Figure 5.4. Every second collection chamber is combined with a volume quantification structure (a) for the measurement of aliquoted volumes. The volume quantification structure is described in detail in Figure 5.9.
5 Multi-liquid aliquoting

Figure 5.9: Volume quantification structure for the 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.

5.6 Experiments

The implementation of the aliquoting process described in this manuscript is depicted in Figure 5.5. The liquid properties of the sample liquids chosen for the characterization of aliquoting can be seen in Table 5.2. The target aliquoting volume is 5 μl. Each inlet of the inner aliquoting structure is filled with 25 μl for generating 4 aliquots. The outer aliquoting is filled with 146 μl for generating 20 aliquots. After filling, the disk is rotated at 90 Hz with an acceleration of 5 Hz s⁻¹. At this high 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 (see Figure 5.10). The used acceleration was 5 Hz/s. Minimum acceleration is typically not critical, since the high fluidic resistance of the inlet channel ensures high
pressure loss and little flow during loading. Very low acceleration rates would be critical because then the pressure loss would not be sufficient to restrict the flow, and the liquid level would rise above the siphon crest, with liquid flowing to the collection chambers prior to metering. The minimum required acceleration was determined via network simulations to be 0.25 Hz/s for inner and 0.5 Hz/s for outer aliquoting.

After the 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 reading out the aliquoted volumes, we chose a rotational frequency of 50 Hz. This ensures a flat meniscus for a precise measurement of volume in the volume quantification structure (Figure 5.9). 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 for outer aliquoting. Photographs from an experiment with a dyed sample are shown in Figure 5.8.

**Table 5.2:** Liquid properties of the aliquoted liquids.

<table>
<thead>
<tr>
<th>Liquid</th>
<th>Viscosity at 25 °C in mPa·s</th>
<th>Density in kg / m³</th>
<th>Advancing contact angle</th>
<th>Receding contact angle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Di water (0.1% Tween 20 v/v)</td>
<td>0.89</td>
<td>1000</td>
<td>62.5° ± 4.8°</td>
<td>12.0° ± 3.2°</td>
</tr>
<tr>
<td>Ethanol</td>
<td>1.1</td>
<td>789</td>
<td>42.7° ± 7.1°</td>
<td>7.1° ± 1.5°</td>
</tr>
<tr>
<td>Fluorinert FC-40</td>
<td>4.1</td>
<td>1855</td>
<td>19.0° ± 1.0°</td>
<td>0°</td>
</tr>
</tbody>
</table>
Figure 5.10: Frequency protocols used for high and low viscosity liquids.
5.7 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 the automation of laboratory assays, variation in the results of centrifugo-pneumatic multi-liquid aliquoting should be within these boundaries. To quantify how robustly centrifugo-pneumatic multi-liquid aliquoting works under varying conditions, we tested both 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 5.2).

We quantify variation as overall variation, intra-run variation and inter-run variation. The overall variation is the coefficient of variation of all measurements of either inner or outer aliquoting for a given liquid and input volume. For the quantification of variation 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. Variation within individual runs can be quantified by intra-run CVs, which is the coefficient of variation within one individual run. Coefficients of variation were calculated using the following formulas. The parameters M and N give the total number of runs and the total number of aliquots within one run, respectively. The volume \( V_{m,n} \) is the volume of aliquot \( n \) in run \( m \).

Overall CV:
\[
CV_{\text{Overall}} = \frac{1}{\sqrt{(M N - 1) \sum_{m=1}^{M} \sum_{n=1}^{N} (V_{m,n} - \bar{V})^2}}
\]

Inter-run CV:
\[
CV_{\text{Inter\ run}} = \sqrt{\frac{1}{M - 1} \sum_{m=1}^{M} (\bar{V}_m - \bar{V})^2}
\]

Intra-run CV:
\[
CV_{\text{Intra\ run, m}} = \sqrt{\frac{1}{N - 1} \sum_{n=1}^{N} (V_{m,n} - \bar{V}_m)^2}
\]

Mean volume of run \( m \):
5 Multi-liquid aliquoting

\[ \bar{V}_m = \frac{1}{N} \sum_{n=1}^{N} V_{m,n} \quad 5.9 \]

Overall mean volume:

\[ \bar{V} = \frac{1}{NM} \sum_{m=1}^{M} \sum_{n=1}^{N} V_{m,n} \quad 5.10 \]

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 inner aliquoting (outer aliquoting). This means both the inner 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 5.3.
Table 5.3: Aliquoting variation for the centrifugo-pneumatic multi-liquid aliquoting. 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 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 [168]. The given intra-run CVs are the mean values 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 volume 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.

<table>
<thead>
<tr>
<th></th>
<th>Input volume</th>
<th>Mean volume in µl</th>
<th>Systematic error</th>
<th>Overall CV</th>
<th>Intra-run CV</th>
<th>Inter-run CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inner aliquoting</td>
<td>0.1% Tween 20 default</td>
<td>5.08</td>
<td>1.6%</td>
<td>0.6%</td>
<td>0.6%</td>
<td>1.1%</td>
</tr>
<tr>
<td></td>
<td>+ 10%</td>
<td>5.09</td>
<td>1.9%</td>
<td>0.5%</td>
<td>0.5%</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>- 10%</td>
<td>5.06</td>
<td>1.1%</td>
<td>0.3%</td>
<td>0.3%</td>
<td>-</td>
</tr>
<tr>
<td>Ethanol</td>
<td>default</td>
<td>5.06</td>
<td>1.2%</td>
<td>1.1%</td>
<td>1.0%</td>
<td>1.2%</td>
</tr>
<tr>
<td>Outer aliquoting</td>
<td>0.1% Tween 20 default</td>
<td>5.07</td>
<td>1.5%</td>
<td>1.4%</td>
<td>0.9%</td>
<td>1.1%</td>
</tr>
<tr>
<td></td>
<td>+ 10%</td>
<td>5.09</td>
<td>1.8%</td>
<td>0.6%</td>
<td>0.6%</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>- 10%</td>
<td>5.11</td>
<td>2.1%</td>
<td>0.5%</td>
<td>0.5%</td>
<td>-</td>
</tr>
<tr>
<td>Ethanol</td>
<td>default</td>
<td>5.08</td>
<td>1.6%</td>
<td>0.9%</td>
<td>0.6%</td>
<td>0.9%</td>
</tr>
<tr>
<td>FC-40</td>
<td>default</td>
<td>5.10</td>
<td>2.0%</td>
<td>1.0%</td>
<td>0.8%</td>
<td>0.5%</td>
</tr>
</tbody>
</table>

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 variation in the range between 20 and 200 µl (ISO 8655-5:2002). In order for a microfluidic aliquoting structure to be robust, it should also tolerate additional variation 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 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- and underfilling (see Table 5.3). Thus, the aliquoting principle tolerates variation in input volumes of at least ± 10%. We determined the maximum permissible variation in input volumes via a network...
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simulation based approach [53, 110]. 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 design presented, 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. 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 (Figure 5.11, 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 the experiments. The corresponding run (Figure 5.11, run no. 9) shows results of the 8 remaining aliquots. The fabrication with high precision steel tools, automated sealing and clean room conditions is expected to further improve the performance of centrifugo-pneumatic multi-liquid aliquoting (as observed by Mark et al. [108] when comparing aliquoting CVs for prototyping and injection molding).
Figure 5.11: 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 5.3. Inner aliquoting (upper row) and outer aliquoting (lower row) were performed for three different liquids (see Table 5.2) of varying contact angles, densities and viscosities. The overfilling by +10% volume and the underfilling by −10% volume confirm the robustness of the aliquoting against different input volumes. The 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.
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5.8 Summary & conclusion

We have introduced centrifugo-pneumatic multi-liquid aliquoting, a new unit operation for metering and aliquoting multiple liquids in centrifugal microfluidics. The aliquoting principle is solely based on pneumatic pressures, centrifugal pressures and viscous dissipation. We have implemented 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 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.

Centrifugo-pneumatic multi-liquid aliquoting is compatible with standard fabrication technologies and does not require sharp corners or surface modifications. Furthermore, we have shown that the aliquoting principle is compatible with a variety of liquids and robust against changes in density and viscosity by more than a factor of two and four, respectively. The aliquoting process 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 aliquoting principles available. Lastly, the structures presented tolerate variation in input volumes by at least ± 10%. For all liquids and volumes tested, 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 the timed sequential addition of metered reagents [110]. Reagent pre-storage can be included by the use of miniature-stick-packs [169], which can be introduced directly in the inlets to further reduce the number of required pipetting steps. Together with centrifugal step emulsification, the aliquoting principle could be used to combine multiple RPA master-mixes with a shared Mg²⁺ solution, prior to the droplet generation for digital droplet RPA [2]. We expect the new aliquoting process to be useful for the 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 the multiparameter analysis of a single sample with different liquid reagents.
6 The microfluidic timer

Frank Schwemmer, Steffen Zehnle, Daniel Mark, Felix von Stetten, Roland Zengerle and Nils Paust

- The following sections have identically been published as an original research paper in Lab on a Chip in 2015 (Volume: 15, Pages: 1545 - 1553). In contrast to the published manuscript, the numbering of sections, figures, tables and references was adapted to the overall numbering of this dissertation. Equations already named in the section fundamentals were deleted from this section and referenced. The abstract was adapted and included in the chapter abstract of this thesis.

Contributions to this publication:

- Frank Schwemmer: Literature search and analysis, conceived the idea for the microfluidic timer, designed the microfluidics, planned, performed and analyzed all experiments, performed the simulations, wrote the manuscript.

- Steffen Zehnle: Set up the network simulations for the fluidics.

