Multilayer Pressure Driven Microfluidic Platform
- µFLATLab

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Danke


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**Own Publications and Patents**


**Publication**: Jochen Rupp, Manuela Schmidt, Roland Zengerle and Martina Daub et.al. 2009: “Rapid microarray processing using a disposable hybridization chamber with an integrated micropump” to be submitted for *Lab-on-a-Chip*.


## Shortforms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
<td>PID</td>
<td>Proportional integral derivative</td>
</tr>
<tr>
<td>CNC</td>
<td>Computerized numerical control</td>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
<td>PMMA</td>
<td>Poly(methyl methacrylate)</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>Double distilled water</td>
<td>RTQ-PCR</td>
<td>Quantitative real time Polymerase chain reaction</td>
</tr>
<tr>
<td>DEP</td>
<td>Dielectrophoresis</td>
<td>SAW</td>
<td>Surface acoustic waves</td>
</tr>
<tr>
<td>DRIE</td>
<td>Reactive ion etching</td>
<td>SEM</td>
<td>Scanning electron microscope</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>LSI</td>
<td>Large scale integration</td>
<td>TPE</td>
<td>Thermoplastic elastomere</td>
</tr>
<tr>
<td>LOC</td>
<td>Lab-on-a-chip device</td>
<td>ssDNA</td>
<td>Single stranded DNA</td>
</tr>
<tr>
<td>MSL</td>
<td>Multilayer soft-lithography</td>
<td>UPEC</td>
<td>Uropathogenic E. coli</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
<td>UTI</td>
<td>Urinary Track Infection</td>
</tr>
<tr>
<td>PDMS</td>
<td>Polydimethylsiloxane</td>
<td>µFLATLab</td>
<td>Microfluidic Flexible Laserwelded Automated TPE based Lab</td>
</tr>
<tr>
<td>Symbol</td>
<td>Unit</td>
<td>Description</td>
<td>Symbol</td>
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<td>--------</td>
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<td>--------------------------------------------------</td>
<td>--------</td>
</tr>
<tr>
<td>$A$</td>
<td>$m^2$</td>
<td>Cross sectional area</td>
<td>$t_s$</td>
</tr>
<tr>
<td>$F$</td>
<td>$N$</td>
<td>Body force</td>
<td>$T$</td>
</tr>
<tr>
<td>$c$</td>
<td>$mol\cdot l^{-1}$</td>
<td>Concentration</td>
<td>$V_{hubmax}$</td>
</tr>
<tr>
<td>$C_t$</td>
<td></td>
<td>Cycle threshold</td>
<td>$V$</td>
</tr>
<tr>
<td>$d_{ech}$</td>
<td>$m$</td>
<td>Valve chamber diameter</td>
<td>$v$</td>
</tr>
<tr>
<td>$d_{pch}$</td>
<td>$m$</td>
<td>Pump chamber diameter</td>
<td>$w_{channel}$</td>
</tr>
<tr>
<td>$d_{spot}$</td>
<td>$m$</td>
<td>Laser spot diameter</td>
<td>$w_s$</td>
</tr>
<tr>
<td>$d_{ss}$</td>
<td>$m$</td>
<td>Seam-to-seam distance</td>
<td>$x, y, z$</td>
</tr>
<tr>
<td>$f_L$</td>
<td>$Hz$</td>
<td>Laser pulse repetition frequency</td>
<td>$\nabla c$</td>
</tr>
<tr>
<td>$f_P$</td>
<td>$Hz$</td>
<td>Pumping frequency</td>
<td>$\Delta Q_p$</td>
</tr>
<tr>
<td>$h_{channel}$</td>
<td>$m$</td>
<td>Channel height</td>
<td>$Q$</td>
</tr>
<tr>
<td>$P$</td>
<td>$Pa$</td>
<td>Pressure</td>
<td>$\gamma$</td>
</tr>
<tr>
<td>$P_C$</td>
<td>$Pa$</td>
<td>Control pressure</td>
<td>$\eta$</td>
</tr>
<tr>
<td>$P_{cap}$</td>
<td>$Pa$</td>
<td>Capillary pressure</td>
<td>$\lambda_L$</td>
</tr>
<tr>
<td>$P_{max}$</td>
<td>$W$</td>
<td>Laser power</td>
<td>$\nu$</td>
</tr>
<tr>
<td>$R_a$</td>
<td>$\mu m$</td>
<td>Mean roughness of absolute values of profile heights</td>
<td>$\Theta$</td>
</tr>
<tr>
<td>$R_p$</td>
<td>$\mu m$</td>
<td>Maximum height of profile above the mean line within the sampling length</td>
<td>$\rho$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>$\sigma_r$</td>
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Summary

In this work a new pressure driven microfluidic platform (µFLATLab) is presented, consisting of a lab-on-a-chip device and a processing instrument for functional control. The disposable lab-on-a-chip device consists of a multilayer polymer stack made of structured polycarbonate (PC) bulk welded to a thermoplastic elastomer membrane (TPE). Fluid management is realized by integrated active membrane microvalves with a high sealing quality and short switching times in the range of 100 ms. The concept of a sealing membrane works reliable and independent from liquid viscosity or surface tension. Membrane micropumps with a broad controllable flow rate from 0.1 µl·s⁻¹ to 55.8 µl·s⁻¹ are realized for liquid transportation and circulation for mixing discrete liquid plugs. Compared to microfluidic platforms based on polydimethylsiloxane (PDMS) the working principle of this platform is characterized by a comparably low actuation pressure and a remarkable level of design flexibility. A full three dimensional fluidic network is created by lasercutting through the membrane layer. Basic operations for microfluidic applications such as valving, pumping and mixing can be realized using this lab-on-a-chip device concept. In addition a liquid reagent storage method is implemented, where a liquid reagent is tightly sealed without any additional production process step. By applying pressure on the storage chamber the seal is broken and the liquid reagent is pushed into the fluidic network. The used materials PC and TPE can be manufactured and structured by mass production processes like injection molding and extrusion. A cost-efficient and production chain consistent way of manufacturing a disposable lab-on-a-chip device is achieved by using laserwelding as a joining technology.

A transportable processing instrument for the control and automation of the lab-on-a-chip device forms the second part of the microfluidic platform. It includes a carrier for the lab-on-a-chip device, eight fluidic and twelve pneumatic interconnections to the macro world for fluid management, two resistive heaters, fan air coolers and temperature sensors for thermal management. Twelve pilot valves are independently controlled for the actuation of the integrated microvalves. Heating rates of 4 K·s⁻¹ and cooling rates of -1.3 K·s⁻¹ enable fast thermal cycling. This portable processing instrument with the size of a shoe box has a graphical user interface (GUI) for programming parameters as temperature, duration and pressure switch for each assay step.
With this microfluidic platform, a lab-on-a-chip device was developed for the processing of a diagnostic example assay. The assay detects the resistance of *Escherichia coli* (*E. coli*) against fluoroquinolone-based antibiotics. The required sub functions include the accumulation of *E. coli* bacteria directly from a 10 ml sample, their thermal lysis, a DNA amplification step using a polymerase chain reaction (*PCR*), before detection of the resistance information with a DNA microarray. An integrated silica filter is used for the accumulation of bacteria out of a 10 ml sample with a filter efficiency of over 90 %. The processable number of bacteria range from $10^4$ to $10^7$. The bacteria lysis is made as an initial thermal step at $95^\circ C$ before the *PCR*. The 31 amplification cycles of the *PCR* are performed in less than 2 h with the processing instrument. The reaction time for the detection microarray could be accelerated from 60 min down to 30 min using an integrated micropump as an active circular mixer. At the same time the signal-to-noise-ratio could be increased. This fully automatable assay for the example application takes only 3 h from sample input to result. A diagnostic relevant bacteria concentration threshold of $10^4$ bac/10 ml could be proven to be detected with the assay integrated on chip. The concept of this cost-efficient mass producible microfluidic platform promises a broad range of future applications with the benefit of a robust, fast and reproducible results.
Zusammenfassung


Die Plattform wird komplettiert durch ein transportables Ansteuergerät für die Automatisierung der Funktionen der lab-on-a-chip Kartusche. Dieses Gerät besteht aus einer Trägerplatte mit einer Vorrichtung für das Einlegen der lab-on-a-chip Kartusche, acht fluidischen und pneumatischen Mikro-zu-Makro-Verbindungen für die fluidische Ansteuerung, zwei Widerstandsheizern mit zusätzlichen Luftkühlern, sowie Thermoelementen für die Temperaturregelung. Somit ist es möglich, bis zu zwölf Pilotventile
1. Introduction

The rate of resistant bacteria against antibiotics is growing rapidly in industrialized nations. Beside natural adaption the frequent use of antibiotics for humans and animals [1] has encouraged the acceleration of this process. For example the use of the fluoroquinolones had increased three times in an emergency room environment in the United States between 1995 and 2002 [2]. In German hospitals more than 25 % [3] of the E. coli bacteria are already resistant against fluoroquinolones. Known products are Baytril from Bayer Corporation (see Fig. 1) or Trovan from Pfizer. The increasing resistance leads to higher costs in health systems, patient sickness and an increasing mortality rate.

![Fig. 1: Resistant Escherichia coli (E. coli) stems against fluorochinolone based antibiotics (Ciprofloxacin) [3].](image)

A high resistance rate of pathogens prevents successful treatment of the patient and leads to a shorter product lifetime of drugs. In modern therapeutic appendages therefore the patient is tested for the characteristics of the pathogen prior to medication. A gold standard for testing is culturing bacteria on agar plates. This method is reliable but it takes 72 h from
sample to result. DNA-based tests including the sample preparation are performed in central laboratories by automated robots such as *cobas systems* from *Roche*. Testing by hand is a time consuming process, while huge automated systems are only economical when there is a high number of samples to process (150 - 160 samples per hour [4]). A benefit for user would be an automated test, where a sample is prepared and tested within hours without transportation to a central lab. Such a test would have to be cost-efficient as well as reliable. With this thought in mind this work has been taken place. As mentioned above one of the main pathogens among hospital germs are the *Escherichia coli* bacteria (*E. coli* see Fig. 2). It is frequently used as a model organism in microbiology studies. Cultivated strains (e.g. *E. coli K12*) are well-adapted to the laboratory environment, and, unlike wild type strains, have lost their ability to thrive in the intestine. *Uropathogenic E. coli* (UPEC) is responsible for approximately 90% of urinary tract infections (UTI) seen in individuals with ordinary anatomy [5]. The level spread of this infection was the foundation of a publically founded project called *Path.Ident* which is short for *Identification of pathogenic E. coli*.

![Fig. 2: SEM pictures of Escherichia coli [6].](image)

The idea was to use a microfluidic system to automate a single step DNA based method. The microfluidic concept comprises of a structured polymer lab-on-a-chip disposable device on which the diagnostic analysis takes place. The steps are performed and controlled by a processing instrument. The vision is to create a handheld device that simply needs the input urine sample which is then analyzed automatically. A microfluidic solution promises portability, fast time-to-result, less laboratory space consumption at lower cost per test compared to established automation [7]. Such a device could provide a fast and cost-efficient solution, independent from central labs, and therefore it could bring enormous economical
success. This development follows the trend for microfluidic cartridges in clinical diagnostics e.g. tests for cardiovascular and bacteriology. Here the main argument for the use of microfluidics is to fasten analysis time by integrating protocol steps within an automatable solution [8].

1.1. Aim of this Work

The main task of this work is to develop a new and generic microfluidic platform for the integration and automation of assays. To proof the performance of this platform one particular example assay is to be integrated which was developed by project partners in parallel to this work1. It includes the sample preparation of $10^4$ to $10^7$ E. coli bacteria from a sample volume in the range of 1 – 10 ml to detect the resistance information against fluorochinolone antibiotics with a DNA based microarray. The duration for processing the integrated assay is supposed to be less than 5 h. The adaption of the sample preparation steps to the microfluidic platform forms another part of the task. The assay required 10 - 20 valves for fluid management as well as other basic operations for liquid transportation. To perform a PCR in less than 3 h, the microfluidic platform must offer a method for thermal cycling between 55 and 95 °C. The basic operations are to be realized and characterized for their functionality by experiment. A production chain consistent and cost-efficient technological approach for a mechanically stable and fluidically tight disposable lab-on-a-chip device is to be developed. This was mainly developed by Manuela Schmidt [73] in parallel to this work. The part investigated in this work is to expand this production process developed by Schmidt to the application specific requirements of particular lab-on-a-chip designs. For example the adaption of a second laserwelding step which is developed in this work, assures the functionality of several microvalves without cross interaction between them during operation. To perform the sub functions of the example assay, a transportable processing instrument has to be developed with the ability to carry

1 Project partners of the publically founded project „Path.Ident“:
Robert Bosch GmbH, Robert Bosch Platz 1, 70839 Gerlingen Schillerhöhe, Germany.
Eppendorf AG, Barkhausenweg 1, 22339 Hamburg, Germany.
Naturwissenschaftliches und Medizinisches Institut, Marktewiesenstraße 55, 72770 Reutlingen, Germany.
Multi Channel Systems GmbH, Aspenhauserstrasse 21, 72770 Reutlingen, Germany.
Institut für Technische Biochemie, Universität Stuttgart, Allmandring 31, 70569 Stuttgart, Germany.
Robert-Bosch-Krankenhaus, Auerbachstraße 110, 70376 Stuttgart, Germany.
one lab-on-a-chip device, hard- and software for **thermal management**, chip to world interconnections as well as the software for the automation of the test.

### 1.2. Overview of Microfluidic Platforms

According to [9] “a microfluidic platform provides a set of fluidic unit operations, which are designed for easy combination within a well-defined fabrication technology. A microfluidic platform paves a generic and consistent way for miniaturization, integration, automation and parallelization of (bio-) chemical processes.” This allows the easy, fast, and cost-efficient implementation of different application-specific processes for handling benefits and reducing costs. A microfluidic platform needs to perform analytical functions including sample preparation, mixing steps, chemical reactions, separation, and detection in an integrated microfluidic network. Miniaturization of the individual analytical tools, like PCR amplification [10], capillary electrophoresis [11], enzyme assays [12], restriction enzyme mapping of DNA [13] and DNA sequencing [14,15] have been successfully realized. Even non pretreated samples like whole blood, fecal samples, live bacteria or urine to perform an on chip sample preparation like lysis, PCR had been realized before [16,17,18,19].

According to [9] the state of the art for microfluidic solutions for assay integration is classified in four platforms according to their dominating main liquid propulsion. A short description according to [9] together with an example application for each platform is described to underline the need for a pressure driven solution presented here.

"In **electrokinetics platforms** microfluidic unit operations are controlled by electric fields acting on electric charges, or electric field gradients acting on electric dipoles. Depending on buffers and/or sample, several electrokinetic effects such as electroosmosis, electrophoresis, dielectrophoresis, and polarization superimpose each other. Electroosmosis can be used to transport the whole liquid bulk while the other effects can be used to separate different types of molecules or particles within the bulk liquid” [9]. Commercially products are available for protein and DNA analysis like the LabChip System (Caliper life Sciences) [20] or the Bioanalyzer (Agilent Technologies) [21].

"In **lateral flow** tests, also known as test strips (e.g. pregnancy test strip), the liquids are driven by **capillary** forces. Liquid movement is controlled by the wettability and feature size
of the porous or microstructured substrate. All required chemicals are pre-stored within the strip. The readout of a test is typically done optically and is quite often implemented as color change of the detection area that can be seen by the naked eye.” [9]. Commercially available products are pregnancy tests like Combur 5 Test Hc (Roche Diagnostics) [22], blood coagulation test like COAGUCHECK (Roche AG) [23] as well as glucose measurement test like GlucoHEXAL (Hexal AG) [24]. The DNA based assay used here includes 1 - 10 ml of sample and a microarray as a detection method. Processing several ml of sample with these three approaches in less than five hours was found to be unsuitable.

“In centrifugal microfluidics all processes are controlled by the frequency protocol of a rotating microstructured substrate. The relevant forces for liquid transport are centrifugal force, Euler force, Coriolis force and capillary force. Assays are implemented as a sequence of liquid operations arranged from radially inward positions to radially outward positions. Microfluidic unit operations include metering, switching, aliquoting, etc.” [9]. Applications realized with this platform are cell or DNA lysis [25], protein based [26] or nucleic acid-based assays [27] in general. This concept was evaluated as a second possible way to implement the example assay within the predetermined time of five hours but was not realized. A microfluidic platform suitable for the integration of this particular assay needs the ability to handle input sample volumes of several ml, thermal processing on chip within five hours and a complex combination of liquid handling steps. The remaining pressure driven platform offers the advantages to handle comparably large volumes in the range of several ml within minutes. It is described by [9]:

“A pressure driven laminar flow platform is characterized by liquid transport mechanisms based on pressure gradients. Typically this leads to hydrodynamically stable laminar flow profiles in microchannels. There is a broad range of different implementations in terms of using external or internal pressure sources such as using syringes, pumps or micropumps, gas expansion principles, pneumatic displacement of membranes, etc. The samples and reagents are processed by injecting them into the chip inlets either batch-wise or in a continuous mode.” Hydrodynamic flow focusing is commonly used with this platform for cell separation [28], flow cytometry or particle counting [29]. Fully integrated on-chip analysis (see Fig. 3) for detection of bacteria including their lysis, DNA isolation and PCR has been published by Sauer et. al. [30].
Introduction

Fig. 3: Example for a passive pressure driven lab-on-a-chip device published by Sauer et. al. for detection of bacteria including their lysis, DNA isolation and PCR have been published in pressure driven platforms [30].

A commonly used pressure driven microfluidic platform is realized with an elastic polydimethylsiloxane (PDMS) as a bulk material. PDMS is structured photolithographically and then bonded by thermal or solvent bonding. The process combines accurate manufacturing at low costs and the ability to integrate active components for fluid management. “The microfluidic channel circuitry contains chip-integrated microvalves based on flexible membranes between a liquid-guiding layer and a pneumatic control-channel layer. The microvalves are closed or open corresponding to the pneumatic pressure applied to the control-channels. More complex units like micropumps, mixers, multiplexers, etc. can be built. With dimensions of some mm² large scale integration (LSI) is realized by combining several hundreds of units on one single chip.” [9].

This platform was first described by [31] and later expanded by Quake by a multilayer soft-lithography process (MSL) [32,41]. Besides optical transparency and biocompatibility, this approach offers high flexibility for robust high throughput testing. The working principle of the fluid management is mainly independent to liquid characteristics like viscosity. Valves, pumps and mixers are easily realized with a high functional integration density and can be combined to a complex fluidic network. PDMS on the other hand is difficult to manufacture in a true mass production process. The here presented approach addresses the advantages of the large scale integration platform by realizing the elasticity with a TPE membrane. The use of TPE in microfluidic analysis has been reported earlier by Waibel et. al. [33] and Stoyanov et. al. [44]. Waibel et. al. used an injection molded TPE sealing lip to successfully realize a piezo-actuated check valve with a diameter of 5 mm to control the flow inside an
electronic fountain pen. Stoyanov et. al. used TPE as a bulk material in which a fluidic network is realized. For fluid management a membrane valve was manufactured by placing a displaceable PDMS membrane on top of a microfluidic channel. The actuation of this membrane microvalve was realized by an external pneumatic pressure source. In the presented platform TPE is used as a membrane layer instead of using it as a bulk material. TPE works as a functional layer to realize basic microfluidic operations like valving and pumping and it is additionally used as a joining layer for the two structured polycarbonate bulks in which the fluidic network is realized. This approach promised a flexible and liquid characteristic independent fluid management principle, similar to PDMS based setups with the possibility for a high throughput structuring technology. The material combination consisting of PC and TPE offers low costs for the materials, chemical inertness as well as optical transparency to fulfill the requirements of a disposable lab-on-a-chip device.
2. Fluidic Theory at a µScale

When the dimensions of a device change from macroscale to microscale some analytical functions are promised to be more rapidly because smaller reagents volumes are needed. In this case microscale means structural dimensions from several 100 µm down to 1 µm. However, scaling theory shows that miniaturization of a device is not a simple matter of reducing its size. Some effects scale well and provide improved performance while others are not as efficient as they are in macroscopic level. For example, the surface to volume ratio increases at small scales and surface forces become more dominant compared to volume forces. A large surface to volume ratio also gives rise to fast chemical reactions because reaction partners can practically react on each other at once. At the same time, however, surface adsorption of biomolecules will be significant, easily resulting in clogging and fouling of biomolecular fluid in microsystems [34]. The theoretical fundamentals which help understanding concepts and experimental results of this thesis are given in the following chapters.

2.1. Navier-Stokes Equation

Microfluidic devices reduce fluid mechanics to micro level. Fluids are classified in Newtonian fluids, where the dynamic viscosity $\eta$ is constant. For non-Newtonian fluids the dynamic viscosity $\eta$ depends on the shear rate or shear rate history showing a non linear shear stress (e.g. blood, molten polymers). The dynamic viscosity is the rate of shear stress $\tau$ to the velocity gradient perpendicular to the layer being moved (Fig. 4).
Fluidic Theory at a µScale

Fig. 4: Schematic linear shear of fluid between a stationary and a moving plate in a channel of length L. For Newtonian fluids a linear velocity gradient \( \frac{\Delta v}{\Delta z} \) between both plates appears. The friction between the fluid and the moving boundaries causes the fluid shear rate \( \tau \). The force required for this action is a measure of the fluid's dynamic viscosity \( \eta \) [34].