- Steffen Zehnle, Daniel Mark, Felix von Stetten, Roland Zengerle and Nils Paust: Scientific advice during experiments and manuscript preparation, proof-reading of the manuscript.
Centrifugo-pneumatic unit operations are one of the major trends in centrifugal microfluidics. In centrifugo-pneumatic unit operations, centrifugal pressure is used to compress air in specially designed pneumatic chambers. When the rotational frequency is reduced, this stored pneumatic energy can be used to pump liquid within the cartridge. This principle has been successfully applied by Zehnle et al. for the inward pumping of liquids [53]. Other groups have presented unit operations for valving [22] and mixing [170]. Furthermore, in chapter 5 an aliquoting principle based on centrifugo-pneumatics was demonstrated. However, all of these unit operations have in common that the release of the pneumatic energy starts immediately with the reduction of the rotational frequency, which can be a limiting factor in the integration of complex laboratory workflows.

One goal of this thesis is to add time control to centrifugo-pneumatic unit operations. For this the microfluidic timer was developed, a unit operation that stores pneumatic energy, which is then suddenly released at a defined time after a reduction in rotational frequency. The time at which the pneumatic energy is released can be engineered by the size of the pneumatic chambers and fluidic resistances. The microfluidic timer can be used as a simple addon for many previously presented centrifugo-pneumatic unit operations. It can be included by simply splitting an existing pneumatic chamber into two pneumatic chambers and adding a fluidic resistance in between the two chambers.

The microfluidic timer has been used in a recently published process chain for “centrifugo-pneumatic sedimentation, re-suspension and transport of microparticles” [109]. Furthermore, the microfluidic timer is now used in multiple LabTube cartridges developed for various applications, including the point-of-care detection of Group B Streptococcus (BMBF project “Kreißsaal-Lab”, project number 031A214C), the point-of-care nucleic acid extraction for the detection of sepsis (BMBF project “EasyTube”, project number 16SV5455) and
6.1 Introduction

Centrifugal microfluidics is a powerful tool for the automation of bio-chemical assays [6–8, 42] with significant advantages when compared to other microfluidic automation concepts: artificial gravity by centrifugation inherently removes bubbles that might interfere with a suitable assay performance, and the centrifugal propulsion allows for the automation of complex assay protocols without any interfaces to external valves and pumps. One important challenge, however, is the automation of the precise timing of fluidic operations, such as reagent supply or valving after an incubation period. The timed addition of reagents for example is required for diverse assay types such as immunoassays, DNA/RNA extraction and amplification, DNA sequencing etc.

In centrifugal microfluidics, timed valving independent of the rotational frequency protocol has been demonstrated using valves actuated by external lasers or infrared light sources [93, 171, 94, 163], external pressure sources [48, 96] or external mechanical actuation [172]. While active valves allow for elegant fluidic automation, the trade-offs are a more complex processing device and in most cases additional fabrication steps for the production of the disposable cartridge.

Timing without employing external means, also referred to as passive timing, can be realized by employing capillary forces. Such passive timing is commonly used in capillary flow-based microfluidics, by designing fluidic resistances in combination with surface tension-based passive pumping or geometric valves [173, 174].

In centrifugal microfluidics, passive valves are typically triggered at increasingly high rotational frequencies. For this purpose, geometric valves [175], hydrophobic patches [160] and centrifugo-pneumatic valves [107, 50, 99] are used. Another passive solution combines a geometric valve triggered at high rotational frequencies with a siphon valve primed by capillary forces at low rotational frequencies [176, 18]. Furthermore, the recently introduced miniature-stick-packs can be used for the sequential release of pre-stored liquids at predefined rotational frequencies [169]. However, all of these passive valves for timed release strongly depend on fabrication tolerances. The geometric valves and hydrophobic patches additionally depend on capillary forces. The accurate control of such dependencies is challenging and leads to considerable variation in rotational burst frequencies, as for
example discussed by van Oordt et al. [169] for reagent release. For a sequential supply of reagents according to the assay protocol, the trigger frequencies have to be sufficiently high to prevent pre-mature release, for example during transport or storage of the cartridge; the frequencies must increase with the release sequence and must not overlap with respect to its variations. As a consequence, the sequential release of more than three liquids is a challenging task, in particular if a robust operation within cartridges that are compatible to cost-efficient mass fabrication technologies is required.

Recently, a new type of passive timing independent of the rotational frequency based on dissolvable films was introduced [50]. The dissolvable films are used to either block a fluidic path or an air vent. Upon contact with the liquid, the film starts to dissolve and after a certain time period, the path opens. By means of a clever combination of dissolvable films with liquid and air routing, Kinahan et al. demonstrated the automation of up to 10 sequential valving steps [51]. However, the fabrication of such cartridges requires an extra fabrication step for introducing the dissolvable films, and the dissolvable film is dissolved within the assay reagents. While the authors could show that for a PCR-based assay and an immunoassay, the dissolved film does not impact the results [50], the use of such valves changes the composition of the assay, which can lead to issues for established assays.

This paper introduces the microfluidic timer as a new fully passive fluidic unit operation for precise temporal control of valving and pumping of typical assay reagents and samples. The timer can easily be combined with any unit operation, where actuation is achieved by compression and decompression of entrapped air volumes. Examples of such pneumatic operations are mixing by reciprocating flow [100, 104], inward pumping [49, 53] or centrifugo-pneumatic cascading [22]. The principle of the microfluidic timer is based on centrifugal pressures, pneumatic pressures and viscous dissipation only. Therefore, the microfluidic timer is not restricted to any specific materials and can be fabricated monolithically with established fabrication technologies such as injection molding or thermoforming. Monolithic fabrication in this context means that the timer can be implemented within the same substrate, without additional fabrication steps, simply by structuring the timer features with the same technology as the rest of the microfluidic features.

We provide a theoretical model that can be used to accurately predict the delay time by network simulations in order to adapt release times to the specific assay needs. Additionally, the model is simplified to enable a more rough analytical prediction of delay times which in our opinion is sufficient for most implementations. The model is validated by comparing
simulated timed valving to experimental data from different liquids that cover a wide range of typical assay reagents.

Experimentally, we discuss a sequential release and a release-on-demand mode. For the latter, within a given design of a microfluidic disk hosting four different liquids, the routing of the four input liquids on-demand in any user-defined sequence is presented.

### 6.2 General fluidic principle and design rules

#### 6.2.1 Functional principle of the microfluidic timer

In theory, the timer can be applied to all microfluidic platforms [42] that provide pressure control. This paper will discuss the application of the microfluidic timer in centrifugal microfluidics, in which pressure control is achieved by centrifugation. The principle of the microfluidic timer is based on the temporary storage of pneumatic energy and the time-delayed sudden release of said energy. The main components of the timer are two pneumatic chambers which are connected to each other by a capillary with a defined fluidic resistance (Figure 6.1). To load the pneumatic energy, a loading pressure is applied to pump liquid into the pneumatic chambers compressing the entrapped air. At a critical filling, the first pneumatic chamber is overfilled; the timing channel is primed and liquid fills the second pneumatic chamber such that the timer is loaded (Figure 6.1A). Subsequently, the loading pressure is turned off and liquid is slowly pushed out of pneumatic chamber 2 by the pressurized entrapped air. The flow rate of liquid is thus limited due to the viscous pressure drop $p_{visc}$ along the timing channel (Figure 6.1B). After a pre-defined time period, all liquid has left pneumatic chamber 2, and the resistance of the timing channel suddenly changes due to the viscosity change from liquid to air (about 50 fold for water/air). At this moment, the timer is released. The flow rate suddenly increases and abruptly releases the stored pneumatic energy. The sudden energy release can be used as a trigger for valving, pumping or other operations (Figure 6.1C) as demonstrated in detail in the experimental section.
Figure 6.1: Functional principle of the microfluidic timer (upper figure) and stored pneumatic energy in the microfluidic timer during different steps of its operation (lower figure). A: Timer loading by applying a loading pressure. The air is compressed within the pneumatic chamber and the pneumatic energy is loaded. After pneumatic chamber 1 is completely filled, liquid overflows into pneumatic chamber 2. The liquid volume transferred into pneumatic chamber 2 sets the timer, which defines the length of delay time. B: Delay time. The applied loading pressure is turned off and the pneumatic pressure pushes liquid out of pneumatic chamber 2. The high viscous dissipation in the timing channel limits the flow rate. During this period, the pneumatic energy is released very slowly. C: Timer release. After the timing channel has been emptied of liquid, the flow rate temporarily increases by the viscosity ratio of liquid and gas and the pneumatic energy is abruptly released. This energy release can be employed to transport liquid e.g. for valving or pumping. One practical example for this is the priming of a siphon in centrifugal microfluidics, as further detailed in the next section (see Figure 6.2).

6.2.2 Theoretical description of the timer

This section describes how the delay time and the stored pneumatic energy of the microfluidic timer can be calculated analytically. The stored pneumatic energy \( E_{pneu} \) which is released abruptly upon timer release can be calculated by:
with \( V_{\text{pneu1}} \) as the volume of pneumatic chamber 1, and \( p_{\text{pneu}} \) as the overpressure inside the pneumatic chambers.

The delay time is the time it takes for all of the liquid to flow out of pneumatic chamber 2. The start of the delay time is defined as the time when the pressure applied for loading and setting the timer is turned off or significantly reduced.

The delay time ends at the timer release, when air replaces the liquid in the timing channel, abruptly changing the fluidic resistance and rapidly releasing the remaining stored pneumatic energy. In case the loading pressure is turned off completely, the delay time can be derived from a pressure balance between the pneumatic pressure difference \( p_{\text{pneu}} \) of the enclosed air and the viscous pressure drop \( p_{\text{visc}} \) along the timing channel:

\[
p_{\text{visc}} \left( \frac{dV_{\text{comp}}}{dt} \right) = p_{\text{pneu}} \left( V_{\text{comp}} \right)
\]

\[
R \frac{dV_{\text{comp}}}{dt} = \frac{p_{0} V_{0}}{V_{0} - V_{\text{comp}}} - p_{0}
\]

This differential equation can be solved for the delay time:

\[
\Delta t_{\text{delay}} = \frac{R V_{0}}{p_{0}} \ln \left( \frac{V_{\text{pneu1}}}{V_{\text{pneu1}} + V_{\text{loaded}}} \right) + \frac{R}{p_{0}} V_{\text{loaded}}
\]

where \( R \) represents the fluidic resistance of the timing channel, \( p_{0} \) is the atmospheric pressure, \( V_{0} \) is the total volume of pneumatic chambers 1 and 2, \( V_{\text{pneu1}} \) is the volume of pneumatic chamber 1 and \( V_{\text{loaded}} \) is the volume of liquid in pneumatic chamber 2 at the start of the delay.

For the design of the microfluidic timer, eqn (6.4) can be used to calculate the delay time (see Table 6.1 and Table 6.2). The parameters used for designing the delay time are the fluidic resistance of the timing channel, the volumes of pneumatic chambers 1 and 2 and the liquid volume in pneumatic chamber 2 at the start of the delay. The fluidic resistance \( R \) of a rectangular channel is given by eqn (2.9).
The microfluidic timer

Table 6.1: Implementations of the microfluidic timer in the microfluidic disk. The length of the timer channel was 13.8 mm. The volume of pneumatic chamber 1 ($V_{\text{pneu1}}$) and the volume in pneumatic chamber 2a at the start of the delay period ($V_{\text{loaded}}$) were 46.7 μl each. All measured and calculated delay times are for DI water at 24 °C.

<table>
<thead>
<tr>
<th>Timer 1</th>
<th>Timer 2</th>
<th>Timer 3</th>
<th>Timer 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cross-section of timing channel in µm x µm</td>
<td>153x125</td>
<td>106x67</td>
<td>80x41</td>
</tr>
<tr>
<td>Total volume of pneumatic chambers $V_0$ in µl</td>
<td>357</td>
<td>278</td>
<td>208</td>
</tr>
<tr>
<td>Delay time estimated by eqn (6.4) in s</td>
<td>1.9</td>
<td>11.2</td>
<td>38.7</td>
</tr>
<tr>
<td>Delay time from network simulation in s</td>
<td>2.2</td>
<td>13.5</td>
<td>43.6</td>
</tr>
<tr>
<td>Measured delay time in s</td>
<td>2.7 ± 0.2</td>
<td>14.0 ± 0.5</td>
<td>43.4 ± 1.0</td>
</tr>
</tbody>
</table>

Table 6.2: Implementation of the microfluidic timer for release-on-demand. The cross-section of the timer channel was 52 µm x 43 µm. The total volume of the pneumatic chambers $V_0$ was 211 µl. The volume of pneumatic chamber 1 ($V_{\text{pneu1}}$) and the volume in pneumatic chamber 2a at the start of the delay period ($V_{\text{loaded}}$) were 46.7 μl each. All experiments were replicated 10 times. All measured and calculated delay times are for DI water at 24 °C.

<table>
<thead>
<tr>
<th>Timer 1</th>
<th>Timer 2</th>
<th>Timer 3</th>
<th>Timer 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length of timing channel in mm</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Delay time estimated by eqn (6.4) in s</td>
<td>5.2</td>
<td>10.3</td>
<td>15.5</td>
</tr>
<tr>
<td>Measured delay time in s</td>
<td>5.8 ± 0.4</td>
<td>11.3 ± 0.5</td>
<td>18.8 ± 0.5</td>
</tr>
</tbody>
</table>

From eqn (6.4) and (2.9) it can be seen that the delay time is linearly related to the fluidic resistance $R$, which in turn scales linearly with the length $l$ of the delay channel. The delay time also scales linearly with respect to the volumes of pneumatic chambers 1, 2a and 2b and the liquid volume in pneumatic chamber 2a at the start of the delay, as long as all three volumes are scaled by the same factor. Obviously, an increase in volume can thus be compensated for by decreasing the length of the delay channel for the design of a specific delay time.
6.2.3 Application of the timer in centrifugal microfluidics

Since the timed sequential addition of reagents is required for many assays, we choose the timed release of liquids as an application example for the microfluidic timer. For this, we implement the timer for the temporally controlled valving of a pneumatic siphon valve as depicted in Figure 6.2A. An inlet structure for reagent supply branches into the timer and a siphon channel, which in turn is connected to a collection chamber. The microfluidic timer is loaded at high centrifugation (f = 85 Hz, Figure 6.2B-C). The resulting high hydrostatic pressure pushes the liquid from the inlet chamber into pneumatic chambers 1 and 2. Upon deceleration to the timer release frequency, the hydrostatic pressure decreases, causing liquid to slowly flow out of chamber 2a and back into the inlet chamber (Figure 6.2D). After a defined delay time, the liquid in the timing channel is replaced with air and the timer is released, causing a high flow rate out of pneumatic chamber 1. The flow out of pneumatic chamber 1 is split into the siphon channel and the inlet channel. If a critical volume $V_{\text{crit}}$ is pumped into the siphon channel, the siphon primes (Figure 6.2E). Since the crest of the siphon is located radially inwards of the inlet channel, priming must be performed against centrifugal forces based on a dynamic principle. The underlying effect is that the sudden increase in the flow rate upon timer release causes an increase in viscous dissipation in the inlet channel. This leads to radially inward flow in the siphon channel as employed for pneumatic inward pumping [53], which allows for priming the siphon even against centrifugal or capillary forces. A detailed description of siphon priming, based on a pressure balance according to Kirchhoff’s law, is provided in the appendix section A6.
Figure 6.2: Implementation of the microfluidic timer into a centrifugal microfluidic disk. Green arrows indicate the liquid flow rate. A: Liquid is pipetted into the inlet chamber. B: At a high rotational frequency, the liquid is transferred from the inlet chamber to the pneumatic chambers. C: The first pneumatic chamber is overfilled, the timing channel is primed and the timer is loaded until the gas overpressure equilibrates the centrifugal pressure. D: Upon lowering centrifugation, the fluidic resistance of the timer channel limits the maximal flow and delays the expansion of the entrapped gas volume. E: Pneumatic chamber 2a has run empty and the timer is released. The fluidic resistance of the delay channel reduces abruptly as the viscosity within the channel drops from liquid to the one of air. This results in a sudden
increase in flow rate. The flow is split towards the siphon channel and the inlet channel. At low centrifugation a critical volume of liquid flows into the siphon channel. The siphon primes and transfers the sample in the collection chamber. F: The sample liquid is transferred to the collection chamber.

After priming, the siphon transfers the sample to the collection chamber (Figure 6.2F). In order to transfer the complete sample to the collection chamber, it is beneficial to maintain a moderate rotational frequency at timer release (about 15 Hz) (Figure 6.2F). The moderate rotational frequency maintains a centrifugal pressure difference from the inlet chamber to the collection chamber and thus allows for transferring the complete liquid volume to the collection chamber. In such configurations, we define the start of the delay time as the time when the moderate rotational frequency is reached. The maintained centrifugal pressure counteracts the pneumatic pressure and reduces the pressure drop across the timing channel. As a consequence, the liquid outflow through the timing channel decreases and the delay time increases. To account for the increase in delay time, the following equation should be solved:

\[
p_{\text{cent}}(V_{\text{comp}}) + p_{\text{visc}} \left( \frac{dV_{\text{comp}}}{dt} \right) = p_{\text{pneu}}(V_{\text{comp}})
\]

This equation includes the centrifugal pressure term \( p_{\text{cent}} \) that depends on the filling heights of the different chambers and thus on the integrated flow rate and geometry of the timer. It can only be solved numerically. For that purpose we use a network simulation based approach which was described previously [53], and which is further detailed in appendix 6. With this network based simulation, we can predict liquid flow and fill levels throughout the fluidic processing. If the centrifugal pressure \( p_{\text{cent}} \) is much smaller than the pneumatic pressure \( p_{\text{pneu}} \), the analytical estimation from eqn (6.4) can be used as a good approximation, as described further in the results section.

### 6.3 Materials & methods

All fluidic structures were designed using SolidWorks (Concord, MA, USA) and fabricated by the Hahn-Schickard Lab-on-a-Chip Design and Foundry Service (www.hahn-schickard.de/fertigung/lab-on-a-chip-design-foundry-service/). Microstructures were milled in a 4 mm PMMA substrate (Plexiglas, Evonik, Germany) using a precision micro-mill (KERN Microtechnik GmbH). The disks were then cleaned by CO\(_2\) blasting, rinsed with DI water and isopropyl alcohol and subsequently dried using nitrogen gas. The PMMA disks were sealed
via lamination of polyolefin adhesive tape (# 900320, HJ Bioanalytik, Germany). Experiments were performed using a prototype LabDisk-Player (Qiagen Lake Constance GmbH) that was modified to a stroboscopic setup (BioFluidix GmbH). Where not noted otherwise, all given viscosities were measured using a rheometer (Anton Paar Physica MCR 101). Experiments were performed at room temperature ((24 ± 1 °C).

Due to the high pressures in the pneumatic chambers, the sealing foil buckles outwards. This deformation of the sealing foil increases the volume of the pressure chamber and decreases the pneumatic pressure. We calculated the actual pneumatic pressure from the rotational frequency and height of the liquid column at maximal rotation. All given volumes for the pressure chambers are measured. All calculations and simulations are based on these measured volumes for the pneumatic chambers.