Most fluids encountered in microfluidic applications may be treated as non-compressible Newtonian for purposes of first approximation. The pressure driven momentum governing equation for non-compressible Newtonian fluid is the 

\[
\rho \frac{\partial v}{\partial t} = -\nabla p + \eta \nabla^2 v + f
\]

Eq. 2-1

<table>
<thead>
<tr>
<th>Fluid density ( \rho )</th>
<th>Dynamic viscosity ( \eta )</th>
<th>( \eta = \frac{\tau}{\Delta v/\Delta z} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body force ( f )</td>
<td>Shear stress ( \tau )</td>
<td></td>
</tr>
<tr>
<td>Velocity ( v )</td>
<td>Pressure gradient ( \nabla p )</td>
<td></td>
</tr>
</tbody>
</table>

The left-hand side of equation Eq. 2-1 represents inertial forces and the right-hand side represents the forces on the fluid due to applied pressure, viscosity and body force like gravity, charge density or electric forces. Given the assumption of a steady pressure driven flow, without convection and gravity, in a long channel compared to its height, the 

\[\text{Navier-Stokes equation results in the Hagen-Poiseuille law (Eq. 2-2).}\]
\[ \Delta p = R_{\text{hydr}} \cdot Q \quad \text{Eq. 2-2} \]

Pressure gradient \( \Delta p \)  
Volumetric flow rate \( Q \)

Hydraulic resistance \( R_{\text{hydr}} \)

Similar to the Ohm’s Law here a hydraulic resistance \( R_{\text{hydr}} \) is introduced which depends on the geometry and the surface characteristics of the channel. Examples for cross sections of channels are given in Tab. 1 [34].

Tab. 1: Hydraulic resistance \( R_{\text{hydr}} \) for smooth-walled channels length \( L \) with different cross sections [34].

<table>
<thead>
<tr>
<th>Shape</th>
<th>( R_{\text{hydr}} ) expression</th>
<th>Shape</th>
<th>( R_{\text{hydr}} ) expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Circle</td>
<td>( \frac{8}{\pi} \eta L \frac{1}{a^3} )</td>
<td>Rectangle</td>
<td>( \frac{12\eta L}{1 - 0.63(h/w) h^3w} )</td>
</tr>
<tr>
<td>Ellipse</td>
<td>( \frac{4}{\pi} \eta L \frac{1+(b/a)^2}{(b/a)^3} \frac{1}{a^3} )</td>
<td>Square</td>
<td>( \frac{12\eta L}{1 - (0.917 \times 0.63) h^4} )</td>
</tr>
<tr>
<td>Triangle</td>
<td>( \frac{320}{\sqrt{3}} \eta L \frac{1}{a^3} )</td>
<td>Two plates</td>
<td>( \frac{12\eta L}{h^3w} )</td>
</tr>
</tbody>
</table>

Adding a no slip assumption to the channel walls leads to a parabolic velocity profile as shown in Fig. 5.
2.2. Reynolds number

The Reynolds number $Re$ is defined as the ratio of the inertial force to the viscous force in a flow. The definition used in a microchannel is described by Eq. 2-3 [36].

$$Re = \frac{\text{Inertial force}}{\text{Viscous force}} = \frac{\rho \cdot v \cdot L}{\eta} = \frac{v \cdot L}{\nu} = \frac{QL}{Av}$$

Eq. 2-3

Inertial force $\frac{\rho \cdot v^2}{L}$
Viscous force $\frac{\eta \cdot v}{L^2}$
Inlet fluid velocity $v$
Characteristic linear dimension $L$

Kinematic viscosity $\nu = \frac{\eta}{\rho}$
Cross-sectional area $A$
Volumetric flow rate $Q = v \cdot A$
Fluid density $\rho$

Due to the dimension feature of only some 100 µm, typical Reynolds number in microfluidic systems are between $0.001 < Re < 100$. Therefore only laminar flow appears for aqueous liquids with a dynamic viscosity similar to water ($\eta_{H,O} = 10^{-3} Pa \cdot s$) and turbulence ($Re > 2300$) does not occur [37].
2.3. Transport Phenomena Convection and Diffusion

In microfluidic applications diffusion limited reactions often take place. Such reactions are for instance surface hybridization reactions between a probe and a microarray spot (see chapter 4.3, p. 88). The governing phenomena which describe and limit such reactions, are convection and diffusion.

Diffusion is the motion of particles or molecules from regions of high concentration towards regions of low concentration. It is a statistical phenomenon caused by random motion on the molecular level. The distance $l_{\text{diff}}$ a particle diffuses over time is given by Eq. 2-4 for a two dimensional movement, where $D$ is the diffusion constant in $m^2 \cdot s^{-1}$ depending the size of a particle in a solution [38]. Some examples for diffusion constants for DNA molecules are shown in Tab. 2.

$$l_{\text{diff}}(t) = \sqrt{2Dt} \tag{Eq. 2-4}$$

<table>
<thead>
<tr>
<th>Diffusion length ($l_{\text{diff}}$)</th>
<th>Time ($t$)</th>
<th>Diffusion constant ($D$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ions in Water [38]</td>
<td></td>
<td>$200 \times 10^{-11} ; m^2 \cdot s^{-1}$</td>
</tr>
<tr>
<td>18-base-pair DNA sequence in water [39]</td>
<td></td>
<td>$10 \times 10^{-11} ; m^2 \cdot s^{-1}$</td>
</tr>
<tr>
<td>200-base-pair DNA sequence in water [39]</td>
<td></td>
<td>$2 \times 10^{-11} ; m^2 \cdot s^{-1}$</td>
</tr>
<tr>
<td>5000-base-pair DNA sequence in water [38]</td>
<td></td>
<td>$0.1 \times 10^{-11} ; m^2 \cdot s^{-1}$</td>
</tr>
</tbody>
</table>

In microchannels with distances in the range of hundreds of micrometer, diffusion brings a significant contribution to the transport of matter [34]. For example an 18 base pair long DNA sequence diffuses a distance of 100 µm to a reaction site within about 2.5 s. A sequence of 5000 base pairs would need 42 min to diffuse the same distance.
A concentration gradient in a solution results in a diffusion current \( J_{\text{diff}} \). For weak solutions the diffusion current is proportional to the concentration gradient \( \nabla c \). The direction of the current is always from a higher to lower concentration which is described by **Fick’s law** shown in Eq. 2-5 [34].

\[
J_{\text{diff}} = -D \nabla c
\]

**Eq. 2-5**

- **Diffusion current** \( J_{\text{diff}} \)
- **Concentration gradient** \( \nabla c \)
- **Diffusion constant** \( D \)

Besides the mass transport caused by a concentration gradient, a molecule can simply be moved by **convection** caused by capillary force, osmosis or a pressure gradient. For example a surface based diffusion limited reaction can be dramatically accelerated by implementing a volumetric flow \( Q \). New reaction partners are constantly transported to the reactive site compensating the depletion of molecules already reacted. The dimensionless **Péclet number** \( Pe \) characterizes the ratio of diffusion relative to convection (Eq. 2-6). A **Péclet number** \( \gg 1 \) indicates that diffusion dominates while convection dominates for \( Pe \ll 1 \).

\[
Pe \equiv \frac{\text{diffusion time}}{\text{convection time}}
\]

**Eq. 2-6**
3. µFLATLab Platform and Production Technology

The trend towards miniaturization and the flexible use of systems such as portable medical devices, micro fuel cells and even micro chemical reactors imposes strong requirements in terms of size, weight, accuracy and the capability for automation. This leads to a growing demand for integrated flow management components such as sensors, pumps and valves [40]. Platforms based on silicon or glass show the highest structural accuracy but the material itself and the structuring technology are expensive. Polymer-based lab-on-a-chip devices fulfill the requirements for disposable devices and have emerged recently. They promise cheaper and faster production cycles. Therefore manufacturing cost-efficient lab-on-a-chip systems by mass production should have great impact in biotech industries, pharmacology, medical diagnostics, forensics, environmental monitoring and basic research [7]. So far most pressure driven polymer platforms are based on polydimethylsiloxane (PDMS) which combine accurate manufacturing at low costs and the ability to integrate active components for fluid management [43,41,42]. However, this approach lacks in a high throughput production technology. The here presented platform combines cost-efficient mass production processes like injection molding and laserwelding together with the ability to create integrated active elements for fluid management.

In this chapter, the concept and working principle of the pressure driven microfluidic platform is presented. This µFLATLab (Microfluidic Flexible Laserwelded Automated TPE based Lab) consists of a lab-on-a-chip device for analysis and a processing instrument (see chapter 3.3, p. 37) necessary for processing the lab-on-a-chip device. The basic microfluidic operations realized with this approach are presented in chapter 3.4 on page 41. The production technologies together with the used materials are described and put in context to comparable existing setups.
3.1. Lab-on-a-Chip Device

The analytical chip or **lab-on-a-chip** device is a structured polymer device with microfluidic channels, reaction chambers, active valves, pumps and mixers. These basic operations are used for fluid management on chip in order to realize sample preparation and detection steps for biochemical assays. The lab-on-a-chip (LOC) device is designed to be a pressure driven system where fluids are transported onto the device by an applied pressure \( P_h \) from processing instrument or by using internal pressure driven elements like a membrane micropump. The assembly is realized as a multilayer stack (Fig. 6) with at least one control and one hydraulic layer each made of microstructured polycarbonate (PC). Between those two bulk layers an elastic membrane made of a weldable thermoplastic elastomer (TPE) with a thickness of 25 µm is placed. The control layer contains channels, which can be pressurized with a control pressure \( P_c \) for deflecting the elastic membrane layer in the middle. The fluidic network is located in the hydraulic layer. When the pneumatic control pressure \( P_c \) is larger than the hydraulic pressure \( P_h \) inside a liquid channel, the membrane is deflected towards the hydraulic layer. The control pressure is externally controlled by macroscopic pilot valves. Based on this, a microvalve or a micropump can be implemented as shown in chapter 3.4 on page 41.

![Control layer](image)

**Fig. 6:** A: Schematic cross section of a lab-on-a-chip device made of a three layer stack with two microstructured polycarbonate bulk layers welded to a thermoplastic elastic (TPE) membrane layer. The control layer contains channels which can be pressurized with a control pressure \( P_c \) for deflecting the elastic membrane layer. B: Design of a lab-on-a-chip used to process an example assay for detection of the resistance of *E. coli*.

The thermoplastic elastomer membrane works as a functional layer for fluid management as well as a joining layer for a mechanically stable and tight part. The bulk layers are joined together with the elastic membrane by laserwelding (chapter 3.2.3, p. 28).
The realization with a here developed multilayer stack, consisting of an elastic thermoplastic elastomer membrane (TPE), was inspired by the PDMS based pressure driven microfluidic platform developed by Quake [41]. The main difference to lab-on-a-chip devices made in PDMS is, that only the membrane is elastic instead of the whole device. TPE has been reported for the use in microfluidics as a sealing or bulk material by Stoyanov et. al. [43,44] or to create a check valve by Waibel et. al. [33]. To the author’s knowledge it hasn’t been reported for the purpose of a functional membrane layer as well as a bonding material layer. In the following chapter the materials and structuring technologies are explained. It will be described why polymers structured by milling and injection molding are chosen as the base of this lab-on-a-chip device.

3.2. Overview of the Manufacturing Steps

An overall view of all production steps for the lab-on-a-chip device itself and the applications needs is crucial, when choosing a concept for a microfluidic platform. The manufacturing concept chosen for the multi layer stack consist of the following steps, which are described in detail later in this chapter:

- **Structuring** of the polymer bulk material using micromilling or injection compression molding (chapter 3.2.2, p. 25).

- Structuring of the TPE membrane using **laser cutting** (chapter 3.2.4, p. 36). Assembly and alignment of the control layer and the TPE membrane.

- **Laserwelding step 1**: Bringing the joining partners in contact and applying a contact pressure. Sealing the control layer (chapter 3.2.3, p. 28).

- **Laserwelding step 2**: Alignment and assembly of the welded control layer and TPE membrane to the hydraulic layer. Sealing the hydraulic layer and join all three layers together (chapter 3.2.3, p. 28).

The two bulk layers can be structured using high throughput fabrication processes such as injection molding [45] but for prototyping, injection compression molding and micromilling is used. This concept considers the need for a cost-efficient and consistent
production chain together with the need for active elements on chip. In the following chapters the used concept is described and put in context to alternatives.

3.2.1. Materials

Lab-on-a-chip devices, like other MEMS applications, have been manufactured in silicon, glass and polymers (Cyclic Olefin Copolymer, Polypropylene, PDMS) [7]. Today polymer based devices cover a market share of 60% in analytical diagnostics, 70% in point of care devices and 40% in devices for total analysis. Silicon based systems, however, dominate the drug delivery market with a market share of 90%. Application examples are inhaler nozzles and silicon micropumps. The total microfluidic market for diagnostics and drug delivery applications reached $800 Million in 2008 [8].

When developing a microfluidic platform the material price, its available structuring techniques as well as the resulting surface properties are to be considered. The choice of the material for example determines the boundary conditions of the lab-on-a-chip device concerning the maximum achievable accuracy, surface properties, structuring and bonding technology and in sum, the costs for manufacturing such a device. Tab. 3 shows an overview of commonly used materials and a rating matrix for corresponding production steps.

Tab. 3: Overview of commonly used materials for lab-on-a-chip devices and their corresponding production characteristics. The ratings are based on lab-on-a-chip devices with dimensions of several cm² and the production of thousands of items including structuring and joining. Ratings: ++ very good/short, + good/short, - poor/long, -- not suitable.

<table>
<thead>
<tr>
<th>Material</th>
<th>Prototyping flexibility</th>
<th>Process time per item</th>
<th>Accuracy and surface quality</th>
<th>Mass production</th>
<th>Costs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silicon</td>
<td>--</td>
<td>-</td>
<td>++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>Glass</td>
<td>--</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>--</td>
</tr>
<tr>
<td>Polymer</td>
<td>++</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>PDMS</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>--</td>
<td>+</td>
</tr>
</tbody>
</table>
The concept of a pressure driven microfluidic platform presented here, with several ml of sample volume, results in a lab-on-chip device with dimensions of some cm². The geometrical accuracy of the hydraulic structures on the other hand is rather low, compared to the accuracy achievable with silicon structuring. Since this work was made in an industrial environment, impact factors like mass producibility and cost efficiency is given special emphasis to. The used polymer materials are chosen with regard to:

- Availability of standard blanks and material.
- Ability to be mass producible by injection molding or extrusion.
- Matching melting temperatures to assure weldability of the material combination.
- Chemical inertness against a broad range of commonly used liquids [46].
- Biocompatibility meaning no functional disturbance or inhibition of assay steps.
- Optical transparency for the readout equipment excitation wavelength (586 nm) and the Cy3 dyes emission wavelength (605 nm).

Also an important criterion is the availability of materials with the same quality over several years to guarantee a consistent production quality. The two structured bulk layers are made of a polycarbonate². When injection compression molding is used as a structuring technique, the parts are the size of a disk with a diameter of 12 mm. When micro milling is used, injection molded PC slides with dimensions of 75.7×25.5×1.5 mm³ are used as blanks. Those blanks are the same size as standard glass slides for microscopy. The thermoplastic elastomer (TPE) membrane³ is an ether-based polyurethane [47] and is extruded to a film with a thickness of 25±2.5 µm on an 80 m long role. The glass transition temperature range of the TPE membrane is between 155 and 170 °C [48] and lies within the range of the transition temperature of PC (145 -290°C [49]). This way the material combination can be welded together successfully. The welding process of TPE and PC has been intensively studied by Schmidt [73] to which the interested reader is forwarded to.

Existing materials used in microfluidic systems are mainly glass, silicon and polymers. Fabrications of microfluidic devices have been reported using glass [143], silicon-glass [10]

² Makrolon®, Bayer MaterialScience AG, Leverkusen, Germany.
³ Walopur®, Epurex films, Bayer MaterialScience AG, Leverkusen, Germany.
and silicon-polymer [50] techniques. To understand why polymers were the material of choice a short overview is given on the following pages.

**Silicon** is the most studied material and the one that can be obtained with the highest purity. It has been intensively studied because of its semiconductor properties. Silicon is widely used for making microprocessors, memory circuits and microelectromechanical systems (MEMS). The **structuring** of silicon is commonly realized by spin-coating a resist which is then structured lithographically. Structures are then created by removing bulk material with etching. Wet chemical etching for example uses acids like Potassium hydroxide (KOH) to create mechanical structures like cavities, micro beams or springs. Another commonly used method is dry etching. Gases react with the silicon bulk to a gaseous product and etch structures in the bulk. Those processes are often plasma enhanced like the deep reactive ion etching (DRIE) also called the *Bosch* process [52]. The process is distinguished by a high etching rate as well as a high aspect ratio. **Functional layers** like metal layers, antistiction layers or passivation layers can be used to create flow rate, pressure or temperature sensors. Structured chips are commonly **bonded** at wafer lever, which means that two or more wafers are bonded together in one step. The technologies are often thermally (fusion bonding, thermal bonding, soldering) and electrically (anodic bonding) enhanced. Silicon offers the highest potential for integrated functionality for a lab-on-a-chip device. However, the lab-on-a-chip device used here has dimensions of at least some cm$^2$. The price per device made in silicon would raise the manufacturing costs per part drastically. Another disadvantage of silicon is the one that makes silicon so easy to structure: its chemical instability to acids and temperature.

**Glass** can be characterized as an amorphous (non-crystalline) solid, which consists mainly of silicon dioxide (SiO$_2$). Glass often contains additives or impurities which modify properties like mechanical stability and glass transition temperature. An endless variety of glass with different chemical compositions and physical properties are in use today. There are optical glasses (e.g. filters and lenses), light sensitive and photochromic glasses, x-ray and gamma-ray absorbing glasses, glasses with coloring and coloring agents [51]. The use of glass for lab-on-a-chip devices instead of silicon is prompted by the unique properties of glass like a high chemical inertness, optical transparency and low background fluorescence. Those characteristics allow optical detection as well as visual inspection. Glass withstands high voltages which are used in electrokinetically driven flows and separations techniques. Other advantages of glass are its hardness, high thermal stability and relative biocompatibility,
which broaden its range of possible applications to DNA separations, enzyme reactors, immunoassays, and cell biology. For lab-on-a-chip devices, borofloat glass, Pyrex® and quartz wafers are mostly used, because they are compatible with many cleanroom processes. Well known structuring technologies like etching or laser treatment are limited in the freedom of design. But they provide the most accurate geometries in the scale of only a few µm. The structuring techniques are very similar to those of silicon. Wet chemical etching, deep reactive ion etching (DRIE), laser patterning or powder blasting are most commonly used [52]. Structured functional layers like electrical conductors can be used to integrate sensors for measuring flow rates, pressures and temperatures. Besides adhesive bonding, bonding techniques are mostly high temperature methods like fusion bonding. Companies like Micronit, NL have been specialized to develop microfluidic systems made of glass.

The need for a disposable device which is going to be in contact with body fluids is obvious [53,54]. The main reason to choose polymer over glass or silicon is the low material price and the production costs for mass fabrication. With dimensions of several cm² which appear here, structuring of silicon or glass is indeed less cost-efficient compared to polymer substrates this size, where structuring is realized with mass production technologies like injection molding, embossing, and casting processes [55,56,57]. One standard polymer material for lab-on-a-chip device for laboratory use is polydimethylsiloxane (PDMS). It was excluded as a material very early in the development process despite its advantages like optical transparency for visible and UV light, chemical inertness to commonly used liquids, elasticity and availability. The disadvantage of this material is that it cannot be injection molded with industrial standard methods. Polycarbonate (PC) as a bulk material in combination with thermoplastic elastomer (TPE) on the other hand combines the functionality similar to PDMS setups and the ability for mass production. Polycarbonates (PC) are a particular group of thermoplastic polymers. They are easily molded, thermoformed or mechanically structured by milling for instance. Because of these properties, polycarbonates have been used in many products like DVDs, automotive headlamp lenses or device cases [58]. It has a glass-like transparency, toughness and resistance-to-fracture. Additionally it is reliable and highly compatibility with body fluids and tissues for contact times of up to 30 days. PC is suitable for medical applications which comply to ISO 10993-1 and the US Pharmacopoeia, Class VI. Devices made in PC can be sterilized by steam at 120°C, by ethylene oxide gas or by gamma irradiation [59]. Other evaluated material combinations include cyclic olefin copolymer (COC) together with a polyethylene based TPE. Cyclic olefin
copolymers (COC) are amorphous polymers with low auto fluorescence. The material combinations together with their characteristics are intensively discussed in the thesis of Schmidt [73]. Polyurethane-based thermoplastic elastomers (TPE) consist of cross-linked elastic phases as well as non-cross-linked thermoplastic phases [60]. They combine characteristics of elastomer (elasticity) and thermoplastics (weldability) and are manufacturable by extrusion or injection molding. The structuring of thermoplastic polymers is rather easy with standard methods described later in this chapter. The transfer from prototyping stage to mass production needs to be kept in mind when micromilling is used as a prototyping method and injection molding is the aspired production technology. For example, the surface properties are different between these two processes. Polymer materials are available with different characteristics regarding their melting point, color, and optical transparency.