6.4 Experimental results

6.4.1 Sequential release

Four different variants of the microfluidic timer were implemented with varying dimensions (Figure 6.3). The volumes of pneumatic chamber 1 and pneumatic chamber 2a were 46.7 μl each. The timer inlets were filled with 100 μl of DI water each. The timers were then loaded at 85 Hz centrifugation. After pneumatic chambers 1 and 2 were filled and a steady state was reached, the centrifugation frequency was reduced to 15 Hz with a deceleration rate of 15 Hz s⁻¹. From the stroboscopic image data, the transferred sample volume to the four collection chambers was measured over time. All experiments were performed in triplicate. The measured delay times during the experimental series are listed in Table 6.1. The sequential release of the four timers can be seen in Figure 6.3. The network simulation based on eqn (6.5) accurately predicted the measured delay times. The analytical estimation from eqn (6.4) underestimates the delay time. This was expected, since the analytical model ignores the counteracting centrifugal force, which acts against the pneumatic pressure. If longer delay times than the reported ~2 min are required, an adaption of the frequency protocol can be applied to release liquids on-demand, as further detailed in the release-on-demand section.
6.4 Experimental results

Figure 6.3: Sequential release with the microfluidic timer. (Upper figure) Release of DI water at RT for the four different timer structures (Table 6.1). The timers were engineered to release at sequential points in time for DI water. (Lower figure) Timing of typical assay reagents and sequential release in opposite order of their viscosity demonstrated employing timers 1, 3 and 4. The delay time and the time to transfer the sample to the collection chamber depend on the viscosity of the sample. All experiments were performed in triplicate. The network simulations are described in detail in appendix 6 (see Figure A6.1). The error bars represent one standard deviation.

6.4.2 Typical assay reagents

The delay time for the microfluidic timer depends linearly on the viscosity of the timed liquid. Thus, for the same timer structure, the liquid with the lowest viscosity would be released first. However, in general, liquids need to be released in any order, independent of their viscosities. To demonstrate viscosity-independent sequential release, we show the timed valving of typical biological reagents in the opposite order of their viscosities. We used timers 1, 3, and 4 with geometries as listed in Table 6.1.

The timers were filled such that the liquid with the highest viscosity (50% w/w glycerol, \( \theta = (78 \pm 6)^\circ \), \( \eta = (5.62 \pm 0.02) \text{ mPa} \cdot \text{s} \), at 24 °C) was released first, then the liquid with medium viscosity was valved (50% w/w ethanol in DI water, \( \theta = (36 \pm 8)^\circ \), \( \eta = (2.56 \pm 0.02) \text{ mPa} \cdot \text{s} \), at 24 °C) and finally, the liquid with the lowest viscosity was released (blood plasma \( \eta \sim 1.68 \text{ mPa} \cdot \text{s} \)). As shown in Figure 6.3, all liquids could be sequentially
6. The microfluidic timer

released in the opposite order of their viscosities, with the delay times calculated by the network simulation (see appendix 6).

To determine the viscosity of blood plasma, we used the microfluidic timer as a rheometer by iterative adaption of viscosity in the network simulation to fit the measured delay time. The resulting viscosity of \( \sim 1.68 \text{ mPa\cdot s} \) falls well within the reference range for a healthy adult, which is reported to be 1.52–1.76 mPa\cdot s at 24 °C [53].

6.4.3 Release-on-demand

For the previous timing events, a minimal frequency protocol has been applied: timer loading at a constant high rotational frequency and subsequent release at a constant low rotation. Using a more complex frequency protocol, the functionality of the timer can be extended to a release-on-demand feature. That means that each of the timing structures can be addressed and released individually and independently from each other. This way valving can be performed in any user-defined sequence allowing for \( N! \) valving combinations. This for example results in 24 combinations with \( N = 4 \) timing structures. The underlying working principle can be explained as follows.

As discussed previously, during timer release, the viscosity within the timing channel suddenly changes from liquid to air, resulting in a temporarily high flow and, consequently, a higher viscous pressure drop in the inlet channel. This pressure drop leads to pumping liquid radially inwards through the siphon channel, against the centrifugal force as employed previously by Zehnle et al. for pneumatic inward pumping [53]. Since the centrifugal force can be adjusted by the frequency protocol upon timer release, it is possible to determine on-demand whether the siphon does or does not prime. If a comparatively high rotational frequency is applied at the moment of timer release, siphon priming can be inhibited because the centrifugal force limits the amount of liquid pumped into the siphon. Under those circumstances the critical volume for priming the siphon is not reached and no liquid is transferred to the collection chamber. Applying a comparatively low rotational frequency at the moment of timer release, siphon priming is promoted as more liquid is pumped into the siphon channel. Under those circumstances the siphon primes and liquid is transferred to the collection chamber (see appendix 6). Consequently, with an appropriate frequency protocol, it is possible to selectively valve exactly one out of multiple timing structures on-demand without influencing all the others.
We demonstrate the valve-on-demand mode with a microfluidic disk containing four microfluidic timers that are released at four different delay times. The layout was designed using eqn (6.4) making use of the linear relationship between the delay time and the lengths of the timing channels (Table 6.2).

The microfluidic timers are designed such that after loading the timer, the microfluidic timers can release at all rotational frequencies below 28 Hz. Moreover, if the timer release happens at a rotational frequency of 14 Hz or below, the siphons prime. In contrast, if the timer release happens at a rotational frequency of 16 Hz and above, the siphons do not prime. That way timer release and valving are independently controllable.

All four timers were filled with 100 μl of DI water and the timers were loaded at 75 Hz rotational frequency. The delay time starts after a reduction of rotational frequency to 20 Hz by 15 Hz s\(^{-1}\). In order to prime one siphon valve, the rotational frequency was reduced to 14 Hz just before the predicted timer release of the corresponding timer. After the priming of the siphon, the rotational frequency was increased back to 20 Hz. This “frequency dip” allowed us to selectively valve the siphon of one of the microfluidic timers, while all siphons of all other timers remained not primed. Figure 6.4 shows the results for the selective priming of siphons of each of the microfluidic timers. All of the experiments were repeated ten times. Selective transfer of the sample from one of the timers could be shown successfully in all 40 experiments. Since the initial loaded state could be restored by rotating at a high rotational frequency of 75 Hz (timer reset), this allowed us to transfer the samples from the microfluidic timers to the collection chambers in any user-defined sequence by simply concatenating frequency protocols 1–4 according to user needs. In the future this may allow us to use one fluidic disk design for multiple assay protocols, by simply adjusting the frequency protocol to a different application.
Figure 6.4: Release-on-demand with four microfluidic timers with increasing delay times: $t_{\text{delay 1}} = (5.8 \pm 0.4) \text{ s}$, $t_{\text{delay 2}} = (11.3 \pm 0.5) \text{ s}$, $t_{\text{delay 3}} = (18.8 \pm 0.5) \text{ s}$, $t_{\text{delay 4}} = (23.4 \pm 1.0) \text{ s}$. After loading the timers at 75 Hz, centrifugation is reduced to 20 Hz. In each of the experiments, one of the siphon valves could be selectively primed by reducing the frequency further to 14 Hz shortly before timer release and increasing it back to 20 Hz shortly after. As depicted, all other siphon valves did not prime and the liquid was transferred back to the inlet chamber instead. This allows us to sequentially release the four microfluidic timers in any user-defined sequence. Each curve corresponds to ten experiments. The data were collected for fixed transferred volumes between 0 and 100 μl in 25 μl steps. Error bars correspond to the time variations when a discrete volume (25 μl) has been transferred. The largest standard deviation was $\pm 1.2 \text{ s}$ measured for frequency protocol 4 in 100 μl volume in the collection chamber. All other error bars are smaller and thus hardly visible in the graphs.
It is worth noting that if one timer is released multiple times in sequence, liquid plugs can form in the siphon channel. Due to contact angle hysteresis this can increase the capillary pressure to a level for which the siphon channel does no longer prime, even below 14 Hz rotation upon timer release. Such liquid plugs can be easily removed from channels by including an intermediate step at a high rotational frequency above the Rayleigh Taylor instability \[107\]. In our case, the critical rotational frequency to remove plugs from the 150 μm diameter siphon channel was \(~\)80 Hz, and an intermediate increase in rotational frequency to 90 Hz was included. The temporarily high centrifugation ensured that no liquid plugs remained in the siphon channel.

6.5 Conclusion

We have introduced a microfluidic timer that can be used for timed release of liquid reagents with different properties such as viscosities and surface tensions. It can easily be integrated with existing unit operations, and does not require surface treatments, external means or additional fabrication steps.

By timing the release of pneumatically stored energy, the microfluidic timer circumvents a major limitation of many state-of-the-art centrifugo-pneumatic unit operations that require fast changes in the rotational frequency. So far, fast frequency changes had to be provided by the processing device \[53\]. In the future, employing a microfluidic timer, fast acceleration or deceleration rates are not required anymore to enable a quick release of pneumatic energy. Thus, the timer allows for centrifugo-pneumatic operations, such as pneumatic inward pumping, to be used in a much wider range of processing devices, e.g. standard laboratory centrifuges as used for example for the LabTube technology \[79\].

Furthermore, the microfluidic timer enables the triggering of valving after a specific delay time and this way enables to reduce or increase the spinning frequencies while the countdown of the timer is running. This creates an additional degree of freedom compared to state-of-the-art valves. For example, combining siphon-based valving with the microfluidic timer allows for applying spinning frequencies during the countdown of the timer which are not accessible for state-of-the-art capillary siphons or geometric- or centrifugo-pneumatic valves because those frequencies would initiate unintended valving.

Finally, the demonstrated capabilities of the timer in release-on-demand mode aim towards a universal cartridge design. With release-on-demand, a generic cartridge can
automate timed valving for different assay protocols, simply by programming the protocol of the rotational frequency.