### 3.2.2. Structuring Technology for Polymer Material

The structures inside the bulk hydraulic layer are used for the transportation of samples, buffers, and other liquids. Nitrogen is used as a medium for applying pressure in the control layer structures. In order to create those structures, several methods are available. Tab. 4 on page 27 shows an overview of structuring methods for use in microfluidics.

**Micromilling** is used as the main prototyping method to face the fact of an iterative change of design. Micromilling is a mechanical method that produces polymer microstructures by mechanically removing the material with a revolving cutting tool (miller, driller). A computerized numerical control (CNC) is used to position the cutting tool, change rotation, or feed rate. The production time for a microstructure lies between several minutes and some hours strongly depending on the feature size and the total cutout volume. Cutting tools are available down to 25 μm and structures down to 10 μm can be fabricated. Those characteristics are sufficient for the structures of the lab-on-a-chip device used here (Fig. 7). This serial process shows a high flexibility for prototyping of a lab-on-a-chip device in the beginning of the development and is used in this work to structure PC blanks. The unstructured PC blanks are injection molded and have dimensions of 75.5 × 25.5 × 1.5 mm³. This geometry is similar to standard glass slides commonly used in biology, e.g., for microarray experiments.
Hydraulic layer
(transparent PC)
Micromilled structures

Fig. 7: Photograph of a 75.5 × 25.5 × 1.5 mm³ hydraulic layer structured using micromilling.

As a second manufacturing method injection compression molding is used. **Injection compression molding** is a well known mass production process which is used for example in the manufacturing process of CDs and DVDs. A master is manufactured by lithography structuring of a 50 µm negative dry film resist⁴ laminated onto an unstructured 6” silicon wafer with 20 mm·s⁻¹ at 85 °C and prebaked for 1 min at 100 °C. The structures are created with a UV 365 nm exposure for 40 s followed by developing supported by ultrasound and a hardbake step for 60 min at 145 °C. Channel depths of 100 µm are achieved by subsequent lamination and processing of a second layer of the same dry film resist. This master is replicated using electroforming to create a metal tool for the injection compression molding of PC (Fig. 8). The black control layer is dyed with carbon for the absorption of the laser energy later in the joining process. This production technology is used to create microvalve structures (see chapter 3.4.1, p. 42) and to optimize the laserwelding process (see chapter 3.2.3, p. 28) which is used for joining of the three layer stack.

In the prototyping state a flexible technology like micromilling is more useful than one with a high throughput like injection compression molding. Micromilling is suitable for the structural dimensions of some 100 µm appearing here. With accuracies of about ± 25 µm it is a sufficiently accurate process. Micromilling shows a high contour accuracy compared to injection compression molding, where a shrink has to be taken in account. The injection compression molded structural height is limited to ≤ 150 µm due to the limitations of the molding process. With ~ 5 € per structured part, injection compression molding only costs a fraction compared to micromilling with costs of several 100 € per part.

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⁴ WPR1050, E. I. du Pont de Nemours and Company (Dupon).
Fig. 8: Photograph of a black control layer, the transparent hydraulic PC layer and the stamp used in the injection compression molding process\(^5\) (DVD production process).

Tab. 4: Overview of the different technologies and characteristics for structuring substrates for the use in microfluidics [61].

<table>
<thead>
<tr>
<th>Method</th>
<th>Time per part</th>
<th>Minimum structure size</th>
<th>Surface Quality</th>
<th>Costs per part</th>
<th>Material</th>
<th>Use for</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micromilling</td>
<td>10-120 min</td>
<td>10 µm</td>
<td>-</td>
<td>High</td>
<td>Polymer, (Glass)</td>
<td>Prototyping early development</td>
</tr>
<tr>
<td>Hot Embossing [62,63]</td>
<td>20-60 min</td>
<td>10 µm to some 100 nm</td>
<td>++</td>
<td>High</td>
<td>Polymer</td>
<td>Prototyping</td>
</tr>
<tr>
<td>Lithography</td>
<td>5-20 min</td>
<td>~100 nm</td>
<td>+</td>
<td>Medium</td>
<td>Polymer (PDMS, PMMA)</td>
<td>Prototyping for laboratorial use</td>
</tr>
<tr>
<td>Laser ablation</td>
<td>1-5 min</td>
<td>100 µm to 2 µm depending on wavelength</td>
<td>--</td>
<td>Low</td>
<td>Polymer, Glass, Silicon</td>
<td>Prototyping</td>
</tr>
<tr>
<td>Casting / Soft embossing [64,65]</td>
<td>2-5 h</td>
<td>10 µm to ~100 nm</td>
<td>+</td>
<td>Very low</td>
<td>Polymer (PDMS)</td>
<td>Prototyping for laboratorial use</td>
</tr>
<tr>
<td>Injection Molding / Injection compression molding [66,67, 68]</td>
<td>0.1-2 min</td>
<td>10 µm to ~100 nm</td>
<td>++</td>
<td>Very low</td>
<td>Polymer</td>
<td>Mass production</td>
</tr>
</tbody>
</table>

\(^5\) arvato digital services GmbH, Carl-Bertelsmann-Str. 161 F, 33311 Gütersloh, Germany.
3.2.3. Laserwelding of the Lab-on-a-Chip Device

When assembling a microfluidic system, the most difficult part is to seal the structures. The main task for sealing such small structures of only some 100 µm is to create a fluidically tight and mechanically stable part while preserving the structural integrity at the same time. It is crucial, that the lab-on-a-chip device does not interact or inhibit the biochemical assay. For instance the presence of some adhesives can inhibit the PCR as well as the adsorption of Taq polymerase to the channel walls [69]. The joining process has to fulfill these application specifications as well as requirements concerning mass production compatibility such as automation and production costs. In the case of a device consisting of thermoplastics, the material properties glass transition temperature $T_g$, optical transparency, coefficient of thermal expansion and polarity are the main characteristics for possible joining technologies. After defining polymer as the material for the lab-on-a-chip device, the remaining joining technologies limited to the following:

**Gluing** is a process where a third material is applied to the joining partners before putting them together. This process is a flexible way to create a mechanically stable part. The glue often contains solvents, hardeners and additional ingredients that can inhibit assay steps performed on the device. The glue has to be put on the joining partners without closing the fluidic structures which is a slow serial process step. Another way to seal a lab-on-a-chip device is to use adhesive foils like sealing tape.

**Solvent bonding** uses an agent to solve one or both joining partners which are in contact to each other. After removing the solving agent the joining partners are permanently bonded. The mechanical properties of these joined parts are almost as high as those of the basic material. However this method compromises the geometric integrity. The usable solvents are different for each material and therefore limit the number of materials to be combined.

**Surface thermal bonding** methods use the plane melting of the joining partners or an additional material to create a mechanically stable part. After solidification the welds tighten the device over the whole contact surface. Lamination uses an additional material on the surface of the joining partners which has a lower melting point as the other material. By

---

6 Advanced Polyolefin Microplate Sealing Tape 9795, 3M.
heating the higher melting material below its glass transition temperature \( T_g \) the other one is melted and joined together with the bulk supported by pressure.

**Ultrasonic welding** or **laser welding** are methods to join material by heating the joining partners locally. Ultrasonic welding commonly uses elevated structures to induce a sufficient amount of energy for melting the material.

In this work **laserwelding** is used as a joining technology, because it is an easily automatable and low cost production process [70,71]. Furthermore additional substances as solvents or adhesives are avoided which minimizes negative effects on processing an assay. Laserwelding allows the creation of bonded and non bonded areas which allows a high freedom of design.

**Working Principle of Laserwelding**

An infrared Ny:Yag laser with a wavelength of \( \lambda_L = 1064 \text{ nm} \), changeable spot diameter \( d_{\text{spot}} = 50 - 200 \mu\text{m} \) and a maximum pulse repetition frequency of \( f_L = 80 \text{ kHz} \) is used. The radiation transmits one bulk layer and induces the biggest part of its thermal energy locally in the absorbing layer forming a weld (Fig. 9).

![Fig. 9: A: Schematic cross section of a lab-on-a-chip device made of a three layer stack with two microstructured polycarbonate bulk layers welded to a thermoplastic elastic (TPE) membrane. B: Top view picture of a laser seam joining a TPE membrane and two bulk polycarbonate (PC) parts.](image-url)
The method is a well-known process and can be described in four steps [72]:

1. The laser passes one transparent layer and is absorbed by a non-transparent layer.

2. The absorbed radiation is transformed into heat. The heat melts the absorbing joining partner locally.

3. The heat conducts into the transparent joining partners and melts all joining partners together.

4. After cooling a form-fit weld seam is created.

In this work a black bulk PC or a black TPE membrane is used as an absorption layer for the laser energy. The other joining partners are transparent or at least partially transparent to the laser wavelength. The weld characteristics are mainly defined by the energy induced per surface area. Therefore, the parameters velocity, laser power and pulse frequency are crucial. The joining partners are assembled and aligned in a cavity as illustrated in Fig. 10 A. A borosilicate glass\(^7\), transparent for the lasers wavelength \(\lambda_L = 1064\), is placed as a cap. This assembly was developed in cooperation with Schmidt [73]. The laser beam is moved at a velocity \(\vec{v}\) to form a continuous weld seam. Heat conduction can only take place, if the surfaces of all layers are in good contact and no air is included between the substrates. To guarantee a homogeneous surface pressure a borosilicate glass with a diameter of 180 mm is pressed against the evacuated cavity by atmospheric pressure. This way, for a surface of a standard slide with a surface of \(75.5 \times 25.5 \, mm = 1925.25 \, mm^2\) a surface pressure of \(\sim 1.3 \, MPa\) is applied on the stack (Fig. 10). The wedge error between glass, cavity, and topology of the joining partners is minimized by an underlying soft silicon strip. To weld different material stacks successfully, the specific melting energy and therefore the optimum laser parameter set has to be adapted for each material combination. The basic parameter investigation was made by Schmidt [73] in parallel to this work. With an elastic TPE membrane thickness of only 25 \(\mu m\), the generated heat is conducted far enough into the

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\(^7\) Advanced Materials, SCHOTT AG, Hildesheimer Straße 35 37581 Bad Gandersheim, Germany.
materials to weld three layers together. An optimum range of parameters for welding two or three joining partners in one process step are shown in Tab. 5. To create a large welded surface the laser is moved in the x and y direction from end to end of the device creating a cross pattern with a seam-to-seam distance $d_{ss}$. 
Fig. 10: A: Schematic of the used setup for laserwelding of three joints partners in an evacuated cavity. A glass is pressed against a three layer stack in an evacuated cavity ($P_{cav}$) by atmospheric pressure $P_0$, creating the contact force. B: The laser is continuously moved over the surface forming a weld cross pattern.

Tab. 5: Overview of the range of used Laser parameters ($\lambda_L = 1064$ nm) for welding a stack of polycarbonate and a TPE. Parameter optimization is evaluated by the measurement of the mechanical strength of the weld [73].†Value in % of the equipment specific maximum power.

<table>
<thead>
<tr>
<th>Material combination (*absorbing layer)</th>
<th>Power† in [%] of $P_{max}$</th>
<th>Velocity $v$ [mm·s$^{-1}$]</th>
<th>Pulse repetition frequency $f_L$ [kHz]</th>
<th>Spot diameter $d_{spot}$ [µm]</th>
<th>Seam-to-seam distance $d_{ss}$ [µm]</th>
</tr>
</thead>
<tbody>
<tr>
<td>TPE (Cutting)</td>
<td>35 ... 45</td>
<td>35</td>
<td>4</td>
<td>40</td>
<td>-</td>
</tr>
<tr>
<td>PC/TPE</td>
<td>25 ... 40</td>
<td>35</td>
<td>4</td>
<td>130</td>
<td>300</td>
</tr>
<tr>
<td>PC/TPE*/PC</td>
<td>25 ... 40</td>
<td>35</td>
<td>4</td>
<td>130</td>
<td>90</td>
</tr>
</tbody>
</table>

**Mechanical Characteristics**

The best mechanical characteristics are achieved with a black TPE membrane as an absorption layer together with two transparent bulk PC layers. Tests made by Schmidt [73] have shown a mechanical strength of 3.89$^{+0.87}$ MPa for a 48 mm long laser welded seam.

A tight lab-on-a-chip device is created by placing several overlapping welds next to each other. The distance $d_{ss}$ between the welding seams is depicted in Tab. 5. The laser beam...
center keeps a secure distance of 300 μm to the microfluidic structures to avoid structural
damage of the TPE membrane. This way misalignment and dimensional discrepancy is
compensated. With a spot diameter of 130 μm, this leads to a distance of only ~ 235 μm
between the welded polymer and the fluidic structure. The elastic TPE membrane is pressed
between the two PC bulk layers during manufacturing and is deformed into the microfluidic
channels. This leads to a very good sealing and inhibits capillary transport of liquids. With
this a sealed device with an integrated chamber of 4 mm in diameter could withstand a
pressure applied inside the chamber of 900 kPa without leakage. The energy needed for
welding is absorbed locally by the black polymer material. This allows minimum thermal
interaction with the micro structures. Liquids can be brought into the channels before the
joining step and even been tightly sealed (see chapter 3.4.9, p. 72). Temperature
measurements by Schmidt [73] showed that a distance of 250 μm from the center of the melt
zone is sufficient for the material temperature to decline below 40°C. If we assume that the
temperature profile around the melt zone is approximately similar in all directions, prestored
liquid or dried reagents, like enzymes or microarray probes would withstand the welding
procedure if a distance ≥ 250 μm to the laser beam is guaranteed.

Two Step Laserwelding of a Stack with Integrated Active Elements

In chapter 3.2.3 on page 28, the principle of joining three partners in one step was
described. The TPE membrane works as a joining and as a functional layer at the same time.
This way a mechanically stable part is manufactured and active elements like microvalves and
membrane micropumps are realized. In order to prevent a cross interaction between two of
these elements, a second laserwelding step has been implemented. As an example the setup in
Fig. 11 is given here, where two separate displacement chambers are located close to each
other. Each displacement chamber has an independent pressure supply. The TPE membrane is
supposed to be welded in all areas except the displacement chambers and their supply
channels.
The manufacturing of this setup within **one laserwelding** step is illustrated in Fig. 12. The laser is moved in cross pattern scanning over the surface avoiding the displacement chambers and their supply channels. Areas with unstructured bulk material lead to a good contact force, hence to a good welding seam between all three joining partners. Areas where a hydraulic channel is in the lower bulk, the TPE membrane is deflected in this hydraulic channel during welding, hence there is only little contact between upper bulk and TPE membrane. As a consequence poorly welded areas are created. For actuation of displacement chamber one, the control pressure is applied displacing the membrane into the hydraulic channel as expected. The poorly welding area between the two displacement chambers causes a leakage path to displacement chamber two. Hence displacement chamber two is actuated as well. This way no reproducible independent activation is possible.

In order to avoid a leakage path, the TPE membrane is welded only to the control layer at first. By using a planar metal plate beneath the membrane, a sufficient contact is achieved. The two steps are shown in Fig. 13 where the first welding path is only in the y-direction. In the **second laserwelding step**, the lower hydraulic layer is welded to the other two layers completing the x-direction of the cross pattern. Since the membrane is already tightly bound to the control layer, no cross interaction between displacement chambers appears.
Fig. 12: A: Schematic of the laserwelding of a lab-on-a-chip device with two displacement chambers in \textbf{one step}. The laser follows a cross pattern at velocity $v_l$. B: Schematic top (B) and cross sectional view (C) of a leakage path between two displacement chambers. An insufficient contact between membrane and bulk causes poorly welded areas and a leakage path between the displacement chambers when a control pressure $P_c$ is applied. No independent actuation of the displacement chambers is possible.

Fig. 13: Schematic laserwelding of a lab-on-a-chip device with two independently controllable displacement chambers in \textbf{two steps}. The laser follows a cross pattern with a seam to seam distance $d_{ss}$ at velocity $v_l$. The TPE membrane is tightly sealed in the first step by creating a sufficient contact between TPE membrane and PC bulk layer (A). In a second welding step (B) the third bulk PC layers is welded to the first two. The cross sectional view (C) shows that when a control pressure $P_c$ is applied no leakage path appears.
The cross pattern was observed to be the most robust setup. The fact, that the membrane is melted twice in some areas showed no negative effect like denaturation or burning of the material. One and two step manufacturing lead to properly working devices, but the additional step increases the yield and allows active elements to be closer to each other. For a slide sized PC bulk with a surface of $75.5 \times 25.5 \text{ mm}^2$, typical duration for the first laserwelding step with a seam to seam distance $d_{ss} = 300 \mu\text{m}$ is 200 s. For the second a $d_{ss} = 100 \mu\text{m}$ is used leading to a process time of 560 s.

3.2.4. Laser Cutting of the TPE membrane

A 25 $\mu\text{m}$ thick black TPE membrane is easily and accurately cut by a laser. The same 1064 nm Ny:Yag laser is used as for welding (chapter 3.2.3, p. 28). Cutting the TPE membrane primarily fulfills the purposes of releasing an $75.5 \times 25.5 \text{ mm}^2$ small piece out of an $80 \times 1 \text{ m}$ large role. Additionally this approach brings several advantages for the functionality and flexibility of the setup (Fig. 14).

Cutting parts out of the black TPE membrane allows optical access which can be used for absorption measurement methods or fluorescent readouts. Cutting also allows vias between functional bulk layers (see Fig. 15). This way the planar channel network is expanded to a three dimensional network with the possibility of bypassing cross channels. For example a high efficient out-of-plane L-shape chaotic advection mixer [74] can be realized, which needs three dimensional structures and hydraulic channels that cross each other. Each bulk layer can work as a control layer, a hydraulic layer or both at the same time. This
improves the integration level and makes the design more flexible. Vias enable a design where all hydraulic and pneumatic interconnections are on one side of the planar lab-on-a-chip device. This approach can be used to manufacture devices with more than two functional bulk layers which can be connected to each other.

Fig. 15: Top view pictures and schematic side view of a hydraulic via created with laser cutting: (1) A blue dyed liquid plug pushed through the top hydraulic channel, (2 & 3) passes the first via into the lower channel (4). An air bubble pushes the plug through the lower channel (5) causing it to rise into the upper channel through the second via (6).

3.3. Processing Instrument

The µFLATLab platform includes a disposable polymer lab-on-a-chip device in combination with a processing instrument. With this instrument, the assay steps are processed on chip. It contains elements for pneumatic control, thermal management and fluidic interfaces. The hard- and software is described in the following chapters.
3.3.1. Hardware

For the functional control of the lab-on-a-chip device a transportable processing instrument is developed which is mounted on a transportable carrier. It includes a mechanical fixation for the lab-on-a-chip device and fluidic interconnections to the macro world. For thermal management, resistive heaters, fan coolers and two thermocouples type K are used. One heater has an electrical resistance of $1 \, \Omega$, consumes up to 6 W and withstands temperature up to 150 °C. To control the integrated microvalves, twelve electromechanically pilot valves are implemented (Fig. 17).

![Diagram of a portable processing instrument with signal paths](image.png)

Fig. 16: Block diagram with signal paths of a portable processing instrument with pilot valves, resistive heating, and input/output measurement control board (I/O-board).
As an actuation medium a 5 bar nitrogen gas supply is connected to a pressure regulator\textsuperscript{8} to provide the control pressure $P_c$ with an output range up to 300 kPa to the pilot valves\textsuperscript{9}. The pilot valves need 8 ms for opening and 10 ms for closing \cite{75}. Additionally a precision flow meter\textsuperscript{10} and two differential pressure sensors are available. Two computer controlled syringe pumps\textsuperscript{11} are used as a source for fluid flow inside the chip. The signals are processed with an input/output measurement and control board (I/O-board) connected via USB to a computer with a LabView\textsuperscript{12}-based control software. The measurement control board was developed by Multichannelsystems (Reutlingen, Germany) as part of the project work in the publically founded project Path.Ident. This portable processing instrument with the size of a shoe box (without the syringe pumps) has a graphical user interface (GUI) for programming each assay step, the corresponding step logic and parameters like duration, temperature and pressure.