### 6.6 Outlook

We expect the microfluidic timer to find broad applications within the centrifugal microfluidic automation of the sequential on-demand release of reagents. Moreover, the timer could also be used as a trigger after stopping the disk enabling the release of the stored pneumatic energy with a defined flow rate with the disk at rest. This is useful for performing measurements under fluid flow with a stationary detector, for example for particle counting or impedance spectrometry on a disk. Furthermore, when used at increased temperatures, the microfluidic timer can be combined with a vapor-diffusion barrier to reduce air pressure and the risk of delamination [106].

Finally, the microfluidic timer can be generalized to other microfluidic areas besides centrifugal microfluidics, since it allows precise flow sequencing for multiple liquids with pressure sources that are comparatively simple, e.g. finger-actuated devices [177].
7 Overall conclusion and outlook

The main goal of this thesis was the development of new centrifugal microfluidic unit operations and process chains for a better integration of complex laboratory workflows. The work can be summarized into three outcomes: (1) a new process chain handling of nanoliter volumes, which was optimized for the screening of protein structures via small-angle X-ray scattering, (2) a new unit operation for aliquoting and combination of multiple liquids on a single fluidic layer and (3) a new unit operation for precise timing of centrifugal microfluidic operations. In the following, the three innovations are critically discussed in the context of the state of the art, and potential future developments are outlined.

7.1 Volume reduction

In chapter 4 a new process chain for the generation of combinatorial dilutions in the nanoliter range was presented. The process chain was developed for preparation of protein samples for small-angle X-ray scattering. However, due to its scalable volume forces, centrifugal microfluidics is in principle very well suited for nanoliter handling in many applications. The newly developed process chain and prototyping techniques lower the entry barrier for future integrations of laboratory workflows in the nanoliter range in centrifugal microfluidics. This may lead to more nanoliter scale applications, thereby saving precious sample volumes and increasing integration in applications beyond the discussed small-angle X-ray scattering.

With regard to the LabDisk for SAXS, the next step is to make the disks available to users of the bioSAXS beamline at EMBL Hamburg for high-throughput screening of protein structures. For this goal 200 disks with 1200 dilution matrices were fabricated and are available to users of the beamline. However, a number of preparation steps are required before regular users can employ the LabDisk for SAXS. So far, the LabDisk for SAXS has been positioned manually within the beamline for each measurement. The automation of this alignment process would not only reduce the risk of misalignments, it would also reduce the time per measured condition from currently ~30 s to ~3 s. Furthermore, to allow for automated high-throughput analyses of the collected SAXS data, the LabDisk for SAXS needs to be included in the data processing pipeline at the bioSAXS beamline. The bioSAXS group
7 Overall conclusion and outlook

at EMBL Hamburg is currently working on programming automated positioning, a graphical user interface and a data analysis pipeline adapted to the requirements of the LabDisk for SAXS are planned for 2016.

One remaining hurdle for the broad application of the LabDisk for SAXS is the high cost per test. The estimated volume of tests for the LabDisk for SAXS is around 10,000 dilution matrices per year. If ordered as multi-layered thermoformed disks at the Hahn-Schickard Design & Foundry service, one dilution matrix with 20 test conditions costs about 40 €. This high cost is mostly related to manual processing steps, in particular the thermoforming of foils and the cleaning of foils before thermoforming and bonding. To reduce these costs in the future, the thermoforming process of the LabDisk for SAXS may be automated using recently purchased automated thermoforming machinery in the ProLab pilot fabrication line at Hahn-Schickard. Since this new machinery is situated in a clean room environment, cleaning steps for foils before and after thermoforming would no longer be necessary. After a systematic process development, this transition to more automated processes could reduce the cost per dilution matrix to about 15 €. To further reduce costs, a simpler single layered fluidics would be essential. Aliquoting and combination of two liquids in a single fluidic layer becomes possible by use of centrifugo-pneumatic multi-liquid aliquoting as presented in chapter 5. For the fluidics of the LabDisk for SAXS to be compatible to this aliquoting principle, the fluidics would need to be reduced to a maximum of two liquids per read-out chamber. Such a simplified LabDisk for SAXS could then be fabricated using injection molding or micro-thermoforming at a significantly reduced cost of less than 5 € per dilution matrix.

7.2 Parallel aliquoting of multiple liquids

For aliquoting and combination of multiple liquids, the centrifugo-pneumatic multi-liquid aliquoting was presented, a new unit operation that can generate precise aliquots both in the radially inward and outward direction. Due to its small footprint centrifugo-pneumatic aliquoting, as presented by Mark et al. [108], will likely still be used for most applications where only one liquid needs to be aliquoted. Centrifugo-pneumatic multi-liquid aliquoting becomes advantageous when multiple liquids need to be aliquoted in parallel. A first application developed in this regard within Hahn-Schickard is the “BoNT Disk” a LabDisk for detection of botulinum neurotoxins (ZIM project “BoNT Disk”, project number KF2146328MD3). By application of centrifugo-pneumatic multi-liquid aliquoting, previous
7.3 Timing of operations

manual pipetting steps were replaced with automatic aliquoting. Together with other improvements, e.g. improved mixing, this resulted in a reduction of the coefficient of variation from previously 14-41% CV with manual pipetting to 1-4% CV. Even though the impact of the new aliquoting on total performance cannot be directly deducted, without precise aliquoting a CV of 1-4% in an enzymatic assay is impossible.

An interesting future application would be to combine centrifugo-pneumatic multi-liquid aliquoting with the microfluidic timer. Each pair of pneumatic and metering chamber could be connected with a different fluidic resistance. Aliquots would then be aliquoted after different delay times. Such timed aliquoting would allow for the timed sequential addition of aliquots into an array of read-out chambers, e.g. for studying reaction kinetics.

7.3 Timing of operations

For the timing of valving and pumping events in centrifugal microfluidics, the microfluidic timer was presented. In the release-on-demand mode, four liquids can be valved in any order solely defined by the frequency protocol. The release-on-demand mode demonstrated that programmability is possible in centrifugal microfluidic disks without external means, which eliminates one of the drawbacks of centrifugal microfluidics (compare Table 1.2). One potential future use of release-on-demand would be to equip the microfluidic timer with large reservoirs that dispense only a small, defined fraction of the reservoir volume per valving event. Using the release-on-demand feature, this would allow for the inclusion of dispensers on disk that can be individually addressed on-demand via specific frequency protocol sequences. Such dispensers would facilitate the integration of complex processes, such as next-generation sequencing protocols, on disk. If combined with sample preparation on disk, the microfluidic timer may even enable fully integrated disks automating the complete process from sample to sequenced answer.
**Glossary**

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ab initio modeling</strong></td>
<td>A computational method in biophysics for the calculation of 3D protein structures.</td>
</tr>
<tr>
<td><strong>Accuracy</strong></td>
<td>The accuracy of a measurement method is described by the combination of two terms “trueness” and “precision”.</td>
</tr>
<tr>
<td><strong>Air vent</strong></td>
<td>The interface for ventilation typically referred to as the interface to atmospheric pressure.</td>
</tr>
<tr>
<td><strong>Aliquoting finger</strong></td>
<td>A metering chamber for volume definition of an aliquot. The Aliquoting finger is at the radially inwards end connected to a waste reservoir. Aliquoting fingers are typically filled from the radially inwards direction.</td>
</tr>
<tr>
<td><strong>Capillary siphon valve</strong></td>
<td>A microfluidic valve that requires a temporary state of low centrifugal pressure to trigger the burst event.</td>
</tr>
<tr>
<td><strong>Capillary valve</strong></td>
<td>A microfluidic valve where the pinning effect of the fluid flow is solely based on the capillary counter pressure.</td>
</tr>
<tr>
<td><strong>Centrifugal microfluidics</strong></td>
<td>Centrifugal microfluidics is a sub-category of microfluidics, defined by its liquid actuation mechanism. In contrast to other microfluidic approaches, centrifugal microfluidics utilizes the rotation of a microfluidic cartridge as one important control parameter for liquid handling. Forces used for liquid manipulation unique to centrifugal microfluidics are the centrifugal force, Euler force and Coriolis force. One centrifugal-microfluidic cartridge can integrate a full laboratory workflow by integration of a sequence of microfluidic unit operations and process chains.</td>
</tr>
<tr>
<td><strong>Centrifugal step emulsification</strong></td>
<td>A process where a step emulsification process is performed under centrifugal force where only the dispersed phase is</td>
</tr>
</tbody>
</table>
moving.

**Centrifugo-pneumatic valve**
A microfluidic valve where the liquid flow is stopped by a combination of the capillary counter-pressure at the interface of a channel to a dead-end chamber and of the counter-pressure of the compressed air inside the dead-end chamber.

**Centrifugo-dynamic inward pumping**
A centrifugal microfluidic unit operation for the pumping of liquids on a centrifugal microfluidic chip. It is based on a pneumatic chamber with an inlet channel with high fluidic resistance and an outlet channel with low fluidic resistance. It can be used especially for pumping liquids to a radially inwards point on a centrifugal microfluidic cartridge.

**Centrifugo-pneumatic aliquoting**
A two-stage aliquoting structure based on centrifugo-pneumatic valves.

**Centrifugo-pneumatic multi-liquid aliquoting**
A process chain for parallel aliquoting of multiple liquids based on pneumatic pumping.

**Collection chamber**
A microfluidic chamber for the final collection of volume after a process step, e.g. inward pumping.

**Combination structure**
A combination structure is a microfluidic structure that combines aliquots in defined ratios for the generation of combinatorial dilutions.