\textsuperscript{8} Pneutronics OEM-EP, Sensortechnics GmbH, Boschstr. 10, 82178 Puchheim, Germany.
\textsuperscript{9} Buerkert 6144D, Christian Bürkert GmbH & Co. KG, Germany.
\textsuperscript{10} Flow Meter ASL-1600 Sensirion AG Laubisruetistrasse 50 CH-8712 Staefa ZH Switzerland.
\textsuperscript{11} Cetoni GmbH, Am Wiesenring 6, 07554 Korbussen, Germany.
\textsuperscript{12} National Instruments LabView, National Instruments Corporation.
3.3.2. Control Loop Strategy for Temperature Management

In the example assay (chapter 4, p. 74) two assay steps need to be thermally controlled. The first step is the hybridization reaction during which the microarray is kept at a steady temperature of 55 °C. The second thermal step is the PCR, where three temperature levels of 92 °C, 55 °C and 72 °C are to be adjusted and repeated several times. These temperature levels need to be controlled precisely with an accuracy of ±1 K. Therefore temperature management is crucial. For this purpose a proportional integral derivative controller (PID) is implemented in a LabView-based control software. The feedback loop (Fig. 18) includes the resistive heater connected to a thermocouple (Type K) to create the feedback signal. This signal is used to calculate the actuation variable \( u(t) \) for the power supply unit connected to the resistive heater (Eq. 3-1). A lab-on-a-chip device shown in Fig. 6 has a PCR chamber with a thermal isolation for faster heating and cooling. The \( K_P \), \( K_I \), and \( K_D \) constants were optimized using the Ziegler Nichols method.

\[
u(t) = K_P e(t) + K_I \int_0^t e(\tau) d\tau + K_D \frac{de(t)}{dt}
\]

Eq. 3-1

Resistive heating is applied from top and bottom to achieve a smaller temperature gradient inside the PCR chamber. Both heaters are independently controlled. The resistive heating is programmed to have an incline ramping of 4.0 K·s\(^{-1}\). For the last 5 K this rate is
reduced to 2.0 K·s\(^{-1}\) to avoid temperature overshooting. An offset of 2 K at the medium level, 4 K at high level and 1 K at low level are set due to the temperature difference measured between resistive heater and the inside of the PCR chamber. The decline of -1.3 K·s\(^{-1}\) is supported by air cooling using a fan. The last 8 K this rate is reduced to -1.0 K·s\(^{-1}\) to avoid undershooting. The duration for changing temperature levels from 72 °C to 92 °C is 9 s, from 92 °C to 55 °C is 33 s and from 55 °C to 72 °C is 8 s. The resulting temperature profile inside the PCR chamber for one PCR cycle with two resistive heaters and two cooling fans is shown in Fig. 19.

Fig. 19: Temperature measurement during a PCR cycle measured inside a PCR chamber and at the heater below and above a chip. Both resistive heaters are independently controlled. Incline ramping: 4.0 K·s\(^{-1}\). To avoid temperature overshooting the incline rate is reduced to 2.0 K·s\(^{-1}\) for the last 5 K. A decline rate of -1.3 K·s\(^{-1}\) is supported by air cooling using a fan. To avoid undershooting during cooling the decline rate is reduced to -1.0 K·s\(^{-1}\) for the last 8 K. Set point offset: 2 K at the medium level, 4 K at high level, 1 K at low level.

The heat capacity and the thermal resistance of the polymer lab-on-a-chip device mainly limit the minimum time for reaching the desired temperature level. Considering a PCR holding time for each temperature level of one minute, this heating strategy leads to total
cycle duration for one cycle of 230 s. This approach reaches ramping times comparable to those measured in commercial available PCR cyclers\textsuperscript{13}.

### 3.4. Basic Operations of µFLATLab

The fluid management and the realization of microfluidic functions are described in this chapter. The realization of these operations is an important issue when it comes to decide which system should be used for a certain application. Each microfluidic platform described in chapter 1.2 on page 8 has different realizations for fluid transportation, actuation and metering. The specification of a certain application can be solved in many ways, but the decision of the suitable platform directly leads to boundary conditions like cost of manufacturing and robustness of the system.

#### 3.4.1. Microvalve

Valving is used to control a flow path or to start and stop a flow. It is therefore one of the basic needs to be fulfilled in microfluidics. Commonly used external devices are electromechanically or piezoelectrically actuated two or three way valves. The devices are usually non integrated stand-alone-parts with a high commercial availability. The functional integration is considered to be “a key requirement, which leads to a growing demand for micro flow management components such as sensors, pumps and valves” \[76\]. In order to get a high integration density into a lab-on-a-chip device, macro-sized stand-alone parts are of limited use because of their relatively large dimensions. Also it would lead to an unacceptable high price for a disposable if the electromechanical devices were thrown away with each lab-on-a-chip device.

Integrated microvalves have been developed as passive check valves, needing no energy input. In contrast active driven microvalves need additional energy input. Commonly used actuation principles for active microvalves include magnetic, electric, piezoelectric or thermal actuation. Passive microvalves are realized as a check valve or uses capillary forces \[77,78\].

\[13\] Eppendorf Master Cycler Gradient, Eppendorf AG, Hamburg, Germany.
For a more detailed overview the reader is forwarded to [79]. There are many requirements that miniaturized valves have to fulfill, such as small dead volume, low energy consumption, good sealing, short response times etc. [80]. Ideally a valve has a negligible flow resistance in the open direction and is completely sealed in the opposite direction even at high back pressures.

**Working Principle and Design Overview of the TPE Membrane Microvalve**

With the integrated elastic membrane presented here, active membrane microvalves are easily realized and represent the central function of the µFLATLab. A microvalve consists of a discontinuous hydraulic channel separated by a support with width $w_s$. The TPE membrane lies on top of a support (Fig. 20). A control displacement chamber with a diameter $d_{vch}$ is located in the control layer. Valving is realized by applying a control pressure $P_c$ inside this control displacement chamber which is located on top of the hydraulic channel. The TPE membrane deforms and is pressed against the support which tightens the hydraulic channel. A similar principle has been shown for several polydimethylsiloxane (PDMS) setups [41,81] or hybrid PDMS-Glass setups [43]. So far this approach has been used mainly in research for a highly integrated and cheap on chip solution. Twelve different geometries as shown in Tab. 6 are investigated. On the hydraulic layer each microvalve has a fluidic inlet and outlet connected by a channel. The microvalve is located in the middle of the channel consisting of a chamber with a support. Each device has a control displacement chamber which has the same diameter as the valving chamber in the hydraulic layer. The geometric variations investigated include the channel width $w_{channel}$, support width $w_s$ and the microvalve chamber diameter $d_{Vch}$. The hydraulic channel length is 18 mm, channel depth is 100 µm for the fluidic and 50 µm for control layer. The operational characteristics are investigated using a constant hydraulic inlet pressure of $P_h = 8$ kPa, generated by an elevated tank filled with deionized water. Each of the twelve microvalves is tested separately.
Fig. 20: Schematic cross section (A), top view (B) and photograph (C) of a membrane microvalve and its working principle. The microvalve consists of a discontinuous hydraulic channel (width $w_{\text{channel}}$, height $h_{\text{channel}}$) separated by a support (width $w_s$). By applying a higher control pressure $P_C$ in the control displacement control chamber (diameter $d_{\text{vch}}$), the TPE membrane is pressed against a support sealing the channel. By releasing the control pressure $P_C$ the membrane can be deflected by the fluid with pressure $P_h$ and a flow is possible.

Tab. 6: Microvalve: overview of the 12 tested geometric variations.
Operational Characteristics of the TPE Membrane Microvalve

The basic functionality of the TPE membrane microvalve is shown in Fig. 21. A constant hydraulic inlet pressure $P_h = 8$ kPa induces a flow $Q$ through the hydraulic channel. The control pressure level is held constant at 10 kPa to make sure $P_c > P_h$. The microvalve is opened and closed periodically at a frequency of 0.25 Hz. When a control pressure is applied the valve stops the fluid flow completely. After releasing the control pressure the flow is induced again.

Fig. 21: Switching operation of a TPE membrane valve. The flow rate $Q$ and the control pressure $P_c$ are normalized with their maximum values being 1. A constant hydraulic pressure induces a flow rate $Q$ which is periodically stopped and started at a frequency of 0.25 Hz.

Switching operation is investigated over a minimum number of 60 cycles of opening and closing the valve while measuring the flow rate. All designs show this basic functionality over several minutes to hours without any loss of sealing quality. A time constant for switching was measured to be between 100 - 200 ms which leads to a maximum switching
frequency of 5 - 10 Hz. The average fluidic flow rate $Q$ varies, depending on inlet pressure, channel width and chamber geometry (Fig. 22).

Fig. 22: Overview of the different resulting average flow rates from investigated geometries of integrated TPE membrane microvalve. The geometric variation (1-12, see Tab. 6, p. 44) includes the channel inlet width $w_{\text{channel}}$ (200 and 400 µm), the chamber diameter $d_{\text{ch}}$ (2000, 1000 µm and same as $w_{\text{channel}}$) and the support width $w_s$ (200 and 400 µm).

Microvalves connected to channels with a cross section of $400 \times 100 \, \mu\text{m}^2$ (design 2, 4, 6, 8, 10, 12) have an increased flow rate compared to those connected to channels with a cross section of $200 \times 100 \, \mu\text{m}^2$ (design 1, 3, 5, 7, 9, 11). The connecting channel therefore is contributing the major part of the total fluidic resistance. The resistance caused by the microvalve depends on the chamber diameter $d_{\text{ch}}$ and decreases with larger chamber diameters (design 1 - 4). The dead volume of the microvalve is calculated to be the volume of the displacement chamber in the hydraulic layer and the volume of the displacement control chamber in the control layer. The height of the displacement control chamber is for every design 50 µm. The largest dead volume therefore is calculated to be 0.5 µl for design 4. The smallest dead volume is realized with design 9 - 12. These microvalves have a dead volume that corresponds to the volume that the TPE membrane can deflect into the displacement control chamber plus the volume of the hydraulic channel in that
area. It is calculated for example for design 12 with a displacement control chamber of \(400 \times 200 \times 50 \, \mu m^3\) and a hydraulic channel volume \(400 \times 200 \times 100 \, \mu m^3\) to be smaller than 20 nl. A large valving chamber (e.g. design 4) works as a displacement pump chamber causing \(\sim 0.3 - 0.6 \, \mu l\) to be pushed out the valving chamber. This results in an overshooting peak during switching. Large valving chambers (design 1 - 4) also tend to trap bubbles. When a bubble is trapped the main function of the microvalve is never affected but the decreased active channel cross section lowers the flow rate depending on the size of the bubble. The 400 \(\mu m\) support has a 10 - 30\% higher resistance to the fluid than the 200 \(\mu m\) support which leads to a decreased average flow rate \(Q\).

**Switching Characteristics of the TPE Membrane Microvalve**

The switching characteristics is measured by increasing and decreasing the control pressure \(P_c\) in the displacement control chamber above and below a constant hydraulic pressure level \(P_h = 5.8 \, kPa\) while measuring the flow rate \(Q\). The control pressure is changed at a rate of 0.5 kPa·s\(^{-1}\) (Fig. 23).
Fig. 23: Normalized switching characteristics for microvalve (design 1, chamber diameter 2000 µm, support 200 µm, hydraulic channel width 400 µm, height 100 µm). The control pressure $P_c$ is increased at a rate of 0.5 kPa·s$^{-1}$ above and below the hydraulic inlet pressure $P_h$ while measuring the flow rate $Q$.

The data is representatively shown for one microvalve design 1 (see Tab. 6) for two ramping cycles from completely open to fully sealed. The switching interval between complete open and complete close is below 3 kPa. The minimum closing pressure shown in Fig. 23 is 2.3 kPa below the fluidic inlet pressure. This could be caused by the TPE membrane being pre-stressed counteracting against the fluidic pressure without any applied control pressure. Hysteresis (direction clockwise) appears in all design variations. It varies between 0.5 kPa and 1.5 kPa. One possible reason for this effect could be the membrane sticking to the support therefore increasing the energy needed for the flow to start. Four microvalves are tested for every design variation listed in Tab. 6 showing similar characteristics. To assure a reproducible complete sealing a minimum closing pressure of 0.72 kPa higher than $P_h$ is necessary. The microvalve works at elevated temperatures up to 92 °C. One application example is the sealing of a PCR reaction chamber, where temperatures of 92 °C appear. The elevated temperature leads to a pressure which pushes fluid outside the chamber inhibiting the proper functionality of the PCR. For a robust functionality of the microvalve, a control pressure up to several 100 kPa can be used, since the lab-on-a-chip device withstanding those pressure levels inside control chambers with diameter in the range of 1000 µm.
The open and close functionality is achieved for every investigated design variation, leading to a minimum size of the microvalve to be in the range of the fluidic channel. Therefore it consumes only some $100 \times 100 \, \mu\text{m}^2$, which is about the same dimensions presented by Unger et. al. and Quake et. al. [41,64] for PDMS based large scale integration valves. Together with a dead volume that is smaller than 20 nl this device is comparable to this technology. The microvalve as a basic processing instrument for flow routing gives the possibility to create a complex fluidic network needed for lab-on-a-Chip device. This concept is a flexible planar setup with high integrated functionality where several microvalves are placed on chip. This way one off chip equipment addresses various lab-on-a-chip devices.

### 3.4.2. Microfluidic Junction

A microvalve as described can be used to define a flow path besides the basic functionality of stopping a flow. A T- or X-junction is realized by placing one or two branches on one side of the microvalve as shown in Fig. 24 B and C.

![Fig. 24: Schematic top view of a microvalve (A) and the design of a T-junction (B), X-junction (C) and an X-junction (D) with a loop structure. The arrows mark the possible flow paths.](image)

One application is the filling of a loop structure. The filling from an inlet to an outlet naturally fills only the path with the lowest hydraulic resistance. One solution to fill the loop
completely is to place a switching microvalve at the in- and outlet, respectively and switch from one path to the other. This method although leaves the risk of trapping a bubble. A second way is to use only one microvalve that separates the beginning and the end of the loop just by its support (see Fig. 24 D). The microvalve is closed leaving only the path through the entire loop open (Fig. 25). After filling it is opened while the in- and outlet microvalves are closed. This way a discrete liquid plug is enclosed. The plug can be circulated for instance inside the loop structure by a micropump. This way a T- or X- junction is created with no bubble trapping. The design shown in Fig. 25 B has a dead volume of only 0.2 µl.

Fig. 25: Schematic sectional (A) and top view (B) of a microfluidic X-Junction. C: Top view picture sequence (1-10) of a microfluidic X junction with a dead volume of 0.2 µl for filling of a micropump on top of a hybridization chamber.
3.4.3. Micropump

Each microfluidic platform has specific fluid movement principles like capillary forces in lateral flow platforms or centrifugal forces (chapter 1.2, page 8) to fulfill the need for a liquid transport within μl volumes. Pressure driven platforms usually take advantage of the fact that fluid movement is easily generated by external forces e.g. syringe pumps to create a pressure gradient. Although some applications cannot be addressed using this principle: e.g. when it comes to circulate discrete liquid plugs, an external pressure source is not applicable (see example assay in 3.4.5 p. 65 and 4.3.4 p. 95). To generate a convective fluid movement, several integrated actuation principles are used in pressure driven microfluidics. Some examples suitable for the µFLATLab developed here are as follows. For a more detailed overview the reader is forwarded to [82,83,84].

- Fluid movement using surface acoustic waves (SAW) [85].
- Fluid movement through surface tension and capillary effects [86].
- Ferro fluidic magnetic micropumps, magneto hydrodynamic (MHD) fluid actuation [87].
- Fluid movement with electrochemically generated gas bubbles [88], electroosmotic induced flows [89], electro hydrodynamic pumping or dielectric forces [90].
- Temperature fields for manipulation of the flow profile and therefore the velocity distribution [86,91].
- Cyclic mechanical displacement of the sample using pneumatic [92], thermo-pneumatic or piezoelectric actuation [93,94].

Thermal, electrical and magnetic principles need either electric circuits or integrated particles. Surface treatment needs additional manufacturing steps and its function is bound to the used liquid. Considering price, complexity and functionality of the principle above here a mechanical principle is realized for this platform.
Working Principle and Design Overview of the TPE Membrane Micropump

The usage of an elastic TPE membrane offers the possibility to displace or suck liquid by applying a control pressure. A pneumatically actuated displacement micropump is easily built by combining two active microvalves (see 3.4.1, p. 42) with a displacement pump chamber (Fig. 26), therefore fulfilling the basic requirement of a microfluidic system to transport small volumes of liquid. Thus the device is categorized as a pneumatically actuated displacement or diaphragm micropump [83,84]. Two designs are investigated:

**Micropump design I** (Fig. 26) has a displacement pump chamber with a diameter of $d_{PCh} = 4\text{ mm}$. The displacement pump chamber height $h_{PCh}$ located in the hydraulic layer is varied between $100\ \mu\text{m}$, $300\ \mu\text{m}$ and $500\ \mu\text{m}$. The support width $w_s$ of the microvalves are $200\ \mu\text{m}$. The displacement control chamber depth is $350\ \mu\text{m}$. It is located in the pneumatic layer, where the control pressure is applied.

![Fig. 26: Top view picture of design I (A) and schematic sectional representation (B) of a membrane micropump with microvalves at the in- and outlet of a displacement pump chamber ($d_{PCh} = 4\text{ mm}$). The displacement pump chamber heights $h_{PCh}$ are 100 , 300 and 500 $\mu\text{m}$. The microvalve support $w_s$ is 200 $\mu\text{m}$. The device is made of a three layers stack consisting the control and hydraulic layer separated by an elastic thermoplastic elastomer membrane (TPE). Initial state: membrane and valves are not actuated.](image-url)
**Micropump design II** (Fig. 27) has a displacement pump chamber diameter of $d_{PCh} = 2$ mm and a displacement control chamber, that is realized as a via through the hole control for a more robust functionality.

The working principle of the membrane micropump contains a deflection and a suction step. The fluid is pushed towards the outlet by actuating the displacement chamber of the micropump while the inlet valve is kept closed, leaving the outlet valve open. The outlet valve is then closed before releasing the membrane of the displacement chamber causing the fluid to reenter through the open inlet valve. Two different pressure levels have been implemented. The release of the displaced membrane is supported by vacuum in the first mode. Vacuum here means an absolute pressure of several 10 kPa, while the control pressure is given as a relative value. In the second mode atmospheric pressure is the surrounding pressure level. The membrane is released here by the reset force caused by its elasticity.
Six Steps Actuation Cycle with Pressure Levels: $P_C$, Vacuum

The membrane is actively pushed by the control pressure $P_C$ and the release is supported by vacuum. The cycle is repeated periodically at a frequency $f_P$. The duration $t_s$ for each step is given in proportion to the total time $T$ for one pump cycle.

Suction of liquid through the open inlet valve.

Step 1: The outlet valve is closed to avoid backflow from the outlet.

\[ \frac{t_S}{T} = 0.3 \]

Step 2: The inlet valve is closed.

\[ \frac{t_I}{T} = 0.1 \]

Step 3: The outlet valve is opened.

\[ \frac{t_S}{T} = 0.1 \]

Step 4: Actuation of the displacement pump chamber pushing the liquid towards the outlet.

\[ \frac{t_S}{T} = 0.3 \]

Step 5: The outlet valve is closed to block any backflow.

\[ \frac{t_S}{T} = 0.1 \]

Step 6: The inlet valve is opened again to initiate the suction step 1.

\[ \frac{t_I}{T} = 0.1 \]
Six Steps Actuation Cycle with Pressure Levels: $P_C$, Atmosphere

The membrane is actively pushed by the control pressure $P_C$, the release of the membrane is caused by the reset force of the membrane resulting from its elasticity. The cycle is repeated periodically at a frequency $f_P$.

**Step 1:** Suction of liquid through the open inlet valve. The inlet valve is not sealed when no actuation pressure is applied. This way fluid can be sucked through the non actuated inlet valve by the released pump membrane. The outlet valve is closed to avoid backflow from the outlet.

**Step 2:** The inlet valve is closed.

**Step 3:** The outlet valve is opened.

**Step 4:** Actuation of the displacement pump chamber pushing the liquid towards the outlet.

**Step 5:** The outlet valve is closed to block any backflow.

**Step 6:** The actuation pressure is turned off to release the closed inlet valve before initiating the suction step 1.
Maximum Pumped Volume per Stroke

The calculated maximum pumped volume per stroke $V_{hubmax}$ is the sum of the displacement pump chamber volume plus half the volume of one microvalve, where the liquid is displaced as well (Eq. 3-2, see Schematic volume transport on page 57). An overview is given in Tab. 7. The volume efficiency is the ratio of the transported volume divided by the maximum volume displaced per stroke. It is calculated as depicted in Eq. 3-3.

\[
V_{hubmax} = \pi \left[ \left( \frac{d_{pch}}{2} \right)^2 + \frac{1}{2} \left( \frac{d_{vch}}{2} \right)^2 \right] - \frac{1}{2} d_{vch} \cdot w_s \cdot h_{pch}
\]

Eq. 3-2

\[
V_{hub} = \frac{Q_{av}}{f_p}
\]

Eq. 3-3

<table>
<thead>
<tr>
<th>Average flow rate $Q_{av}$</th>
<th>Valve support width $w_s$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actuation frequency $f_p$</td>
<td>Valve chamber diameter $d_{vch}$</td>
</tr>
<tr>
<td>Pump chamber diameter $d_{pch}$</td>
<td>Height of structure $h_{pch}$</td>
</tr>
</tbody>
</table>

Tab. 7: Overview of the calculated maximum volume per stroke for a membrane micropump.

<table>
<thead>
<tr>
<th></th>
<th>Micropump Design I</th>
<th>Micropump Design II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Displacement pump chamber height $h_{PCh}$ [µm]</td>
<td>100 300 500</td>
<td>300</td>
</tr>
<tr>
<td>Displacement pump chamber diameter $d_{PCh}$ [mm]</td>
<td>4 4 4</td>
<td>2</td>
</tr>
<tr>
<td>Microvalve diameter $d_{valve}$ [mm]</td>
<td>2 2 2</td>
<td>0.6</td>
</tr>
<tr>
<td>Microvalve support width $w_s$ [µm]</td>
<td>200 200 200</td>
<td>200</td>
</tr>
<tr>
<td>Maximum volume per stroke $V_{hubmax}$ [µl]</td>
<td>1.39 4.18 6.97</td>
<td>1.13</td>
</tr>
</tbody>
</table>
Schematic volume transport. Half of the volume of the inlet valve is added to the transported volume per stroke from the pump chamber (step 2-4). Because of (step 2). The same volume is not pushed back by the displacement of the outlet valve in step 5 because the actuated pump chamber has a high fluidic resistance.