**Coriolis switch**
A microfluidic switch that exploits the Coriolis force in order to route the liquid.

**Dilution matrix**
Each LabDisk for SAXS contains six dilution matrices. One dilution matrix comprises microfluidic structures for the generation of 20 measurement conditions for SAXS measurements of 240 nl each. Each measurement condition is generated from different combinations of three input liquids.
<table>
<thead>
<tr>
<th><strong>Glossary</strong></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>External means</strong></td>
<td>Use of assistive equipment in the processing device to allow fluidic functionalities. An example is the use of lasers in the processing device for valving.</td>
</tr>
<tr>
<td><strong>Feeding channel</strong></td>
<td>A feeding channel connects aliquoting fingers or metering chambers with the inlet channel and chamber and the waste reservoir.</td>
</tr>
<tr>
<td><strong>Footprint</strong></td>
<td>The surface area of an object usually referred to the horizontal surface area.</td>
</tr>
<tr>
<td><strong>Functional materials</strong></td>
<td>Materials other than the substrate material of the cartridge, which are integrated specifically to allow for additional fluidic functionalities, e.g. wax valves.</td>
</tr>
<tr>
<td><strong>Inlet chamber</strong></td>
<td>The chamber taking the sample/reagents on a LabDisk cartridge. Typically accessible with a pipette.</td>
</tr>
<tr>
<td><strong>Inlet channel</strong></td>
<td>A channel that connects the inlet chamber with downstream fluidic elements.</td>
</tr>
<tr>
<td><strong>LabDisk</strong></td>
<td>A disposable polymer disk that contains (micro-)fluidic structures to perform a liquid handling procedure in a centrifugal device.</td>
</tr>
<tr>
<td><strong>LabDisk for SAXS</strong></td>
<td>A LabDisk for the high-throughput screening of protein structures using minimal input volumes via small angle X-ray scattering (SAXS).</td>
</tr>
<tr>
<td><strong>LabDisk player</strong></td>
<td>A portable centrifugal device that processes a LabDisk by controlling temperature, rotation and readout.</td>
</tr>
<tr>
<td><strong>Liquid routing</strong></td>
<td>Transport of liquid through the desired path.</td>
</tr>
</tbody>
</table>
**Metering chamber**
A chamber for metering a liquid volume, e.g. the volume defining chambers during aliquoting.

**Microfluidic chip / microfluidic cartridge**
The microfluidic chip which is often referred to as microfluidic cartridge is the substrate that provides structures like chambers, channels etc. with a hardware implementation of the fluidic unit operations. For most applications, microfluidic chips are disposed after use to avoid cross-contamination and/or save costs for regeneration.

**Microfluidic platform**
According to Mark et al. [42] “a microfluidic platform comprises an easily combinable set of microfluidic unit operations that allows assay miniaturization within a consistent fabrication technology” and thereby facilitates the efficient integration of laboratory workflows and applications.

**Microfluidic timer**
A centrifugal microfluidic unit operation for the timed delayed sudden release of liquids.

**Nucleic acid**
Macromolecules, the major organic matter of the nuclei of biological cells, made up of nucleotide units, and hydrolysable into certain pyrimidine or purine bases (usually adenine, cytosine, guanine, thymine, uracil), d-ribose or 2-deoxy-d-ribose and phosphoric acid.

**Nucleic acid extraction**
Refers to a process in which an organism or virion is lysed to recover DNA or RNA from the same followed by a nucleic acid purification.

**One-stage aliquoting**
An aliquoting process where the liquid volume is metered directly in the final collection chambers.

**Pneumatic chamber**
A non-vented chamber that can be used to compress a compressible fluid, using centrifugal pressure.
<table>
<thead>
<tr>
<th><strong>Point-of-care</strong></th>
<th>Laboratory diagnostic testing performed at or near the site where clinical care is delivered.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Polymerase chain reaction (PCR)</strong></td>
<td>A laboratory technique to rapidly amplify pre-determined regions of double-stranded DNA. Generally involves the use of a heat-stable DNA polymerase.</td>
</tr>
<tr>
<td><strong>Precision</strong></td>
<td>The closeness of agreement (degree of scatter) between a series of measurements obtained from the multiple sampling of the same homogenous sample under the prescribed conditions.</td>
</tr>
<tr>
<td><strong>Process chains</strong></td>
<td>Strohmeier et al. [9] define process chains as “assemblies of fluidic unit operations and external means that represent laboratory workflows on a higher level of integration”, e.g. blood plasma separation or nucleic acid extraction.</td>
</tr>
<tr>
<td><strong>Processing device</strong></td>
<td>The processing device is reusable hardware that provides additional means to operate the microfluidic chip. This may comprise the main actuator to control the fluids as well as auxiliary means such as temperature control and/or magnetic, electric, optic, pneumatic or mechanic features including means for detection/read-out.</td>
</tr>
<tr>
<td><strong>Reproducibility</strong></td>
<td>Describes the possibility to replicate, within measurement errors, the result of an experiment or entire study.</td>
</tr>
<tr>
<td><strong>Read-out chamber</strong></td>
<td>A microfluidic chamber that is structured in such a way that it supports read-out of a liquid volume or a surface with an external detector.</td>
</tr>
</tbody>
</table>
Sample

A generic term encompassing controls, blanks, unknowns and processed samples, as described below. Blank: A sample of a biological matrix to which no analytes have been added that is used to assess the specificity of the bioanalytical method. Quality control sample (QC): A spiked sample used to monitor the performance of a bioanalytical method and to assess the integrity and validity of the results of the unknown samples analyzed in an individual batch. Unknown: A biological sample that is the subject of the analysis.

Sample/reagent supply

The process of loading the sample/reagent onto the microfluidic cartridge.

Screening agent

A biochemical solution, e.g. a solution of different pH or salt concentration, that, when mixed with a protein solution, can cause a conformational change of said proteins in solution.

Sedimentation

The process of separating particles from a suspension by gravitation or centrifugation.

Shake mode mixing

A microfluidic mixing principle that relies on continuous changes in the spin speed. The angular momentum caused by repetitive acceleration of deceleration induces a layer inversion of liquids in a microfluidic chamber.

Small angle X-ray scattering

Small-angle X-ray scattering (SAXS) is an important technique in structural biology for the analysis of macromolecules in the range of 1-100 nm [135].

Suspension

A liquid in which solid particles are dispersed.

Timer delay time

The countdown time of the centrifugal microfluidic unit operation called “microfluidic timer”.

Timer release

Timer release happens when the countdown time of the centrifugal microfluidic unit operation called “microfluidic
timer” ends. The pneumatic energy is released abruptly when the 2nd compression chamber and the timing channel of the microfluidic timer are empty.

**Timer setting**
Setting the countdown time of the centrifugal microfluidic unit operation called “microfluidic timer”. The countdown time is defined by the liquid volume transferred to the 2nd compression chamber of the microfluidic timer.

**Timing channel**
The channel that connects pneumatic chamber 1 and pneumatic chamber 2 in the centrifugal microfluidic unit operation called “microfluidic timer”.

**Trueness**
The “trueness” of a measurement refers to the closeness of agreement between the arithmetic mean of a large number of test results and the true or accepted reference value.

**Two-stage aliquoting**
An aliquoting where liquid is first metered in a metering chamber and separated from the collection chamber by a valve.

**Unit operation**
A microfluidic unit operation is an implementation of a basic fluidic functionality, such as valving, switching and metering [42]. Depending on the setting, the same functionality can be realized using different unit operations, e.g. depending on the application different types of valves may be appropriate.

**Volume quantification structure**
A microfluidic structure designed for the precise read-out of liquid volumes, e.g. during the evaluation of aliquoting.
Abbreviations

%-v/v  Volume percent
%-w/w  Weight percent
COC    Cyclic Olefin Copolymer
COP    Cyclic Olefin Polymer
CV     Coefficient of variation
DI     De-ionized
DNA    Deoxyribonucleic acid
ELISA  Enzyme-linked immunosorbent assays
IP     Intellectual property
ISO    International Organization for Standardization
PDMS   Polydimethylsiloxane
PMMA   Poly(methyl methacrylate)
PCR    Polymerase chain reaction
R_g    Radius of gyration
RNA    Ribonucleic acid
RPA    Recombinase Polymerase Amplification
SAXS   Small-angle X-ray scattering
Physical measures

Physical and mathematical constants

\[ \pi \quad \text{Pi} = 3.141 \]
\[ \text{Mol} \quad 6.022 \times 10^{23} \]

Units

- ° Degree
- °C Degree Celsius
- Hz Hertz
- kg Kilogramm
- m Meter
- s Seconds
- min Minutes
- Pa Pascal