Step 1: Suction of liquid through the open inlet valve.

The elasticity the membrane offers a capacitance for the volume displaced in step 1. Pump displacement chamber buffers the displaced volume from the inlet valve.

Step 2: Pump displacement chamber blocks the backflow from the displaced volume of the closed output valve.

Step 3: The outlet valve is opened.

Step 4: The outlet valve is closed to block any backflow.

Step 5: The outlet valve is closed to avoid backflow. The actuation pressure is turned off to release the closed inlet valve before initiating the suction step 1.
Characteristics of the TPE Membrane Micropump Design I

The micropump is characterized by increasing the actuation frequency $f_P$ from 0.2 Hz up to 8.0 Hz measuring the average pump rate $Q_P$. The average flow rate is the average of 20 actuation cycles for each frequency $f_P$. One Hz here means one full six step actuation cycle in one second. A control pressure $P_c = 50$ kPa is periodically applied to the active microvalves and the displacement pump chamber as well.

The frequency characteristics of a membrane micropump (design I) is shown in Fig. 28 for an actuation cycle with $P_c$ and vacuum as actuation pressure levels. Since a control chamber in the pneumatic layer has a depth of 350 µm, an additional volume of 6.5 µl has to be taken into account when vacuum is applied during suction stroke.

Fig. 28: Average pump rate $Q_P$ vs. actuation frequency $f_P$ of a membrane micropump with a displacement pump chamber diameter $d_{PCh} = 4$ mm and a chamber height $h_{PCh} = 100$, 300 and 500 µm (design I). Pressure level: $P_c = 50$ kPa, vacuum (absolute some 10 kPa). The data shown are the average pump rate $Q_P$ of 20 pump cycles for each frequency. Ramping is repeated five times. Symbols are measurement data, lines are linear fit.

**Pump rates** can be controlled between 3.4 µl·s$^{-1}$ and 55.8 µl·s$^{-1}$ ($h_{PCh} = 500$ µm), 2.2 µl·s$^{-1}$ and 46.1 µl·s$^{-1}$ ($h_{PCh} = 300$ µm), 1.8 µl·s$^{-1}$ and 28.5 µl·s$^{-1}$ ($h_{PCh} = 100$ µm) at actuation frequencies between 0.2 Hz and 8.0 Hz respectively. The devices show a high reproducibility with a standard deviation of < 4 % ($h_{PCh} = 500$ µm), < 4 % ($h_{PCh} = 300$ µm),
and < 2 % \( (h_{Pch} = 100 \, \mu m) \). The average \textbf{incline pump rate} \( \Delta Q_P \) between 0.2 Hz and 8.0 Hz is 7.8 µl·s\(^{-2}\) for \( h_{Pch} = 500 \, \mu m \), (coefficient of determination \( R^2 = 0.94 \)), 6.5 µl·s\(^{-2}\) for \( h_{Pch} = 300 \, \mu m \), \( R^2 = 0.95 \) and 5.4 µl·s\(^{-2}\) for \( h_{Pch} = 100 \, \mu m \), \( R^2 = 0.95 \). The \textbf{volume efficiency}\(^{14}\) of the transported volume per stroke is > 90 % \( (h_{Pch} = 500 \, \mu m) \) for all three pump chamber depths at low actuation frequencies. It declines with increasing frequency to 50 % at 8.0 Hz. The transported liquid increases therefore with larger displacement volumes at the price of a lower efficiency per stroke.

The frequency characteristics of a membrane micropump \textbf{design I} for an actuation cycle with \( P_c \) and \textbf{atmospheric} pressure as the two actuation pressure levels is shown in Fig. 29.

---

\(^{14}\) Values are calculated using the measured displacement chamber depth \( (524 \, \mu m, 330 \, \mu m, 162 \, \mu m) \) and the control chamber depth \( (350 \, \mu m, 282 \, \mu m, 330 \, \mu m) \).
Pump rates are measured to be between 0.5 µl·s⁻¹ and 2.6 µl·s⁻¹ ($h_{Pch} = 500 \ \mu m$), 0.5 µl·s⁻¹ and 1.7 µl·s⁻¹ ($h_{Pch} = 300 \ \mu m$), 0.1 µl·s⁻¹ and 1.0 µl·s⁻¹ ($h_{Pch} = 100 \ \mu m$). As depicted in Fig. 29 the maximum flow rate appears at a peak between 2.0 and 4.0 Hz. At higher frequencies the average pump rate declines. This could be due to the fact, that the membranes mechanical characteristics limit the time it takes the membrane to deflect itself into initial state. Therefore, at frequencies higher 2.0 Hz the membrane is deflected into the hydraulic layer at the next cycle before it could finish elevating itself.

The volume efficiency declines rapidly at frequency above the maximum flow rate as shown in Fig. 29. The measured flow rates vary strongly between different micropumps of the design I. Adhesion of the TPE membrane to the bottom of the displacement pump chamber was observed. This causes a flow rate of almost zero for some devices regardless of the actuation frequency. It seems there is less adhesion for rougher surfaces, which explains the poor reproducibility between tested devices. An increase of the membranes stiffness or reset force is one way to release the membrane more reliably. This could be realized by increasing the aspect ratio of the displacement pump chamber. Here the TPE membrane could be deflected a larger distance into the pump displacement chamber with the result of a higher reset force. Another strategy is to minimize the contact surface by placing pillars or changing the surface characteristics. Structures with one, four and six pillars at the bottom of a displacement pump chamber with a height of 200 µm were investigated. The yield of functional micropumps was increased, although the adhesion of the TPE membrane is not sufficiently avoided for every device.

Increasing the membranes stiffness by a smaller displacement chamber diameter is realized with micropump design II. The mechanical tension $\sigma_r$ of a circular plate with a thickness $h$ caused by a pressure $p$ over the entire surface is proportional to the square radius $R^2$ [95]. The diameter of the membrane in design II is with 2 mm half the size of the 4 mm, therefore the stiffness is four times higher. An increase of the membrane thickness $h$ would lead to the same effect but was not implemented because of the influence a thicker TPE membrane would have on the laserwelding process (see 3.2.3, p. 28) of the lab-on-a-chip device.
\[
\sigma_r = 1.24 \cdot p \frac{R^2}{h^2}
\]

Eq. 3-4

Mechanical tension of a circular plate: \(\sigma_r\), Membrane thickness: \(h\), Radius of a circular membrane: \(R\), Pressure: \(p\)

**Characteristics of the TPE Membrane Micropump Design II**

The frequency characteristic of a micropump **design II** with a displacement chamber of 2.0 mm in diameter is shown in Fig. 31.

**Pump rate** can be controlled between 0.3 \(\mu\)l\(\cdot\)s\(^{-1}\) and 5.7 \(\mu\)l\(\cdot\)s\(^{-1}\) at actuation frequencies between 0.20 Hz and 8.0 Hz respectively. The device shows a high **reproducibility** with a standard deviation of < 6%. The average **incline pump rate** \(\Delta Q_p\) between 0.20 Hz and 8.0 Hz is 0.8 \(\mu\)l\(\cdot\)s\(^{-2}\) with a coefficient of determination of \(R^2 = 0.98\). The control chambers in design II are realized as vias. The membrane is deflected into the control chamber during the
suction stroke by the applied vacuum. The transported volume therefore depends on the applied vacuum. This additional volume is not particularly defined as in design I, where the control chamber height is 350 µm, which is why the efficiency is not shown in Fig. 30.

The frequency characteristics of a micropump design I are shown in Fig. 29. Here the pressures levels used for actuation are $P_c$ and atmospheric pressure.

![Fig. 31: Average pump rate $Q_P$ vs. actuation frequency $f_P$ of a membrane micropump with a displacement chamber diameter of 2 mm and a chamber depth of 300 µm (design II). Left: Actuation cycle: six steps, pressure level: $P_c = 50$ kPa, atmospheric pressure. Right: Pump efficiency. The data shown are from 20 pump cycles per frequency. Ramping is repeated three times. Symbols are measurement data, lines are linear fit.](image)

**Pump rate** can be controlled between 0.1 µl·s⁻¹ and 3.5 µl·s⁻¹. The flow rate saturates with frequencies from 5.0 Hz to 8.0 Hz showing no significant decline of the pump rate $Q_P$ at higher frequencies. The reset force at a diameter of 2 mm is four times higher than the one appearing at 4 mm diameter in design I. This way the force for deflection of the membrane during the suction stroke is sufficient for a reproducible flow rate with a deviation in flow rate of < 3%. The average incline pump rate $\Delta Q_P$ between 0.2 Hz and 3.0 Hz is 0.8 µl·s⁻² with a coefficient of determination of $R^2 = 0.99$. The efficiency is with ~ 70% of the theoretically transported volume per stroke stable between 1.0 and 2.0 Hz and declines at higher frequencies.
The data shown is from one micropump representative for the designs characteristic. All other tested devices (Design I: \( h_{\text{Pch}} = 500 \, \mu\text{m}, \) number of tested devices \( n = 11, \) \( h_{\text{Pch}} = 300 \, \mu\text{m}, \) \( n = 11, \) \( h_{\text{Pch}} = 100 \, \mu\text{m}, \) \( n = 10; \) Design II: \( n = 3 \) ) show similar behavior. Differences in pump rates are the result of milling manufacturing tolerances. The dominant influences are the dimension of the devices and here mainly the depth of the displacement pump chamber. The differences between set and actual chamber depths are: \( 39^{\pm 15} \, \mu\text{m} \) (\( h_{\text{Pch}} = 500 \, \mu\text{m}, \) number of measurements \( n = 21 \) ), \( 43^{\pm 18} \, \mu\text{m} \) (\( h_{\text{Pch}} = 300 \, \mu\text{m}, \) \( n = 21 \) ) and \( 54^{\pm 11} \, \mu\text{m} \) (\( h_{\text{Pch}} = 100 \, \mu\text{m}, \) \( n = 21 \) )

This basic microfluidic operational unit is suitable for many tasks in a LOC system. The flow rate mainly depends on the micropump geometry which makes the principle robust and flexible. The characteristics of the elastic membrane offer the possibility to gain a flow with one pressure level with limitations in adjusting of the maximum flow rate. A sufficiently stiff membrane is essential to avoid adhesion of the \( \text{TPE} \) membrane and to achieve reproducible results. An introduction of a lower second control pressure level (vacuum) increases the bandwidth and the reproducibility of the flow rate tremendously. This mode is used in the application example described in chapter 3.4.5 (Active Rotary Mixer using a Membrane Micropump on page 65) and 4.3.4 (Acceleration of the Microarray Hybridization by an Overlaying Flow on page 95).

3.4.4. Mixing

A mixer is one of the basic building blocks in microfluidics. Mixing is essential for providing a homogeneous and reproducible environment for chemical or biological components to interact with each other. In the context of microfluidics, mixing is the process through which uniformity of concentration is achieved. Depending on the application, the concentration may refer to that of solutes like ions, small molecules, biomolecules, solvents, or suspended particles such as beads or colloids. As described in chapter 2.3 on page 16 molecules in solution undergo random motions, giving rise to the process of diffusion. Under a concentration gradient, diffusion results in flux \( J \) of molecules that tend to homogenize the concentration \( c \) of that molecular species [38]. Under typical microfluidic conditions with a laminar flow it is difficult to mix liquids or it takes hours for the molecules to diffuse even
the small distances appearing in microfluidics. As a basic element, the possibilities of implementing a mixer in µFLATLab is described. An overview of the mixing principles which have the potential to be integrated into this microfluidic platform are the following:

- **Hydrodynamic flow focusing**: one stream with a small flow rate is to be diluted into another stream with a larger flow rate [96,97].

- **Lamination**: a stream is splitted into multiple streams. The streams are interdigitated and brought back together in order to be mixed. Example: parallel lamination of two or more streams [98], T and Y flow configurations [99], multilamination pattern [100], triangular interdigital mixer [101].

- **Chaotic advection**: fast lamination by exponential thinning of the striation length. Example: splitting and recombine [102,103] herringbone Mixer [104], plug serpentine mixer [105], dean mixer [106], viscoelastic/electrokinetic instability mixer [107].

- **Two phase flow**: introducing circulating flows inside fluid segments of droplets which can be used to convert the inherently two-dimensional flows to three dimensional chaotic flows under suitable conditions. Example: serpentine mixer [98].

- **Pressure/velocity field disturbance mixers**: external pressure or velocity disturbance causes a change in the flow pattern inside the main channel enabling fast mixing [108]. Example: pressure perturbation mixer [109], centrifugal micromixer [110].

- **Rotary mixer**: active elements like pumps drive a fluid flow in a circular microchannel resulting in a linear stretching of the fluid [101].

- **Induced field electroosmosis mixers**: uses electroosmotic flow generated under AC excitation an electrolyte solution due to tangential migration of induced charges using planar electrodes. Example: electrohydrodynamic mixer [111].
• **Other:** dielectrophoretic mixing [112] (migration of polarizable particles under an electric field gradient), electrokinetic mixing [113] (fluid flow driven by migration of charges at microchannel surfaces und an electric field), acoustic actuation [114], thermally generated bubbles [115], magnetohydrodynamics [116] (flow in a current carrying fluid in a magnetic field induced by Lorentz forces).

### 3.4.5. Active Rotary Mixer using a Membrane Micropump

The integrated membrane micropump works as an active rotary mixer by circulating liquid inside a chamber. In the example assay (4.3.4, p. 95) a hybridization probe is circulated inside the chamber where a microarray is located to accelerate the diffusion limited hybridization reaction. To illustrate the mixing of two liquids, the setup in Fig. 32 is used.

---

**Fig. 32:** Schematic top (A) and sectional (B) view a membrane micropump with a chamber arranged on top. Initially the pump is filled with 4 µl dye and the chamber contains 33 µl water. The dye is pumped in a circle from the micropump into the chamber through via I and from the chamber back into the micropump through via II.

A membrane micropump with a displacement chamber with a diameter of 2 mm and a structure depth of 300 µm is used (see micropump design II, 3.4.3, p. 52). An additional passive capacity is put in series for balancing out the transported volume during the displacement and suction stroke. The balancing capacity offers a volume for the displaced liquid after the displacement stroke. Otherwise the micropump would work against a high...
hydraulic resistance inside the closed network. Fig. 33 illustrates the mixing of the dye with water at different times by circulation of the two liquids (4 µl fluorescent dye\textsuperscript{15} and 33 µl H\textsubscript{2}O) at a pump rate of 5.7 µl·s\textsuperscript{-1} at a pump actuation frequency of \(f_p = 8\) Hz. Initially the pump is filled with fluorescent dye. The dye is pumped in a circle from the micropump into the chamber through via I and from the chamber back into the micropump through via II.

![Fig. 33: Picture sequence of two liquid plugs mixed by a membrane micropump. The dye is pumped in a circle through via I located on the right into the chamber arranged on top of the micropump and back through the left via II.](image)

The dyed fluid plug is dispersing in the water over time with a distinct parabolic flow profile (Fig. 33, picture at time 0.662 s). The dye is distributed, indicated by an increasing homogenization of the color. The color is used to qualify the homogenization process. Fig. 34 shows a color phase analysis of the dye-color over time. The characteristic of the surface with that particular color phase leads to a steady state after approximately 8 s indicating, that the two plugs are homogeneously distributed.

\textsuperscript{15} Fluorescein (MW 332).
Fig. 34: Normalized color analysis of the dyed surface for an active mixing of 4 µl fluorescein dye (FITS) and 33 µl water. The measured data is compared to a normalized transient simulated data for diffusion of 4 µl FITS inside a 10×10×0.25 mm³ chamber at room temperature. The diffusion starts at one front end at a rate $D_{FITC} = 0.6 \times 10^{-9} \text{m}^2\text{s}^{-1}$ [117]. 90 % of the asymptotic steady state value is reached for simulated diffusion after 350 s, for active mixing after only 8 s.

### 3.4.6. Phaseguides

During the filling of a chamber that has a large width to height ratio and a comparably small punctual liquid entrance, bubbles are easily trapped in corners. The liquid spreads according to the surface characteristics which vary according to material and roughness. Milled structures have a rough surface compared to injection molded parts for instance. Optical measurements of milled surfaces lead to an average surface roughness $R_a = 1.7 \mu\text{m}$ and an average maximum peak height of $R_p = 15.2 \mu\text{m}$. Those values indicate a much higher roughness compared to injection molded surfaces where $R_a = 0.1 \mu\text{m}$ and $R_p = 1.9 \mu\text{m}$ were measured. Therefore the flow profile varies strongly over a wide chamber with a milled surface. This way a part of one liquid front can pass another part causing a bubble to be trapped. A bubble trapped in the hybridization chamber for example, explained in 4.3 after...
page 88, leads to spots without liquid contact. This lowers or even avoids a proper signal of microarray spots.

An established method for a bubble free filling of a chamber are obstacles that guide the liquid. Those “Phaseguides are stripes of material that control the advancing or receding liquid/air interface. They act as capillary pressure barriers that span over the complete length of an advancing or receding liquid/air meniscus” [118]. The principle is shown in Fig. 35.

![Fig. 35: Schematic sectional view of a phaseguide. The liquid moves from left to right forming a contact angle between the solid and gas phase $\Theta$. The phaseguide is filled (A) until the meniscus front reaches the end of the phaseguide (B). The liquid pins at the 90° edge causing a defined liquid front. The fluid has to be moved further until it overflows the barrier when the capillary pressure $p_{cap}$ is overcome (C).](image)

The filling process of a $10 \times 10 \times 0.4 \text{ mm}^3$ chamber and phaseguides with a height of 200 $\mu$m is shown in picture series in Fig. 36. The obstacles work as a pressure barrier and force the liquid front to align at defined distances. After completely filling the volume behind one phaseguide barrier the pressure increases until it reaches the value to overcome the barrier. This pressure is given by the capillary pressure $p_{cap}$ (Eq. 3-5).
\[ P_{\text{cap}} = \frac{2\gamma_{\text{H}_2\text{O}}}{r} = 720 \text{ Pa} \]

Eq. 3-5

Radius \[ r = 200 \mu m \]

Capillary pressure \[ P_{\text{cap}} \]

Surface tension \[ \gamma \]

Surface tension \( \text{H}_2\text{O}, 25^\circ \text{C} \) [119]

\[ \gamma_{\text{H}_2\text{O}} = 71.97 \frac{mN}{m} \]

Contact angle between \( \text{PC}/\text{H}_2\text{O} \) with surrounding air [120]

\[ \Theta_{\text{PC}/\text{H}_2\text{O}} = 84^\circ \]

Fig. 36: Top view picture sequence of a bubble free filling of a \( 10 \times 10 \times 0.4 \text{ mm}^3 \) chamber. Initial state: chamber is filled with air. The dyed water stops at each pressure barrier (1,3,8,10,11,12) leading to a defined liquid front.
3.4.7. Metering Fluid Volumes

The metering of fluids is realized by transporting a certain amount of fluid volume into the chip either by an automated syringe or by hand. Although metering is not investigated in particular in this work, this function is a basic part of a microfluidic platform and therefore a possible realization is described. µFLATLab offers a solution for metering an exact volume on chip as shown in Fig. 37.

A metering chamber is connected to two in- and outlet channels with two microvalves at each end. The volume of the metering chamber is completely filled through the first branch from inlet to waste. The in- and outlet channel is then closed by microvalves leaving an exact volume of liquid inside the chamber. Through a second branch this volume can now be emptied by pushing a second medium, for instance air, through the second branch. The accuracy only depends on the dead volume of the microvalve and the structuring precision of the chamber. This method can be used, when an exact ratio of several liquids needs to be provided.
3.4.8. Flow Profile Homogenization Pillar

The variation of the flow velocity inside a wide reaction chamber leads to different kinetics for surface based reactions in a constant flow. To level this effect an obstacle can be used to homogenize the flow velocity over a distinct area. A transient simulation\textsuperscript{16} of a flow velocity in a hybridization chamber with dimensions of $0.25 \times 10 \times 10 \text{ mm}^3$ is given in Fig. 38.

\textbf{Fig. 38:} A: Schematic top view of a chamber with dimensions of $0.25 \times 10 \times 10 \text{ mm}^3$ without (I) and with flow profile homogenisation structures: pillar radius of 0.5 (II) and 1 mm (III). B: Stationary 3 D simulation of the velocity profile inside the chamber. The liquid properties of H$_2$O with a dynamic viscosity $\eta = 1 \text{ mPa} \cdot \text{s}$, density $\rho = 1000 \text{ kg} \cdot \text{m}^{-3}$ at room temperature and atmospheric pressure are used. The liquid enters the inlet at a constant flow rate of $Q = 5.7 \mu\text{l} \cdot \text{s}^{-1}$. A no slip boundary condition at the chamber walls is used to solve the Navier-Stokes equation for an incompressible Newtonian fluid. C: Velocity profile inside a chamber at a height of 100 µm with a flattened velocity profile by homogenisation structures.