General measures

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \alpha )</td>
<td>Dimensionless constant used to calculate the fluidic resistance of a channel. Depends on width and height of the channel.</td>
<td></td>
</tr>
<tr>
<td>( \lambda )</td>
<td>Wavelength</td>
<td>m</td>
</tr>
<tr>
<td>( \sigma )</td>
<td>Surface tension</td>
<td>N/m</td>
</tr>
<tr>
<td>( \theta )</td>
<td>Contact angle</td>
<td>°</td>
</tr>
<tr>
<td>( \theta_a )</td>
<td>Advancing contact angle</td>
<td>°</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
<td>Unit</td>
</tr>
<tr>
<td>--------</td>
<td>------------</td>
<td>------</td>
</tr>
<tr>
<td>θ_r</td>
<td>Receeding contact angle</td>
<td>°</td>
</tr>
<tr>
<td>ρ</td>
<td>Density</td>
<td>kg/m³</td>
</tr>
<tr>
<td>ω</td>
<td>Angular velocity</td>
<td>rad/s</td>
</tr>
<tr>
<td>ω̇</td>
<td>Change in angular velocity</td>
<td>rad/s²</td>
</tr>
<tr>
<td>η</td>
<td>Dynamic viscosity</td>
<td>Pa s</td>
</tr>
<tr>
<td>f</td>
<td>Rotational frequency</td>
<td>Hz</td>
</tr>
<tr>
<td>f_{cent}</td>
<td>Centrifugal force density</td>
<td>N/m³</td>
</tr>
<tr>
<td>f_{Coriolis}</td>
<td>Coriolis force density</td>
<td>N/m³</td>
</tr>
<tr>
<td>f_{Euler}</td>
<td>Euler force density</td>
<td>N/m³</td>
</tr>
<tr>
<td>r</td>
<td>Radius of liquid element</td>
<td>m</td>
</tr>
<tr>
<td>r₁</td>
<td>inner radius</td>
<td>m</td>
</tr>
<tr>
<td>r₂</td>
<td>outer radius</td>
<td>m</td>
</tr>
<tr>
<td>w</td>
<td>Width of channel</td>
<td>m</td>
</tr>
<tr>
<td>h</td>
<td>Height of channel</td>
<td>m</td>
</tr>
<tr>
<td>l</td>
<td>Length of liquid plug / channel</td>
<td>m</td>
</tr>
<tr>
<td>l_{iso}</td>
<td>Isoradial length of a liquid plug</td>
<td>m</td>
</tr>
<tr>
<td>p_{cent}</td>
<td>Centrifugal pressure</td>
<td>Pa</td>
</tr>
<tr>
<td>p_{Euler}</td>
<td>Euler pressure</td>
<td>Pa</td>
</tr>
<tr>
<td>p_{visc}</td>
<td>Viscous pressure drop</td>
<td>Pa</td>
</tr>
<tr>
<td>p_{inertial}</td>
<td>Inertial pressure</td>
<td>Pa</td>
</tr>
<tr>
<td>p_{cap}</td>
<td>Capillary pressure at a liquid meniscus</td>
<td>Pa</td>
</tr>
<tr>
<td>p_{cap_hyst}</td>
<td>Capillary pressure over a liquid plug due to contact angle hysteresis</td>
<td>Pa</td>
</tr>
<tr>
<td>p_{pneu}</td>
<td>Pneumatic pressure</td>
<td>Pa</td>
</tr>
<tr>
<td>p₀</td>
<td>Atmospheric pressure</td>
<td>Pa</td>
</tr>
<tr>
<td>R</td>
<td>Hydraulic resistance</td>
<td>Pa s / m³</td>
</tr>
<tr>
<td>Q</td>
<td>Flow rate</td>
<td>m³ / s</td>
</tr>
<tr>
<td>Ḫ</td>
<td>Change of flowrate</td>
<td>m³ / s²</td>
</tr>
</tbody>
</table>
# Measures related to the microfluidic timer

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>$E_{\text{pneu}}$</td>
<td>Pneumatic energy stored in the microfluidic timer</td>
<td>J</td>
</tr>
<tr>
<td>$V_0$</td>
<td>Initial air volume in a pneumatic chamber (or a pair of pneumatic chambers for the microfluidic timer)</td>
<td>m$^3$</td>
</tr>
<tr>
<td>$V$</td>
<td>Volume of air in pneumatic chamber during compression</td>
<td>m$^3$</td>
</tr>
<tr>
<td>$V_{\text{pneu}1}$</td>
<td>Total volume pneumatic chamber 1 of the microfluidic timer</td>
<td>m$^3$</td>
</tr>
<tr>
<td>$V_{\text{pneu}}$</td>
<td>Total volume of the pneumatic chamber in the multi-liquid aliquoting</td>
<td>m$^3$</td>
</tr>
<tr>
<td>$V_{\text{loaded}}$</td>
<td>Volume of liquid in pneumatic chamber 2 of the microfluidic timer</td>
<td>m$^3$</td>
</tr>
<tr>
<td>$V_{\text{comp}}$</td>
<td>Total liquid volume loaded into the microfluidic timer</td>
<td>m$^3$</td>
</tr>
<tr>
<td>$t$</td>
<td>Time</td>
<td>s</td>
</tr>
<tr>
<td>$\Delta t_{\text{delay}}$</td>
<td>Delay time of the microfluidic timer</td>
<td>s</td>
</tr>
</tbody>
</table>
### Measures related to the multi-liquid aliquoting

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{\text{ali}}$</td>
<td>Aliquoted volume</td>
<td>m$^3$</td>
</tr>
<tr>
<td>$V_{\text{meter}}$</td>
<td>Volume of the metering chamber</td>
<td>m$^3$</td>
</tr>
<tr>
<td>$V_{\text{out}}$</td>
<td>Liquid volume in the transfer channel during the metering phase</td>
<td>m$^3$</td>
</tr>
<tr>
<td>$V_{\text{bf}}$</td>
<td>Volume backflow towards the inlet during the transfer phase</td>
<td>m$^3$</td>
</tr>
<tr>
<td>$V_{\text{in}}$</td>
<td>The liquid volume remaining in the inlet channel after aliquoting</td>
<td>m$^3$</td>
</tr>
<tr>
<td>$V_{\text{input}}$</td>
<td>Liquid volume pipetted by the user into the inlet per aliquot</td>
<td>m$^3$</td>
</tr>
<tr>
<td>$V_{\text{m,n}}$</td>
<td>The volume of aliquot n in run m</td>
<td>m$^3$</td>
</tr>
<tr>
<td>$V_{\text{m}}$</td>
<td>Mean volume of run m</td>
<td>m$^3$</td>
</tr>
<tr>
<td>$\overline{V}$</td>
<td>Mean aliquot volume over a set of M runs</td>
<td>m$^3$</td>
</tr>
<tr>
<td>$R_{\text{in}}$</td>
<td>Fluidic resistances of the inlet channel</td>
<td>Pa·s / m$^3$</td>
</tr>
<tr>
<td>$R_{\text{out}}$</td>
<td>Fluidic resistances of the transfer channel</td>
<td>Pa·s / m$^3$</td>
</tr>
<tr>
<td>$f_{\text{max}}$</td>
<td>Maximal rotational frequency</td>
<td>Hz</td>
</tr>
<tr>
<td>$r_{\text{of}}$</td>
<td>Position of the meniscus at the connection of the metering chamber to the pneumatic chamber</td>
<td>m</td>
</tr>
<tr>
<td>$r_{\text{out}}$</td>
<td>Radial most inwards position of the transfer channel</td>
<td>m</td>
</tr>
<tr>
<td>$M$</td>
<td>Total number of runs</td>
<td></td>
</tr>
<tr>
<td>$N$</td>
<td>Total number of aliquots within one run</td>
<td></td>
</tr>
<tr>
<td>$CV_{\text{Overall}}$</td>
<td>Overall CV</td>
<td></td>
</tr>
<tr>
<td>$CV_{\text{Inter run}}$</td>
<td>Inter-run CV</td>
<td></td>
</tr>
<tr>
<td>$CV_{\text{Intra run, m}}$</td>
<td>Intra-run CV for run m</td>
<td></td>
</tr>
</tbody>
</table>
Appendix to chapter 4

Liquid loss due to cornerflow

Figure A4.1: Liquid loss due to cornerflow in the first and last aliquoting fingers of the LabDisk for SAXS. Displayed is an older version of the LabDisk for SAXS fabricated as a milled disk in PMMA and sealed with adhesive film. In this version of the disk liquid loss was clearly visible in the first and last aliquoting finger after long holding times.
Buffer subtracted curves collected with the LabDisk for SAXS

Figure A4.2: Buffer subtracted curves collected with the LabDisk for SAXS: the curves are offset for clarity. Protein concentration: 1.8 mg/ml (black), 3.7 mg/ml (red), 5.5 mg/ml (red), 11 mg/ml (yellow). NaCl concentration (from bottom to top): 0 mM, 83 mM, 166 mM, 250 mM, 333 mM.

Dead volumes

Table A4.1 shows which amount of protein volume is used for fluidic processing and which amount of protein volume ends up in the actual read-out chambers. Reduction of total sample volume without change to the read-out chambers could be achieved by reducing the pipetting tolerance, reducing the size of the feeding channel or reducing the excess volume in the read-out chambers.

Another way to reduce total sample volume would be to shrink the size of all volumes. However, this would include reduction of the size of the read-out chambers. In the current design the read-out chamber in the frontside foil has an aspect ratio of 1. Reducing the diameter of the read-out chamber would lead to an aspect ratio larger than 1, which is not recommended for micro-thermoforming. Reducing the depth of the read-out chamber...
would decrease the height of the measured liquid column, 860 µm in the current design, and consequently reduce the data quality.

**Table A4.1:** Calculation of the protein volume used for SAXS analysis. Out of the total input protein volume of 2.5 µl, 680 nl are used for the actual SAXS analysis. Additional liquid volume is required to tolerate pipetting errors, to ensure proper aliquoting and to ensure complete filling of the read-out chambers.

<table>
<thead>
<tr>
<th>Type</th>
<th>Dead volume</th>
<th>Remaining protein volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total input protein volume</td>
<td>-</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>Extra volume included for pipetting</td>
<td>500 nl</td>
<td>2.0 µl</td>
</tr>
<tr>
<td>tolerance</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extra volume required to ensure</td>
<td>~680 nl</td>
<td>1.32 µl</td>
</tr>
<tr>
<td>proper aliquoting (limited by the size</td>
<td></td>
<td></td>
</tr>
<tr>
<td>of the feeding channel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Excess volume included to ensure</td>
<td>49%</td>
<td>680 nl</td>
</tr>
<tr>
<td>complete filling of the read-out chambers</td>
<td>(123 nl per read-out chamber)</td>
<td></td>
</tr>
</tbody>
</table>

**Calculation of \( \chi^2 \)**

\( \chi^2 \) can be calculated from eqn (A4.1), where \( n \) is the number of data points and \( \sigma \) is the experimental error [178].

\[
\chi^2 = \frac{1}{n-1} \sum_{k=1}^{n} \left( \frac{I_{\text{exp}}(q_k) - I_{\text{calc}}(q_k)}{\sigma(I_{\text{exp}}(q_k))} \right)^2
\]  

A4.1
Appendix to chapter 6

The following description of the network-based simulation is an adaption from the description in the ESI material of Zehnle et al. [53]. The network model can be seen in Figure A6.1. Table A4.1 describes the function of the individual fluidic elements depicted in Figure A6.1. Figure A6.2 shows one implementation of the timer structure. The figure highlights the implementation of the individual fluidic elements as described in Figure A6.1 and Table A4.1. Each fluidic element has 6 ports. These ports are used to transfer information between neighboring fluidic elements. Two or more ports are combined at one node.