The liquid properties of H$_2$O with a dynamic viscosity $\eta = 1 \text{ mPa} \cdot \text{s}$, density $\rho = 1000 \text{ kg} \cdot \text{m}^{-3}$, at room temperature and atmospheric pressure are used. The circular homogenization pillar is placed at the center axis directly after the inlet. The liquid enters the

\textsuperscript{16} Comsol Multiphysics, COMSOL Multiphysics GmbH, Germany.
inlet at a constant flow rate of $Q = 5.7 \mu l \cdot s^{-1}$ which is similar to the average flow rate of the micropump (design II at 8 Hz) described in chapter 3.4.3, p. 51. A no slip boundary condition at the chamber walls is used to solve the Navier-Stokes equation for an incompressible Newtonian fluid. The cross section of the simulated flow velocity in the middle of the chamber shows a peak shaped profile for a chamber without a pillar (see Fig. 38 C). This way the reaction condition for surface based reactions would vary, depending on the location inside the chamber. For example microarray spots placed on the center axis would have a faster reaction as spots located next to the center axis. This would result in different signal intensities for equal spots. By placing a circular homogenization pillar with a radius of 500 µm in the center axis, the resulting velocity profile in the chamber flattens. The homogenized velocity profile leads to more homogenized reaction conditions. This homogenization effect is increased by a larger pillar with a radius of 1 mm but the total hydraulic resistance is increased with larger pillars as well.

3.4.9. Liquid Storage on Chip

A liquid storage structure with an integrated actuation method is realized by a liquid reservoir connected to a sealed microvalve (Fig. 39). The membrane is displaced into the liquid reservoir chamber, pushing the liquid towards the outlet. This working principle is similar to the one used for actuating a micropump (chapter 3.4.3, p. 51).

Six storage chambers, each with a total volume of 57 µl, are filled with 50 µl of water and sealed using laserwelding. First the control layer is welded to the TPE membrane. The control layer is then placed on top of the hydraulic layer which contains the prefilled liquid reservoir. To avoid capillary forces transporting liquid between the bulk layers, the storage chambers are not completely filled. During the next laserwelding step the outlet microvalve is sealed. The triangular shape of the predetermined breaking point leads to a stress concentration in the peak of it, causing the weld to break at this point. The sealed outlet microvalve is then broken at the predetermined breakage structure by applying an actuation pressure pushing 85 % of the liquid towards a measurement channel. Every seal could be broken. The actuation pressure needed varied between 100 to 200 kPa. The shape and the laser parameters power, frequency and velocity define the weld quality and therefore the
pressure needed for breaking the seal. Therefore the minimum breaking pressure can be varied in a broad range, but is to be further investigated. The shape of the storage chamber is cylindrical which limits the maximum volume that can be emptied. A more optimal profile would be a more spherical shape to gain less dead volume in the corner of the storage chamber.

Fig. 39: Schematic side and top view of a liquid chamber for storage (A) and actuation (B) on chip. The filled liquid reservoir is connected to a sealed outlet microvalve which is broken when a critical actuation pressure is overcome. The liquid is then pushed to the outlet channel.

This proof of principle shows that µFLATLab provides the pre-storage of a liquid on chip combined with its actuation. Besides the liquid filling, no additional production step is required. Waste is also storable on chip with this approach, thus avoiding contamination of the outside world and the user. For a lab-on-a-chip device this technology reduces hydraulic interconnections leading to a more convenient use of the system.
4. Example Assay

To proof the performance of µFLATLab, an example assay is implemented in a lab-on-a-chip device. This molecular diagnostic assay is for DNA based test of the resistance of *E. coli* against fluoronchinolone-based antibiotics. It is based on extracting DNA from pathogenic bacteria and characterizes a gene for a polymorphism using a microarray. The results of the individual sub functions bacteria accumulation, extracting genomic DNA by bacteria lysis plus an amplification of the *gyrA* gene using *PCR* on chip followed by a readout of a microarray, are investigated separately (Fig. 40).

This particular assay was developed within the framework of the publically founded project called *Path.Ident*. The microarray development and layout was mainly the work of the *Institut für Technische Biochemie*, Universität Stuttgart, the *Robert Bosch Krankenhaus* Stuttgart and the *Eppendorf AG*, Hamburg. The goal is to adapt and integrate assay steps which are normally performed by hand in a lab environment. Each step is explained in the following chapters.

Step 1: Bacteria accumulation with silica filter  
Step 2: Thermal lysis, amplification and fluorescence labeling (*PCR*)  
Step 3: Signal readout

![Diagram of assay steps](image)

*Fig. 40: Schematic overview of the assay steps for the application example of the microfluidic platform (µFLATLab): bacteria accumulation, extracting genomic DNA by thermal bacteria lysis, plus an amplification of the *gyrA* gene using *PCR* on chip followed by readout with a microarray.*
For this example assay a fully integrated lab-on-a-chip device is designed to automate all functions. Such a lab-on-a-chip device is shown in Fig. 41. The sample preparation steps can be performed on chip, including filtration, thermal cycling for PCR and microarray hybridization. The concept includes the sample and reagents input to the lab-on-a-chip device by hand or a syringe pump. The fluid paths on chip are controlled by eight microvalves, which are controlled by the processing instrument as described in chapter 3.3 on page 37. The microarray is located on a surface modified glass substrate\(^\text{17}\). This glass slide is bound removable to the lab-on-a-chip device with an adhesive frame\(^\text{18}\). After hybridization the glass slide with the microarray is removed from the lab-on-a-chip device. The readout of the microarray is then performed with a stand-alone microarray laser scanner\(^\text{19}\).

\textbf{Accumulation, lysis & PCR}

\textbf{Microvalves}

\textbf{Micropump}

\textbf{Control layer}

\textbf{TPE membrane}

\textbf{Hydraulic layer}

\textbf{Hybridization chamber}

\textbf{Hydraulic / pneumatic interface}

\textbf{Insulation against heat loss}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{lab-on-a-chip-device}
\caption{Design of the lab-on-a-chip device used to perform a three step assay for the analysis of \textit{E. coli} resistance against fluoronchinolone-based antibiotics. Left: schematic drawing. Right: picture of an assembled chip.}
\end{figure}

\begin{footnotes}
\textsuperscript{17} \textit{Nexterion Slide}, Schott AG Schott Jenaer Glas GmbH, Jena, Germany.
\textsuperscript{18} \textit{Geneframe}, ThermoScientific.
\textsuperscript{19} \textit{ScanArray Express}, Perkinelmer Life & Analytical Sciences Shelton, CT, USA.
\end{footnotes}
4.1. Step 1: Accumulation using a Solid Phase Silica Filter

Prior to molecular-based diagnostics, pathogens must frequently be enriched from the host sample. Filtering bacteria mechanically using a solid phase silica filter is a well established and simple method [121] and is used in this work (Fig. 42). The main advantage of an integrated filter is the possibility to handle volumes in the ml range and is considered to address a broad range of applicable samples like urine, blood or saliva.

![Silica filter In- outlet](image)

Fig. 42: Photograph of functional unit including six filter chambers (diameter = 7.2 mm, depth = 0.8 mm, volume = 34 µl). The chip is used for filtering 10 ml *E. coli K12* suspension and determination of the filter efficiency. After filtration a PCR is performed on this chip with the filtered bacteria.

There are several other methods which can be used for accumulation of *E. coli* of which two are described as an example for an alternative approach:

**Dielectrophoresis (DEP)** is based on the principle that bacteria are held back in an alternating electrical field while passing the electrodes in a continuous flow gaining a high efficiency around 90% [122]. An advantage of this method is the ability of sorting dead from living cells [123,124]. The low forces and the need for narrow hydraulic channels to gain sufficient electric fields allow only slow flows in the range of some µl·s⁻¹. For a milliliter-sized sample this leads to an accumulation time of several 10 minutes to hours. The efficiency strongly depends on the pH of the sample and therefore is not suitable for real world urine samples where this value varies between pH 5-8 [125,126]. The realization in a lab-on-a-chip device demands a cost intensive metallization step, because the electrode has to be inside the hydraulic channel to gain sufficient DEP forces.

**Antibody coated beads** specifically bind bacteria or viruses [127] surrounding them in a sample. Large sample volumes can be handled by a corresponding large number of used
beads. Accumulation is realized by extracting those beads using magnetic forces or filters. The higher costs compared to a filter based method is the major disadvantage.

**Accumulation Efficiency of a Solid Phase Silica Filter**

For evaluation of the filter efficiency 10 ml bacteria suspension of *E. coli K12* in 1 x phosphate buffered Saline (PBS) are filtered at a rate of 0.5 ml/min using a six-chamber chip (Fig. 42, chamber diameter 7.2 mm, depth 0.8 mm, volume 34 µl). A solid phase silica filter with a diameter of 7.6 mm and a height of 0.9 mm is used to hold back bacteria mechanically. Bacteria concentrations are $10^3$, $10^4$, $10^5$, $10^6$ and $10^7$ bacteria per 10 ml with $10^5$ bacteria per 10 ml being the threshold for an urinary tract infection [128,129]. The filter efficiency is investigated by comparing cell colony growth counting of the bacteria sample prior and after filtration. The results are shown in Tab. 8. 100 µl of bacteria suspension is plated on an agar medium and incubated overnight at 37 °C, followed by the counting of evolving colony forming units (CFU see Fig. 43). The use of artificial urine was ruled out by the fact, that commercially available products contain antibiotics for sterility reasons and therefore would inhibit bacteria colony growth as a measurement method.

![Petri dish Colony forming unit](image)

**Fig. 43:** Different states of *E. coli* colony forming units (CFU) on agar plate. The remaining bacteria after filtration are colonized. The number of CFU is used to determine the filtering efficiency of the silica filter.
Tab. 8: Silica filtering efficiency for different initial concentration of \textit{E. coli K12}. The filtered sample volume is 10 ml. The remaining bacteria after filtration are colonized. The number of \textit{CFU} is used to determine the filtering efficiency of the silica filter.

<table>
<thead>
<tr>
<th>Initial bacteria concentration per ml (desired value)</th>
<th>(1 \times 10^3)</th>
<th>(1 \times 10^4)</th>
<th>(1 \times 10^5)</th>
<th>(1 \times 10^6)</th>
<th>(1 \times 10^7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of \textit{CFU} without filtration</td>
<td>(4.25 \times 10^3)</td>
<td>(0.59 \times 10^4)</td>
<td>(0.58 \times 10^5)</td>
<td>(5.69 \times 10^6)</td>
<td>(0.45 \times 10^7)</td>
</tr>
<tr>
<td>Number of \textit{CFU} after filtration</td>
<td>(0.03 \times 10^3)</td>
<td>(0.07 \times 10^4)</td>
<td>(0.04 \times 10^5)</td>
<td>(0.03 \times 10^6)</td>
<td>(0.01 \times 10^7)</td>
</tr>
<tr>
<td>Average filter efficiency [%]</td>
<td>(99^{\pm1})</td>
<td>(85^{\pm22}_{27})</td>
<td>(92^{\pm8}_{17})</td>
<td>(94^{\pm5})</td>
<td>(98^{\pm1})</td>
</tr>
<tr>
<td>Number of experiments</td>
<td>4</td>
<td>8</td>
<td>8</td>
<td>6</td>
<td>10</td>
</tr>
</tbody>
</table>

Although \textbf{cell colony counting} is a measurement method with limited accuracy\textsuperscript{22}, the reproducible high efficiencies shown in Tab. 8 proof the efficacy of the accumulation step. A sufficient slow flow of \(Q < 0.5\) ml·min\(^{-1}\) as well as a bypass free integration of the silica matrix is crucial for a working filtration. The filter chamber production tolerance must not deviate from the designed dimensions more than 50 µm. A chamber with a height 100 µm smaller than the height of the compressible silica filter is used to assure that the silica filter is pressed properly against the chamber walls. This way bypasses are minimized. Another improvement is given when using support structures clamping the silica filter locally. For example a support with a height of 150 µm clamps the silica filter only locally without compressing the whole filter. This way bypasses are avoided and the increased hydraulic resistance caused by the compression of the whole filter is reduced. Bypasses can be optically detected by adding 10 µl of dye before preparing the bacteria suspension. The silica filter holds back bacteria and cells caused by the high density of the crossed fibers. This way the hydraulic resistance during the filtering is constantly low compared to e.g. nylon membranes that are faster clogged by the particles they filter. In order to function properly the filter has to be large enough. This criterion is sufficiently fulfilled with the used silica filter which has a diameter of 7.2 mm and a height of 0.8 mm. This design criterion was not optimized in particular, but a filter with a smaller diameter of 4 mm and a height of 0.8 mm was found to be too small for proper filter efficiency. Another advantage is that gas bubbles do not clog this silica matrix. A disadvantage though is the sponge characteristic retaining the liquid in the chamber. This can lead to a significant loss of reagent volume, when the chamber content needs to be transported to another location in a following step. An additional advantage is that

\textsuperscript{20} Genomed GmbH, Löhne, Germany.
\textsuperscript{21} E.g.: Bio-Rad Laboratories, Inc., Liquichek Urine Chemistry Control includes Gentamicin.
the filter holds back bigger cells like mammalian cells, which thereby cannot interfere with
the later assay steps. Compared to other continuously working methods, like dielectrophoresis
\( (DEP) \), this method is faster and cheaper to implement. Additionally it is more independent
from influences like the pH of the sample or the surrounding temperature. Accumulation
methods based on antibody coated beads, represent a smart and flexible accumulation method.
Bacteria species can be accumulated specifically according to their surface characteristics.
One disadvantage is the comparably high price for the beads. Also a way of accumulating
those small particles (e.g. by a filter, electric or magnetic forces) would be necessary to be
implemented in the lab-on-a-chip device.

\[ \text{4.2. Step 2: Lysis and PCR on Chip} \]

In order to extract genomic DNA withholding the resistance information from bacteria,
the cell membrane is destroyed by thermal disruption of the \( E. coli \) membrane at 95 °C for
4 min [130]. The method is efficient, reliable and needs no additional lysing fluids or
electrode structures. Therefore no additional hydraulic connections or expensive production
processes, like structured metallization, are needed to realize this method in a lab-on-a-chip
device. An overview of the cell lysis methods is given as follows derived from [131]:

- **Mechanical lysis:** mechanical disruption, rotating blades [132], manual grinding
  [133], repeatedly freezing [134], sonoporation [135,136]. It is an efficient method
  which can destroy particularly robust spores or gram-positive bacteria\(^{23}\). The method
  is rather complex to integrate in a lab-on-a-chip device.

- **Electrical lysis:** rapid disruption using electroporation of cell membranes in a high
electrical field in the range of \( \text{kV} \cdot \text{cm}^{-1} \) [137,138,139,140]. Easy but cost intensive
  integration in a lab-on-a-chip device in form of electrodes inside channels.

\(^{22}\) One colony forming unit is not necessarily evolved from one initial bacterium.

\(^{23}\) Gram-positive bacteria are stained dark blue or violet by Gram staining indicating a more stable cell
structure.
- **Thermal lysis**: disruption of the cell wall with a laser or a conventional heater with high efficiency [141,142,143]. The disadvantage is the evaporation of gas appearing at temperatures above or near the dissolving point. It is easy and cost-efficient to implement in a microfluidic platform with a high lysis efficacy for gram-negative bacteria like *E. coli*.

- **Chemical or biological lysis** destroys the cell membranes by reacting with detergents or biological enzymes which causes a release of the intracellular materials. The reactions can take place under comparably mild conditions such as room temperature [144]. For the integration in a lab-on-a-chip device additional liquid interface, channels and chambers are needed as well as additional assay steps.

### 4.2.1. Amplification of DNA Template using a *PCR*

The filtration of bacteria from sample is used to gain genomic DNA, which contains the resistance information of the bacterium. A minimum number of $10^{11}$ genomic DNA molecules, and therefore *E. coli* bacteria, are needed to reach the detection threshold of the microarray. This number was investigated during the publically funded project *Path.Ident* by project partners. In order to fulfill the detection limit of the diagnostically relevant concentration of $10^4$ bacteria per ml, a calculated total sample volume of 10000 l would be necessary. Since this amount is out of reach, the assay includes the increase of detectable probe molecules with an amplification step using a *PCR*. The *PCR* used in this example assay copies the part of the genomic DNA, which contains the resistance information of the *E. coli* against fluoronchinolone-based antibiotics. This information is coded in the *gyrA* gene [145]. The *PCR* increases a 200 base pair (Bp) long DNA template of this gene exponentially. A dye (Cy3) is built in every *PCR* product. It is used to produce a fluorescence signal later on the microarray (see 4.3 p. 88), where the template molecules are specifically bound to the probes immobilized on the surface.

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24 *Institut für Technische Biochemie, Universität Stuttgart, Allmandring 31, 70569 Stuttgart, Germany.*

*Eppendorf AG, Barkhausenweg 1, 22339 Hamburg, Germany.*

*Robert-Bosch-Krankenhaus, Auerbachstraße 110, 70376 Stuttgart, Germany.*
Polymerase Chain Reaction and Quantitative Realtime PCR

This chapter gives a brief description of the two PCR processes appearing in this work. Note that first described PCR is used as a part of the example assay, while the RTQ-PCR is used as a measurement method for an assay step.

PCR is a molecular biological technique for addressing and exponentially amplifying a DNA fragment and has been demonstrated in miniaturized lab-on-a-chip systems frequently [146,147]. It is mostly based on three temperature cycles with an enzymatic amplification step (Fig. 45) [148]. A PCR process with the following parameters has been implemented:

- **Denaturation** at 94 °C for 10 s: disruption of the hydrogen bonds of the DNA template between complementary bases, yielding single strands of DNA (ssDNA).

- **Annealing** of primers at 55 °C for 20 s to the single stranded DNA template. The polymerase binds to the primer-template hybrid and begins DNA synthesis. The primer is labeled with a fluorescent dye (Cy3).

![Diagram of E. coli bacteria with genomic DNA](image)

Fig. 44: Schematic of *E. coli* bacteria with genomic DNA. After thermal lysis genomic DNA with sequence containing resistance information is released (*gyrA* gene).
• **Elongation** at 72 °C for 20 s: the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs\(^\text{25}\) that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the nascent (extending) DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified.

Under optimized conditions, a single template DNA molecule is sufficient for starting the process. DNA fragments up to about 10,000 base pairs can be amplified by PCR. It is a well known and established standard tool in biology with a broad range of applications like other bacteria, viruses or fungi and their multiplexing to more than one DNA template [149].

A **quantitative real time PCR (RTQ-PCR)** monitors the signal increase during its PCR cycle in real time by using fluorescence techniques [150,151,152]. The major advantages are the possibility to **quantify** with a high reliability over a wide dynamic range from single DNA

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\(^{25}\) dNTP: Desoxynucleotide or nucleoside triphosphates (dATP, dGTP, dCTP, dTTP) are the structural units of
templates to more than eight orders of magnitude [153]. Although a comparison of a measured DNA sample to a standard dilution will only give a fraction or ratio of the sample relative to the standard, allowing only relative comparisons between different tissues or experimental conditions. The results from these experiments can only be interpreted as a relative correlation because of the lack of a probe, working as reference probe with a known number of initial genetic materials and the knowledge of the efficiency of the RTQ-PCR for the combination of DNA and primers.

4.2.2. Quantification of the Lysis and PCR on Chip

The characteristics of the lysis and the PCR are investigated together. The steps are performed on chip as well as off chip with the standard protocol as a reference.

The PCR on chip is investigated with a functional unit, including six chambers (diameter 7.2 mm, depth 0.8 mm, volume 34 µl). The PCR is performed on a thermal slide cycler26. Each chamber contains an integrated silica membrane filter as described in chapter 4.10 on page 76. Since the filter efficiency is performed by looking at the waste, the bacteria held back in the filter can be further processed. The filter chamber therefore is used for accumulation, lysis and PCR altogether. The chambers are used for filtering (see chapter 4.1, p. 76) 10^3, 10^4, 10^5, 10^6, 10^7 E. coli out of a 10 ml probe equal to 5.4 pg, 54 pg, 540 pg, 5.4 ng and 54 ng of genomic DNA. To completely fill the chamber including the in- and outlet, 40 µl of PCR master mix (Tab. 9) is used. One chamber contains PCR master mix without additional DNA as a negative control. One chamber contains a positive control with 54 ng of purified DNA, which is prepared off chip.

The thermal lysis step is performed at 95°C for 5 min. Afterwards 31 PCR cycles are performed on a thermal cycler26 followed by a terminal elongation step at 72°C for 10 min (see 4.2.1 p. 80). The filters are then released by destroying the chip and the PCR product is diluted with 20 µl of double distilled water (ddH₂O) by centrifugation. The gained product (12-15 µl) is then qualified using a gel electrophoresis and quantified by a quantitative real Time PCR (RTQ-PCR, for description see p. 81). The results are depicted in Tab. 10, p. 85.

RNA and DNA.

26 FlexCycler, Biozym Scientific GmbH Steinbrinksweg 27, 31840 Hessisch Oldendorf, Germany.
The same lysis and PCR protocol (see 4.2.1 p. 80) is performed in parallel off chip. This way the performance of the assay steps, processed on chip, can be compared to standard processing off chip. To gain comparable boundary conditions 40 µl of each sample is processed. The number of bacteria is varied between $10^3$, $10^4$, $10^5$, $10^6$, $10^7$ E. coli bacteria. This describes the same concentration as if the accumulation step on chip would filtrate 100% of the bacteria out of the 10 ml sample.