- Pressure and flow rate is transferred via two hydraulic ports (black ports)
- The wetting state (wet / dry) at the beginning and the end of a fluidic element is transferred and received via a total of four digital ports (blue ports)
- The analog input port (green port) receives the stimulus from the signal generator, in our case the analog port receives the rotational frequency of the system.

Any ports which are not connected are defined as „dry“, since they are connected to ground.

For each fluidic element, the flow rate \( q \) can be calculated numerically from the pressure differential \( \Delta p \) between the ends of the element. The pressure across a radial element is defined as:

\[
\Delta p(q) = p_{\text{centrifugal}} + p_{\text{viscous}}(q) + p_{\text{inertial}}(q) + p_{\text{capillary}} \tag{A6.1}
\]

For an isoradial element the pressure differential is defined as:

\[
\Delta p(q) = p_{\text{Euler}} + p_{\text{viscous}}(q) + p_{\text{inertial}}(q) + p_{\text{capillary}} \tag{A6.2}
\]

The fill level and the resulting volume of liquid for each element are calculated by integrating the flow rate through the fluidic element:

\[
V_{\text{liquid}}(t) = \int_0^t V_{\text{liquid}}(t') \, dt' \tag{A6.3}
\]
Figure A6.1: Fluidic network as simulated in the network simulation.
Table A6.1: List of functional elements as modeled in the network simulation.

<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>Ground</td>
<td>Models ambient pressure; reference pressure: 1013 hPa</td>
</tr>
<tr>
<td>1b</td>
<td>Ground</td>
<td>Models the air vent in the inlet chamber; reference pressure: 1013 hPa</td>
</tr>
<tr>
<td>1c</td>
<td>Ground</td>
<td>Models the air vent in the collection chamber; reference pressure: 1013 hPa</td>
</tr>
<tr>
<td>2</td>
<td>Ideal gas</td>
<td>Fluidic capacitance: Models the enclosed air as an ideal gas</td>
</tr>
<tr>
<td>3</td>
<td>Pressure chamber 2</td>
<td>Models compression chamber 2 as a fluidic element with radial orientation</td>
</tr>
<tr>
<td>4a-4e</td>
<td>Timing channel</td>
<td>Models the timing channel as a series of channels with radial or tangential orientation</td>
</tr>
<tr>
<td>5</td>
<td>Pressure chamber 1</td>
<td>Models compression chamber 1 as a fluidic element with radial orientation</td>
</tr>
<tr>
<td>6a – 6b</td>
<td>Connection channel</td>
<td>Models the channel connecting compression chamber 1 with the inlet channel and the siphon channel as a series of radial and tangential channels</td>
</tr>
<tr>
<td>7</td>
<td>T-connector</td>
<td>Collapses all connected ports to nodes</td>
</tr>
<tr>
<td>8</td>
<td>Inlet channel</td>
<td>Models the timing channel as a fluidic element with radial orientation</td>
</tr>
<tr>
<td>9</td>
<td>Inlet chamber</td>
<td>Models the inlet channel as a fluidic element with radial orientation</td>
</tr>
<tr>
<td>10a-10d</td>
<td>Siphon channel</td>
<td>Models the siphon channel as a series of radial and tangential channels</td>
</tr>
<tr>
<td>11</td>
<td>Collection chamber</td>
<td>Models the collection chamber as an element that takes up incoming liquid</td>
</tr>
<tr>
<td>12</td>
<td>Signal generator</td>
<td>Models the rotational frequency: It provides the stimulus for the fluidic elements as a piecewise linear function</td>
</tr>
</tbody>
</table>
Figure A6.2: Illustration of siphon priming. The flow from compression chamber 1 is split into the siphon and the inlet channel. The siphon primes if a critical volume flows into the siphon. The volume that flows into the siphon is defined by equations 1–4 and can be calculated by numerical integration.

The pressure at point P3 and P4 is the atmospheric pressure $p_0$. Since the viscous dissipation of air in the timing channel can be neglected, the pressure at point P1 is equal to the pneumatic pressure in the pressure chamber $p_{pneu}$:

$$p_{P1}(V_{comp}) = p_{pneu}(V_{comp}) = \frac{p_0 v_0}{\nu - \nu_{comp}}$$  \hspace{1cm} \text{A6.4}

The pressure at point P2 is then defined as:

$$p_{P2}(V_{comp}) = p_{P1}(V_{comp}) + p_{cent}(r_{P1}, r_{P2}) - p_{visc}(V_{comp}, R_{P1\rightarrow P2})$$  \hspace{1cm} \text{A6.5}

with $p_{cent}(r_{P1}, r_{P2})$ being the centrifugal pressure that accumulates between P1 and P2, $V_{comp}$ being the total flow rate between P1 and P2 and $p_{visc}(V_{comp}, R_{P1\rightarrow P2})$ being the viscous dissipation between P1 and P2:

$$p_{visc}(V_{comp}, R_{P1\rightarrow P2}) = \dot{V}_{comp} R_{P1\rightarrow P2}$$  \hspace{1cm} \text{A6.6}
From the pressure difference between P2 and P3, and P2 and P4 it follows that:

\[
p_{P1}(\text{comp}) + p_{\text{cent}}(r_{P1}, r_{P2}) - p_{\text{visc}}(\text{comp}, R_{P1 \rightarrow P2}) = p_{\text{atm}} + p_{\text{cent}}(r_{P2}, r_{P3}) - p_{\text{visc}}(\text{inlet}, R_{P2 \rightarrow P3})
\]

\[
p_{P1}(\text{comp}) + p_{\text{cent}}(r_{P1}, r_{P2}) - p_{\text{visc}}(\text{comp}, R_{P1 \rightarrow P2}) = p_{\text{atm}} + p_{\text{cent}}(r_{P2}, r_{P4}) - p_{\text{visc}}(\text{siphon}, R_{P2 \rightarrow P4})
\]

If we insert the viscous pressure drop from eq. 3 into eqn (A6.4) and eqn (A6.5), we receive:

\[
\dot{V}_{\text{siphon}} = \frac{p_{P1}(\text{comp}) + p_{\text{cent}}(r_{P1}, r_{P2}) - p_{\text{visc}}(\text{comp}, R_{P1 \rightarrow P2}) - p_{\text{atm}} - p_{\text{cent}}(r_{P2}, r_{P4})}{R_{P2 \rightarrow P4}}
\]

\[
\dot{V}_{\text{inlet}} = \frac{p_{P1}(\text{comp}) + p_{\text{cent}}(r_{P1}, r_{P2}) - p_{\text{visc}}(\text{comp}, R_{P1 \rightarrow P2}) - p_{\text{atm}} - p_{\text{cent}}(r_{P2}, r_{P3})}{R_{P2 \rightarrow P3}}
\]

According to the continuity equation for incompressible liquids, the flow rate between P1 and P2 has to be the same as the sum of flow rates in the inlet channel and the siphon:

\[
\dot{V}_{\text{comp}} = \dot{V}_{\text{inlet}} + \dot{V}_{\text{siphon}}
\]

The radial positions \( r_{P1}, r_{P3} \) and \( r_{P4} \), as well as the fluidic resistance \( R_{P2 \rightarrow P4} \), are dependent on the fill level of the respective channels and chambers and thus can be expressed by a volume to height function in dependence of the total volume in the compression chamber \( V_{\text{comp}} \). If this is taken into account, the total volume flowing into the siphon channel can be calculated by a numerical integration of the differential equations eqn (A6.6), eqn (A6.7) and eqn (A6.8):
The siphon primes if the volume \( V_{\text{siphon}}(t) \) at any point in time becomes larger than \( V_{\text{crit}} \). \( V_{\text{crit}} \) is the volume required to fill the siphon across the siphon crest and further so that centrifugal gravity causes the transport of liquid to the outlet.

This condition is fulfilled if the meniscus in the siphon has passed the crest and subsequently moves to a position radially outwards of the liquid level in the inlet chamber (P3).

The volume in the siphon \( V_{\text{siphon}}(t) \) can be calculated from eqn (A6.9) in combination with eqn (A6.6) to eqn (A6.8). Since the volume transported into the siphon depends on centrifugal acceleration as indicated by the term \( p_{\text{cent}}(r_{P_i}, r_{P_j}) \), the rotational protocol can be used to control whether the siphon does or does not prime. A numerical solution of eqn (A6.9) for two different rotational protocols, one that inhibits and one that ensures siphon priming, is depicted in Figure A6.3.

![Figure A6.3: Simulation of siphon priming for timer #3 in the release-on-demand disk. The volume pumped into the siphon is described by eqn (A6.6) to eqn (A6.9) and depends on the splitting of flow between the inlet channel and the siphon channel, based on fluidic resistances and centrifugal pressures within the siphon channel and the inlet channel. The timer release is displayed at the time 0 s, the volume in the siphon channel quickly rises after timer release. If more than the critical volume is filled into the siphon, the siphon is primed and liquid is transferred to the collection chamber. At 14 Hz centrifugation, the siphon primes and liquid is transferred. At 20 Hz the centrifugal counter pressure from the siphon channel inhibits siphon priming and no liquid is transferred to the collection chamber.](image-url)
Pursuing a Ph.D. can be very stressful at times and therefore I’d like to thank those, who supported me during my time.

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