Additionally the processing off chip can give helpful informations when it comes to failure analysis after an unexpected result of an experiment. For example if the polymerase of the PCR master mix is inactive because of inappropriate storage conditions the PCR does not work. This failure will appear on chip as well as in the standard protocol off chip. The fact that the standard protocol does not work either indicates a global reason independent from chip related properties such as chip material.

Tab. 9: PCR master mix composition for amplification of genomic E. coli DNA on an integrated silica filter.

<table>
<thead>
<tr>
<th>Description</th>
<th>Concentration</th>
<th>Volume</th>
<th>Supplier and remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forward primer</td>
<td>0.2 µM</td>
<td>1.6 µl</td>
<td>Metabion AG&lt;sup&gt;27&lt;/sup&gt;; gyrA cy3 labeled</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>0.2 µM</td>
<td>1.6 µl</td>
<td>5'-CGCCATGAACGTACTAGGCA-3'</td>
</tr>
<tr>
<td>dNTPs</td>
<td>0.1 mM</td>
<td>0.8 µl</td>
<td>Metabion AG&lt;sup&gt;27&lt;/sup&gt;; gyrA cy3 labeled</td>
</tr>
<tr>
<td>Taq Polymerase</td>
<td>5 U/µl</td>
<td>0.8 µl</td>
<td>5’ CAGAGTCGCCGTCGATAGAA 3’</td>
</tr>
<tr>
<td>Taq Buffer</td>
<td>1x</td>
<td>4 µl</td>
<td>Metabion AG&lt;sup&gt;27&lt;/sup&gt;; gyrA cy3 labeled</td>
</tr>
<tr>
<td>MgCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>1.5 mg/ml</td>
<td>10 µl</td>
<td>Fermentas GmbH&lt;sup&gt;28&lt;/sup&gt;</td>
</tr>
<tr>
<td>HPLC-H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>17.1 µl</td>
<td></td>
<td>Sigma Aldrich&lt;sup&gt;29&lt;/sup&gt;</td>
</tr>
<tr>
<td>BSA</td>
<td>5 mg/ml</td>
<td>10 µl</td>
<td>Sigma Aldrich&lt;sup&gt;29&lt;/sup&gt;</td>
</tr>
<tr>
<td>Genomic DNA</td>
<td>54 pg, 540 pg, 5.4 ng, 54 ng</td>
<td>For use in standard protocol; purification using a DNA tissue Kit S with a QuickGene Mini80, Fuji Film&lt;sup&gt;30&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

For the quantification of the lysis and PCR product a quantitative real time PCR (RTQ-PCR) is used as described on page 82. Prior the RTQ-PCR quantification, the PCR product gained on chip, is dilutied 1:10<sup>6</sup> in ddH<sub>2</sub>O. As an off chip reference the product

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<sup>27</sup> metabion international AG, lena-christ-str. 44/1 82152 Martinsried, Germany, www.metabion.com.
<sup>28</sup> Fermentas GmbH, Opelstraße 9, 68789 St. Leon-Rot, Germany, www.fermentas.de.
gained from the standard protocol PCR is performed and diluted 1:10^6 in ddH₂O as well. Three redundant probes (V = 2 µl) are processed. From the resulting real-time curves the cycle threshold $C_t$ is measured. This value defines the cycle number after which the fluorescent signal significantly outreaches the background fluorescent signal. This threshold indicates the point where an efficient exponential growth of the DNA product is initiated. The lower the $C_t$ value is, the higher the initial DNA concentration was in the sample.

Tab. 10: Quantification of the product after Filtration, Lysis and PCR step and the efficiency of these three steps in series. The PCR is performed on a thermal slide cycler (FlexCycler, Biozym). A real time quantitative PCR was performed with 2 µl of a diluted (*1:10^6) product.

<table>
<thead>
<tr>
<th>Initial bacteria concentration per 10 ml (desired value)</th>
<th>$1 \times 10^3$</th>
<th>$1 \times 10^4$</th>
<th>$1 \times 10^5$</th>
<th>$1 \times 10^6$</th>
<th>$1 \times 10^7$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycle threshold $C_t$ (Processing with standard protocol*)</td>
<td>21.9^{2.1}</td>
<td>19.2^{2.4}</td>
<td>16.8^{2.7}</td>
<td>17.3^{2.4}</td>
<td>16.1^{2.4}</td>
</tr>
<tr>
<td>Number of experiments n</td>
<td>4</td>
<td>8</td>
<td>8</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>Cycle threshold $C_t$ (Processing on chip*)</td>
<td>24.2^{2.3}</td>
<td>22.0^{0.9}</td>
<td>21.8^{2.6}</td>
<td>19.6^{2.1}</td>
<td>18.5^{2.5}</td>
</tr>
<tr>
<td>Number of experiments n</td>
<td>4</td>
<td>8</td>
<td>8</td>
<td>6</td>
<td>10</td>
</tr>
</tbody>
</table>

The PCR on chip works for every initial bacteria concentration as well as the reference in the standard protocol, although the efficiency for the standard protocol is higher. Assuming an ideal duplication of PCR product for each cycle, the decrease of $C_t$ values between initial bacteria concentration is theoretically 3.3^{10} for each order of magnitude. The lower change in $C_t$ values for initial bacteria concentration > $10^5$ could be due to the saturation effects. But there is a consistent reciprocal correlation for both experiments between the numbers of bacteria used prior the PCR and the $C_t$ values resulting from the RTQ-PCR. The assay steps lysis and PCR are not investigated separately. Together they get robust and reproducible results.

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31 Biorad IQ SybrGreen Supermix on a Biorad IQ5 Cycle, Bio-Rad Laboratories GmbH, 80901 München, Germany.
32 ld(10)=3.3219...
4.2.3. Material influence on the PCR in the Lab-on-a-Chip Device

The large surface of the silica filter as well as a rough channel surface binds DNA template and therefore inhibits a PCR. Surface saturation with proteins from Bovine serum albumin (BSA) or Q-Solution [33,154,155,156] added to the master mix avoids inhibition of the PCR. BSA concentrations of 0, 5, 15, 25 mg·ml⁻¹ are tested. The PCR works with a BSA concentration that is higher than 5 mg·ml⁻¹. Two different Taq-Polymerases [35] are tested. Both provided a properly working PCR, whereas the Fermentas Taq was preferred because of the lower price. A list of reagents used for the PCR master mix is shown in Tab. 9 on page 84.

4.2.4. Influence of the Temperature on the PCR in the Lab-on-a-Chip Device

The temperature level of each PCR step (see 4.2.1, p. 80) is crucial for its functionality. The denaturation temperature needs to be high enough to melt the DNA without the risk of evaporation of the liquid. This leads to a temperature bandwidth between 100 °C > T1 > 90 °C. In a planar lab-on-a-chip device, evaporation can lead to displacing liquid from the heated area. This lowers the cycled volume and can even inhibit the PCR. With a closed in- and outlet microvalve during cycling evaporation can be minimized. The higher the annealing temperature the more specific the primers bind. A chamber temperature is kept at 55 °C for 20 s. For lower temperatures, primers bind more unspecific to the DNA template with a consequence of unspecific PCR products. At higher temperatures, they do not bind at all. The temperature level for proper functionality depends on the primer and is here in the range of ± 1 K. The polymerase during elongation has a temperature set level of 72 °C. This step is more robust to temperature variation and works even when the temperature level varies in the range of ± 1.5 K. In order to guarantee an accurate temperature with a discrepancy of only ± 1 K, a closed feedback control loop is implemented as described in chapter 3.3.2 on page 40. As a rule-of-thumb, at its optimum temperature, the DNA polymerase will polymerize a thousand bases per minute [149] so the times for the temperature levels used is much longer than the actual time for the reaction of a 200 bp long

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[33] Protein that has numerous biochemical applications and does not affect other enzymes.
[34] Q-Solution changes the melting behavior of DNA and will often improve a suboptimal PCR caused by templates that have a high degree of secondary structure, Qiagen AG, Germany.
[35] Phusion High-Fidelity DNA Polymerase and Taq DNA Polymerase, Fermentas GmbH, Germany.
ssDNA. The durations are chosen to provide a minimum temperature gradient deviation in the chamber to improve the efficiency of the PCR.

The PCR experiment showed that the reactants are degrading over time during thermal cycling. The polymerase for instance had lost its activity during a PCR experiment that took 4.5 h. As a consequence the maximum total time for the PCR is limited. Experiments performed in a thermal cycler\(^\text{36}\) indicated a maximum time of 3 h. This fact needs to be taken into account when designing a lab-on-a-chip device. The temperature in a comparably bulky lab-on-a-chip device with a total thickness of 3 mm reaches its final level very slowly when a big thermal mass is thermally cycled. The time for heating 72 °C to 93 °C inside a PCR chamber on chip is in the range of 2 min, when the whole lab-on-a-chip device is heated. For the standard protocol in a tube cycler\(^\text{37}\) this only takes about 30 s. To accelerate the temperature change inside the lab-on-a-chip device (Fig. 46) it includes a thermal insulation of the PCR chamber.

It reduces the thermal mass and accelerates heating and cooling durations. This strategy leads to total duration for 31 PCR cycles of 118 min. A microvalve is closed at the in- and

\(^{36}\text{FlexCycler, Biozym Scientific GmbH Steinbrinksweg 27, 31840 Hessisch Oldendorf, Germany.}\)
outlet with a control pressure $P_c = 50$ kPa during the PCR. This provides a tight barrier against evaporation or displacement of the liquid. Thermal contact is provided by clamping the lab-on-a-chip device in between two resistive heaters. The aluminum heat contact plates are the size of the PCR chamber and include thermocouples for a local temperature measurement.

### 4.3. Step 3: Hybridization and Microarray Signal Readout

The final step of the assay is the readout of a fluorescent signal from a microarray. Microarrays are miniaturized two-dimensional arrays containing hundreds up to thousands of capture agents, such as nucleic acids (e. g. oligonucleotides, genes, gene fragments) or proteins (e. g. antibodies). Those are immobilized on a solid substrate in small chemical reaction areas (spots). Typical substrates are glass slides or silicon wafers. The complex biological interactions are identified by the specific location of the capture molecules on the microarray. The arranged multitude of biological agents acts as capture probes to bind molecules out of a sample. Microarray based analytics is expected to have a broad range of applications such as Desoxyribonucleic acid (DNA), protein and antibody detection from blood, cell or tissue extract [157,158]. Microarrays can be regarded as an analytical method that combines small size with the capability to detect several hundreds of different biomolecules simultaneously.

The working principle of the used microarray is shown in Fig. 48. The microarray contains spots with the investigatory capture probes. The point mutation (single nucleotide polymorphism SNP) at position 248, 259 and 260 of the gyrA gene (Fig. 47) are commonly significant for the fluorochinolone resistance of the *E. coli* [159].

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37 Eppendorf Master Cycler Gradient, Eppendorf AG, Hamburg, Germany.
Fig. 47: Schematic of a single stranded DNA template from a *gyrA* gene with investigatory point mutations at position 248, 259, and 260 significant for fluorochinolone resistance of the *E. coli*. [159].

Each spot contains one sort of capture probes. The different spots vary the point mutation of a single nucleotide (A, C, T, G). The Cy3 labeled DNA template specifically binds to the capture probes, depending on which mutation is present at which position (248, 259, or 260). The intensity of the fluorescent signal correlates to the number of bound DNA template. The position of the signal gives the information of the point mutation state. Together this results in the resistivity information of the sample bacteria.

Fig. 48: Schematic working principle of a hybridization reaction between surface immobilized probes and fluorescence labelled ssDNA. A difference in a single base pair (SNP) is detected.
4.3.1. Microarray Layout

The microarray comprises of 10 x 9 spots (Fig. 49), where each spot has a diameter of $w_s = 120 \mu m$ and a spot to spot distance of $d_{ss} = 320 \mu m$. Row 1 and 10 comprises 9 spots with probes for hybridization, process and spotting control. Spot 10/b is empty for orientation. The positive hybridization control should always bring a signal when hybridization conditions are good thus the negative control should never enhance.

Three redundant spots of every investigatory probe are present. Row 2-5, column a-f, include the target capture probes with point mutations of A, C, T and G at position 259 of the gyrA gene. Row 6-9, column a-f, includes the target capture probes for point mutations at position 260. Row 2-5, column g, h, i are spots with capture probes for point mutations at position 248. Column a-c and d-f investigate the influence of a silent mutation in the gyrA gene which is not relevant for the results presented here. Therefore only SM:C results are discussed.
An *E. coli* K12 type is used as a sample. This antibiotic sensitive species (not resistant) leads to a microarray signal as depicted in Fig. 49. The perfect matches appear in row 3 / g-h, 4 and 6 / a-f. The perfect matches result in a peak fluorescent signal, while the mismatches in row 2, 3, 5 and 7-9 should bring low signals compared to the specific reaction of those coming from perfect matches.

4.3.2. Results of the Microarray Hybridization

The product coming from bacteria accumulation, lysis and *PCR* on chip are used as a sample for the microarray. These steps are described in chapter 4.2 starting on page 79. To characterize the performance for the different initial bacteria concentrations $10^3$, $10^4$, $10^5$, $10^6$ and $10^7$ bacteria per 10 ml, the fluorescence intensity of the microarray spots is used. The same lysis and *PCR* protocol (see 4.2.1 p. 80) is performed in parallel off chip. Those products are used as samples for the microarray as well. This way the results from on chip processing can be compared to those coming from standard processing off chip.

The microarray is spotted on a surface modified glass substrate\textsuperscript{39} using an automated contact spotting robot\textsuperscript{40}. A 65 µl hybridization chamber is generated with an adhesive frame\textsuperscript{41} and a cap. After hybridization of a 65 µl sample for 1 h at 55 °C, the microarray is immediately washed in three steps each 10 min and dried with N\textsubscript{2} afterwards. The hybridization buffer used is a 6 x SSPE buffer and a 5 x Denhardts solution with a DNA concentration of $7.58 \times 10^{-4}$ µM. After hybridization the microarray is immediately washed in three steps each 10 min and dried with N\textsubscript{2} afterwards. Data are obtained by acquisition of fluorescence signals from a microarray laser scanner\textsuperscript{42} with a constant amplification factor from a *photo multiplier tube (PMT)*\textsuperscript{43} of 60 for all experiments.

\textsuperscript{38} Layout and capture probes: Institut für Technische Biochemie, Universität Stuttgart, Allmandring 31, 70569 Stuttgart, Germany and Robert-Bosch-Krankenhaus, Auerbachstraße 110, 70376 Stuttgart, Germany.

\textsuperscript{39} Nexterion Slide, Schott AG Schott Jenaer Glas GmbH, Jena, Germany.

\textsuperscript{40} Microgrid II, Biorobotics Ltd, Cambridge, UK.

\textsuperscript{41} Geneframe, ThermoScientific.

\textsuperscript{42} ScanArray Express, Perkinelmer Life & Analytical Sciences Shelton, CT, USA.

\textsuperscript{43} Extremely sensitive detectors of light in the ultraviolet, visible and near-infrared ranges of the electromagnetic spectrum. These detectors multiply the current produced by incident photons according to the acceleration voltage used.
The **signal intensity** of the perfect matches (gyrA 259 G) increases with higher initial bacteria concentration. The values for on chip processing and samples processed according to standard protocol off chip give similar results. The low value for a standard protocol with an initial bacteria concentration $10^7/10$ ml result from a bubble trapped over the microarray, which avoided proper hybridization conditions. Signal saturation is reached at initial bacteria concentrations higher than $10^6/10$ ml. The sample preparation therefore brings feasible products for the microarray detection. An exact conclusion from the fluorescent signal backwards to the number of bacteria in a sample is hardly possible. Because of the variation of bacteria filter efficiency in chain with the variation of the PCR efficiency the exact number cannot reproducibly be calculated. But the assay leads to a conclusion whether a
diagnostically relevant threshold of $\sim 10^5 \text{Bac/10 ml}$ is reached. Therefore it provides the information whether a patient is healthy or not.

**Qualification by Discriminating between a Perfect Match and a Mismatch**

Each microarray spot contains one sort of capture probes. The different spots vary the point mutation of a single nucleotide (A, C, T, G). Only spots for one point mutation (perfect match) should highlight for one sample. As a negative control (mismatch) the other three spots should bring comparably low signals. Comparing the signals of these spots (see Fig. 49) to the specific perfect matching spot indicates the level of sensitivity of the array. This **discrimination ratio** is therefore used as a qualification indicator for the microarray experiment. Tab. 11 lists the discrimination ratio by comparing the signals of the perfect match and the highest mismatch reaction for all four point mutations C, T, G and T for the different initial bacteria concentrations.

<table>
<thead>
<tr>
<th>Bac / 10 ml</th>
<th>$1 \times 10^4$</th>
<th>$1 \times 10^5$</th>
<th>$1 \times 10^6$</th>
<th>$1 \times 10^7$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>gyrA 259 G SM:C</strong></td>
<td>15</td>
<td>4</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td><strong>gyrA 259 G SM:T</strong></td>
<td>16</td>
<td>4</td>
<td>14</td>
<td>12</td>
</tr>
<tr>
<td><strong>gyrA 260 A SM:C</strong></td>
<td>5</td>
<td>4</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td><strong>gyrA 260 A SM:T</strong></td>
<td>9</td>
<td>1</td>
<td>9</td>
<td>7</td>
</tr>
</tbody>
</table>

The multitude for probes with a perfect match is up to 16 times higher than signals from the highest mismatch signal. This ratio is decreasing for higher initial bacteria concentrations of $10^6$ and $10^7 \text{bac/10 ml}$. The absolute signals for mismatches and perfect matches increase with higher bacteria concentration. The saturation of the fluorescent sensor is reached at $10^6 \text{bac/10 ml}$ for the perfect match which then remains constant. The signal for mismatches is far lower but increases with higher bacteria concentrations. Therefore the discrimination ratio decreases at higher initial bacteria concentrations. The ratio for the threshold range
around $10^5$ bac/10 ml has a discrimination ratio of at least five. Thus it is suitable to discriminate resistant from non resistant species.

4.3.3. Influences on the Hybridization Reaction

A higher hybridization **temperature** leads to a more specific reaction similar to the polymerase reaction described in chapter 4.2.1 on page 80. Since only one modified base pair in the *gyrA* gene indicates the resistance, the temperature level (55 °C) is crucial for a differentiation between a perfect match and a mismatch. The washing procedure after the hybridization reaction is crucial to produce feasible results without smear or salt residuum. Bubbles over a spot need to be avoided. A bubble trapped over a spotted area prohibits probes to diffuse to the reactive spots.

The time-to-result of this heterogeneous assay is limited by the time a target molecule needs to diffuse from the bulk liquid to the probe immobilized at the bottom of a hybridization chamber. For large molecules, like the 200 bp long DNA fragments used here, it takes several minutes to hours to bring feasible results [160]. As described in several modeling works [161,162] this time can be dramatically reduced by superimposing this diffusion limited process with a convective flow (chapter 4.3.4 on page 95). Several stand-alone instruments are commercially available for that purpose. They generate the convective fluid movement by surface acoustic waves [163], with electrochemically generated gas bubbles [164] or cyclic mechanical displacement of the sample [165]. In addition, fully integrated microfluidic chips have been proofed to enhance the quality and time-to-result of microarray based analytics. They employ convective flow by integrated peristaltic micropumps or inertial forces during centrifugation [166,167,168,169,170]. This principle is additionally used in the final step of this application example by integrating a membrane micropump as presented in chapter 3.4.3 on page 61 to create a convective flow in the hybridization chamber.
4.3.4. Acceleration of the Microarray Hybridization by an Overlying Flow

To investigate the influence of an overlying flow to the reaction kinetics of the hybridization reaction (chapter 4.3, page 88) two experiments are compared:

- **Static**: the sample is processed without fluid movement, the reaction is purely diffusion driven.

- **Dynamic**: the sample is pumped in a circle with a flow of $Q_p = 5.7 \, \mu\text{l}\cdot\text{s}^{-1}$. A micropump is arranged on top of a hybridization chamber (Fig. 51) and is used to circulate the sample liquid within the hybridization chamber with dimensions of $0.25 \times 10 \times 10 \, \text{mm}^3$. This leads to an average flow velocity inside the chamber of $v = 2.3 \, \text{mm} \cdot \text{s}^{-1}$.

![Fig. 51: Schematic of a membrane micropump assembled on top of a substrate with a microarray.](image)

The used microarray is similar to the one described in chapter 4.3.1 on page 90. For sample preparation, genomic DNA from susceptible *E. coli* strain was extracted. A fluorescence labeling within a polymerase chain reaction (PCR) is followed by a purification step. 0.1 ng·µl$^{-1}$ DNA (equals 7.6 mM) is used as a sample. A thermomixer is used to reach the reaction temperature of 55 °C. The hybridization chamber has a volume of 25 µl. To fill the micropump volume, an additional volume of 4 µl is needed. Since the reaction kinetics

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44 *Eppendorf Thermomixer Comfort, Eppendorf AG, Hamburg, Germany.*
strongly depends on the reactants concentration $c_0$ in spite of the total volume, this additional volume is considered to be negligible.

The static two dimensional simulations in Fig. 52 shows the concentration over reactive microarray spot after 180 s.

Fig. 52: Static 2 D simulation of the depletion zone over a reactive microarray spot after 180 s. Spot diameter $w_s = 120 \, \mu m$, diffusivity of the target molecules $D = 2 \times 10^{-11} \, m^2 \cdot s^{-1}$ [161]. Sample DNA solution concentration $= 7.6 \, mM$. A: The solution is pumped at a volumetric flow rate of $Q_p = 5.7 \, \mu l \cdot s^{-1}$ through a channel of height $h = 250 \, \mu m$ and width $w_c = 10 \, mm$ over a microarray. A small steady depletion zone of 10 $\mu m$ is formed when molecules are actively transported to the spot. B: The solution is not in motion. A larger depletion zone is formed which continuously increases over time. The small depletion zone (A) leads to a large concentration gradient and therefore a higher flux and a faster microarray reaction.

With the assumption that all molecules react on the spot surface a concentration of zero appears at the spot surface. The concentration therefore decreases from the bulk solution to the reactive surface. The distance between the maximum concentration and the spot surface is called the depletion zone. When a constant flow with a velocity of $v = 2.3 \, mm \cdot s^{-1}$ is present (Fig. 52 A), only a small visible depletion zone of 10 $\mu m$ in the $z$-direction over the reactive spot appears. If diffusion is the only transport mechanism, the depletion zone over the reactive spot is larger and it is continuously increasing (Fig. 52 B). A smaller depletion zone leads to a
larger concentration gradient over the reactive area. The resulting higher flux $J_{\text{Diff}}$ (see Eq. 2-5) of transported molecules leads to an acceleration of the reaction time.

The absolute fluorescence signals shown in Fig. 53 proof, that the overlaying flow increases the signal intensity approximately by a factor 2 for all investigated durations. It is possible to reach signal intensity after 30 min with pumping similar to signal appearing after 60 min without pumping.

![Graph showing fluorescent signal intensity](image)

**Fig. 53:** Fluorescent signal intensity of a microarray with an active flow compared to a pure diffusion driven reaction over time.

The discrimination ratio between a perfect match and the highest mismatch over time is shown in Tab. 12. The higher ratio for the dynamic experiment shows, that the active flow shifts the reaction kinetics to a more specific binding between probe and target. For a static hybridization reaction, a discrimination ratio of 33 appears after 60 min. With pumping the perfect match signal is already 36 times higher than the signal from the highest mismatch after 30 min.
Tab. 12: Ratio of perfect match to the highest mismatch for static and dynamic reaction over time.

<table>
<thead>
<tr>
<th></th>
<th>5 min</th>
<th>15 min</th>
<th>30 min</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Static</td>
<td>4</td>
<td>14</td>
<td>14</td>
<td>33</td>
</tr>
<tr>
<td>Dynamic</td>
<td>12</td>
<td>26</td>
<td>36</td>
<td>52</td>
</tr>
<tr>
<td>Increase (x-times) of dynamic compared with static</td>
<td>3.0</td>
<td>1.9</td>
<td>2.6</td>
<td>1.6</td>
</tr>
</tbody>
</table>

An overlaying flow therefore leads to faster microarray hybridization together with a more specific binding between probes and target molecules. The more specific binding leads to a higher sensitivity of the microarray. The sensitivity is the distinction between the signal of perfect matches and mismatches. The increase (x-times) of dynamic compared to static has an average factor of two (see Tab. 12 average of last row). This is comparable to other work done by Liu et. al. [168]. They reported an increase factor of 1.6 and 2.1 for two microarray experiments. They proofed that this fact could be further increased by chaotic mixer where they reached an increase factor of 6.9.
5. Discussion and Outlook

In this work, a pressure driven microfluidic platform was developed. This µFLATLab (Microfluidic Flexible Laserwelded Automated TPE based Lab) consists of a lab-on-a-chip device for analysis and a processing instrument to control the functionality on chip.

The lab-on-a-chip device is made of a multilayer stack of two bulk polymers separated by an elastic membrane. The material combination was polycarbonate (PC) as a bulk layer and polyurethane based thermoplastic elastomer as a membrane layer. These materials are mass producible by injection molding and foil extrusion. Together with laserwelding as a joining technology, this concept forms a consistent low cost production chain for a high throughput fabrication of a disposable lab-on-a-chip device. Since the black TPE membrane works as an absorbing layer between two transparent bulk PC layers, a three layer stack could be easily extended to four, five or more layers, forming a three dimensional fluidic network. For joining, laser scanning velocities in the range of some ten mm·s⁻¹ are used. With high throughput method like a mask laserwelding process, a disposable the size of a microscopic slide could be joined in less than five seconds. For a three layer disposable a price less than 3 € per piece is expected.

Fluid control is realized by applying a control pressure on a channel crossing a hydraulic structure to deform the elastic membrane. A similar working principle for pressure driven PDMS-based setups has been developed earlier by Quake [41,64], leading to a accurate, flexible and highly integrated microfluidic platform. In microfluidics TPE has been reported by Stoyanov et. al. [43,44]. They used TPE as a bulk material in which a fluidic network is realized. For fluid management they manufactured a membrane valve by placing a displaceable PDMS membrane on top of a microfluidic channel. The actuation of this membrane valve was realized by an external pneumatic pressure source. Waibel et. al. [33] used an injection molded TPE sealing lip to successfully realize a piezo actuated check valve with a diameter of 5 mm to control the flow inside an electronic fountain pen.

In the here presented µFLATLab TPE is used as a membrane layer. It works as a functional layer to realize basic microfluidic operations like valving and pumping. Additionally it is utilized as a joining layer between the two structured polycarbonate bulk
layers in which the fluidic network is realized. The platform presented here has its own intrinsic advantages and disadvantages compared to \textit{PDMS} based setups. The \textbf{actuation principle} is similar to pressure driven \textit{PDMS} based platforms with the simplicity and reliability of a pneumatic actuation of microvalves and -pumps on chip, with the disadvantage of a bulky equipment like pilot valves and pressure sources. Solvent or plasma activated bonding in between \textit{PDMS} or \textit{PDMS/Glass} is commonly used. Areas where the materials are in contact, are bonded together with these methods. This way contact areas which should not be bound together, are not manufacturable. The advantage of a local heat application of a laser is that areas with and without bonding are possible. This is useful when a normally closed membrane microvalve design is needed. The bonding strength can be influenced with different laser parameters. An application would be an adjustable breaking threshold of a predetermined breaking well for the use of a sealed liquid storage on chip. The laser welding \textbf{joining process} was developed by Schmidt [73] in parallel to this work who successfully investigated laser parameters for the used materials to construct a tight and mechanically stable microfluidic lab-on-a-chip device. This joining process was adapted to application specific lab-on-a-chip designs and functions.

\textbf{Laser cutting} was used to cut vias through the \textit{TPE} membrane to create a three-dimensional microfluidic network. This is hard to implement e.g. with soft embossed \textit{PDMS}. The method solves problems like channel crossing and enables functional units like high efficient split and recombine mixers. Laser cutting offers more freedom of design, a higher functional integration, and therefore leads to a more cost efficient disposable device.

The prototyping path from idea to device contains computer aided design, creating vector files for structuring the bulk, cutout of the \textit{TPE} membrane and welding patterns for each step. It is a consistent and straight forward process which contains little error sources and is partially automatable. Micromilling was used as the main \textbf{prototyping} method, with rough surfaces and limited geometrical accuracy compared to soft embossing of \textit{PDMS}. The aim is to move the processes form prototyping to injection molding.

The \textbf{mechanical strength} of the setup is suitable for low and middle pressure applications - even at elevated temperatures over 90 °C. The softening temperature of the \textit{TPE} membrane mainly limits the maximum temperature stability which is in the range of 110 - 140 °C. Leakage has been observed in some lab-on-a-chip devices at pressures higher than 30 kPa and a temperature of over 90 °C. To maximize the mechanical strength of the
stack, further optimizations of the laserwelding parameters regarding mechanical strength, leakage mechanism and stress minimization are recommended. A second approach should be to strengthen the multilayer stack with construction methods like increasing joined surface by interdigitating structures.

A distance between laser weld and structure of 300 µm is necessary. This distance, although not yet fully optimized, limits the integration density of active elements. Each active element on chip consumes some mm². Compared to PDMS based large scale integration where only some 100 µm² are needed, this is a lower integration density.

Due to the fact that the bulk material itself is not elastic and does not deform like PDMS, it is easy to manufacture dimensionally robust chambers with a high width to height aspect ratio and comparably large dimensions in the mm range. This way a liquid storage of 50 µl on chip could be realized corresponding cavities have been sealed successfully. The sealing is implemented during the laserwelding joining process and needs no additional and cost-intensive process steps. The TPE membrane could be used to pressurize the prestored liquid, crack a predetermined breaking weld, and successfully displace 85 % of the liquid into the fluidic network. For most assays, manual preparations are the most time consuming and error-prone steps. Therefore the ability to store reagents on chip is one of the most important features for a successful product. This represents an important step towards highly integrated lab-on-a-chip devices, because it reduces the number of hydraulic interfaces, in best case, to only one (sample) input. It facilitates the assay by decreasing the number of manual filling steps with pre-stored and/or pre-mixed reagents on chip. Fewer fluidic interconnections are required, leading to a more convenient use of the system. The reproducibility of the assay is increased and the influence of the operator is minimized by standardized and quality controlled filling during the production process. Waste is storable on chip as well, thus avoiding possible contamination of the outside world or the user. Because of the gas permeability of PDMS, no such approach has been reported for PDMS based platform to the author’s knowledge at this point. The influence for a longer storage period and the influences of temperature change, storage conditions, humidity and evaporation of the reagents should be investigated in the future. Attention should be turned to long term material behavior, especially when using polymers like PC where additives tend to dissolve over time and interact with the content stored on chip. SiO₂ diffusion barrier layers have been investigated by Schmidt in parallel to this work [73]. This could be the initial point for further activities like investigating the storage conditions for liquids other than water, e.g. bio active liquids.
including enzymes, sensible assay reagents like dNTPs, DNA or buffers. The following list is a suggestion of parameters to be investigated in the future:

- Long time stability of the stored liquid reagents and influences between chip material and stored content, UV stability of the lab-on-a-chip device and its content and temperature dependency for different storage conditions (storage at room temperature, cooled or freezeed storage, temperature cycle).

- Storage of liquid and non liquid content like freeze-dried enzymes.

- Interaction of the bulk material PC as well as the TPE membrane with the stored reagents.

- Evaporation of the liquid over time.

- Contamination risk during laserwelding.

PC has good optical characteristics, like transparency to wavelength from visible light to UV. It is manufacturable in optical surface quality, e.g. for spectral lenses. Compared to PDMS, it has higher auto fluorescence when using UV readout methods like UV laser microarray reader.

A membrane microvalve has been successfully realized. Experimental characterization proofed the basic functionality of several different designs. The characteristics showed low actuation pressures of only some 10 kPa and a robust functionality independent from the liquid used to control. In contrast to concepts where local surface treatment is used to stop liquid, this mechanical element for fluid management is mostly independent of wetting properties and viscosity. The principle of a discontinuous hydraulic channel extends the simple start and stop functionality of a valve to a T- and X-junction which enables loop structures to be filled without bubble trapping and little dead volume.

The laserwelding joining process was expanded with a sequential second laserwelding step. The first step made sure that the TPE membrane is properly welded to the pneumatic control layer. With the second laser step the hydraulic layer was joined. This way cross interaction between two or more separate active structures could be successfully avoided. This way independently working microvalves could be realized which form the fundament for a complex fluidic network at the cost of an additional production step. The TPE membrane is
melted twice with this sequential process. No change in mechanical behavior or any other characteristics were observed, which would indicate a negative influence from melting the material twice.

A membrane micropump was realized and experimentally characterized as well as showing broad tunable flow rates from 0.1 µl·s⁻¹ to 3.5 µl·s⁻¹. One pump cycle included six steps. The actuation was made with one actuation pressure level during the displacement stroke but without a vacuum applied during the suction stroke. Adhesion of the TPE membrane to the micropump chamber bottom limits the dynamic of this actuation method. Since the tested devices were all micro milled with a comparably rough surface, this effect should worsen when the device is made by injection molding with a smooth surface. This problem could be avoided by the cost of an additional (under) pressure level to actively suck the membrane from the chamber bottom. With this method, pump rates from 0.3 µl·s⁻¹ to 5.7 µl·s⁻¹ could be reproducibly realized with the same pump design within actuation frequencies from 0.25 to 8.0 Hz. By using an under pressure the maximum flow rate could not yet be reached with the used equipment. Spot tests with the maximum actuation frequency of the pilot valves, which is equivalent to a pumping actuation frequency of 50 Hz, reached a maximum pump rate of 55.8 µl·s⁻¹. This broad dynamic range shows that this micropump could be used for various applications. In a second design the stiffness of the membrane was increased. This way the force for deflection of the membrane during the suction stroke was sufficient to overcome the adhesion of the membrane to the chamber bottom. This led to a controllable flow rate between 0.1 µl·s⁻¹ and 3.5 µl·s⁻¹. This solution provides a reproducible fluid transport without the need for an additional under pressure level. The micropump forms the second, yet more complex, basic element and was used to successfully show the principle of active micromixing of two discrete liquid plugs of 4 µl and 33 µl within eight seconds. The micropump is also capable of transporting liquid from off chip liquid storage, replacing the functionality usually performed by a syringe pump. Together with the shown liquid storage method on chip, this functional unit promises a high integration with less surrounding equipment usually used for pressure driven microfluidic platforms. The range of possible control pressures from under pressure of only some 10 kPa absolute to 900 kPa at room temperature guarantees a liquid independent transportation. Gas bubbles can be strongly compressed and moved through the pump displacement chamber reliably.

A processing instrument was implemented and constantly adapted to the requirements of the fluid management and the assay implementation on chip. It included the pilot valves for
pneumatic actuation, the measurement and control board\textsuperscript{45} and the software for implementing assay processing on chip. A switching logic of 8 or more microvalves with 10 different static (switch) and dynamic (pumping) states was realized. The interfaces for hydraulic and pneumatic functions were realized by tubing. This is a reliable method for lab environment, but is not suitable for a successful product. Tubes being manually connected to a liquid reservoir where reagents are stored off chip lack the concept of a fast automated test because of the high manual effort. Further developments should lead to a reliable pneumatic interface between the lab-on-a-chip device and the processing instrument. An example solution would be a convenient “click and connect” solution with a silicone gasket seal between equipment and the lab-on-a-chip device. A standardized connection pattern has already been implemented in the design of the devices. The goal is to pre-store all reagents on chip as a liquid or in form of a solid powder. A heating concept with a resistive heater, fan cooler and temperature control was realized. The temperature is controlled by a software implemented PID. Thermal processes of the example assay (lysis and PCR) could be realized within the required temperature accuracy of 1 K. The attained heating rates of $+4 \text{ K\cdot s}^{-1}$ and cooling rates of $-1.3 \text{ K\cdot s}^{-1}$ are similar to those of commercial available thermo cyclers. The equipment was mounted on a transportable setup to be able to experiment in facilities of project partner for testing the biochemical assay. A graphical user interface (GUI) was developed for a convenient user interaction. Data and software documentation is a key factor during the prototyping stage of the assay implementation on chip, because of the complex parameter interaction such as PID factor optimization for heating, pump actuation cycles etc.

The processing instrument is the first step towards equipment needed for the implementation of every biochemical assay. The size of this laboratory setup implies that a small desktop apparatus half the size of a shoe box is realistic. Further miniaturization of the processing instrument towards the size of a handheld is thinkable. Although the principle of a pneumatic actuation needs the implementation of an integrated pressure source which limits the minimum size and weight of such a device. A change of the actuation method like a thermo-pneumatic actuation \cite{171} would lead to a very small processing instrument with dimensions of a smart phone.

A method to realize and integrate fluid tracking could be central to further activities. Possible applications are assays where a feedback control is needed to provide more accurate

\textsuperscript{45} Developed by Multi Channel Systems GmbH, Aspenhauser Straße 21, 72770 Reutlingen, Germany.
and reproducible liquid coordination. An example application is the processing of several equal assays simultaneously on one device. The resistance of a hydraulic channel is subject to its geometry and therefore the manufacturing variance. The resulting flow velocity of a certain liquid varies with the resistance. When two flows need to be coordinated, a fluid tracking could measure the actual position of a liquid or the crossing of a check point. Optical methods are the most commonly used and reliable strategies to fulfill this requirement. As a measurement signal the different light absorption between an air-filled and a liquid-filled channel can be used. Light is transported to the check point by optical fibers and the absorption of the channel is measured. Another method would be to utilize the difference in the refractive indices between an air-filled channel and a liquid-filled channel. Total internal reflection (TIR) would lead to a binary signal between a filled and an unfilled channel [172]. Although the integration demands optics and additional hard- and software, both measurement methods could be implemented in the µFLATLab and are suggested as further investigations.

The materials showed no negative interaction for the biochemical reactions before or after joining by laserwelding. Therefore the material and the production process can be entitled suitable for the biochemical liquids used in this particular application example. The assay had to be adapted to the silica filter by increasing BSA as a blocking agent to improve PCR performance.

The aim of the example assay was to perform a sample preparation from an urinary tract infection caused by E. coli for the identification of a possible resistance with a given microarray. The goal of the assay was to gain DNA sample for a microarray experiment by enriching E. coli bacteria. The assay was developed by project partners during a publically founded project (Path.Ident). It was adapted for the integration into a lab-on-a-chip device. Filtering a sample was chosen as a robust, simple and universal method for accumulation. Thermal lysis is robust and easily integrated as an extra thermal step prior the PCR. The PCR itself as a standard method in biology offers a wide range of thinkable applications besides E. coli analysis. The evaluation of the different methods for each task of the assay was chosen by its biochemical functionality together with the capacity to be realized in a cost-efficient microfluidic platform. Since this was an iterative process until the end of this work, these

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46 Institut für Technische Biochemie, Universität Stuttgart, Allmandring 31, 70569 Stuttgart, Germany. Eppendorf AG, Barkhausenweg 1, 22339 Hamburg, Germany. Robert-Bosch-Krankenhaus, Auerbachstraße 110, 70376 Stuttgart, Germany.
three steps are only one possible way of performing this particular analytic task, and therefore do not claim to be the most efficient one. The realization of filtering a sample and performing a PCR in the same chamber has some elegance and advantages, though.

The need for handling ml sample volumes could be successfully realized by filtering. The sample volume is only limited by the time the user is willing to spend for the application and the filter capacity. The processing of 10 ml with $10^7$ E. coli bacteria took about 10 min and turned out to be below the capacity limit of the used silica filter (diameter 7.6 mm, height 0.8 mm). This fast processing of such a sample volume, being large for microfluidic dimensions, is a key factor for applications where the species to be identified are scarcely present in the sample. During a blood infection caused by Salmonella for instance only one bacteria per milliliter blood is present [173]. If only a small 10 µl blood droplet is used as a sample, there is only 0.1 calculated species in this sample, meaning that there is a high probability that there is no detectable species in the sample at all. A disadvantage of a filter is its sponge characteristic, conserving liquid inside the filtering chamber. This makes the further transport of the sample more difficult. A future work should include the processing of urine samples to investigate influences of mammalian cells, blood, proteins and other substances appearing in real world samples.

The thermal lysis together with the PCR in the filter chamber on chip is an elegant way of integrating those sub functions. This way one liquid transport step on chip is avoided which always leads to some loss. The fluid handling includes only the application of the PCR master mix. Thermal lysis is an effective way to treat E. coli but is not an universal approach. For example, gram-positive bacteria or fungal spores are unlikely to be cracked with this method. The PCR was performed after filtration of susceptible E. coli suspension on a filter integrated in a chip. The results showed that the material did not inhibit or interact with the PCR. This was one major concern because it couldn’t be excluded that the materials coming from a manufacturer, particularly the TPE membrane, didn’t contain additives or production artifacts interacting with the PCR. PCR as a biochemical tool has a high potential for other applications beyond this one.

The filter efficiency strongly depends on the geometry of the filter chamber. Therefore the filter efficiency deviation can be minimized by quality management of the chamber dimensions. A minimization of the chamber size and chamber walls would lead to an acceleration of the time consuming PCR by reducing the thermal resistance and capacity.
The minimum size depends on the minimum sample needed for the test. Therefore this value must be defined prior to change. A more powerful heating/cooling strategy, e.g. by using Peltier elements, is another way to accelerate sequent cycling. Much of the time consumed during a PCR is the transition between reaction temperatures. A promising way for reducing the transition duration is a **parallel heating strategy**. Here the fluid is moved between three constantly heated zones. Only the thermal mass of the liquid is cycled, leading to a thermal time constant below one second. The total duration for a 31 cycle PCR as described in (4.2.1, p. 80) would be reduced from 2h down to 25 min. This heating concept could be easily realized with µFLATLab by placing three displacement pump chambers in series each of them at a constant temperature of 94, 55, and 72 °C. With an adequate actuation sequence, the fluid plug is then periodically transported between the three temperature levels.

The final **readout** with a **microarray** was realized by further processing of the PCR product. The reproducible results for all tested initial bacteria concentrations ($10^4$-$10^7$ bac/10 ml) showed the functionality of the assay integrated in a lab-on-a-chip device. For a better quantification and a qualification of the sample preparation, an internal reference control should be processed parallel to the sample. For a lysis control, species could be integrated which are harder to destroy, like fungal spores for example. Those would need to be detectable on the microarray, if successfully destroyed. If the lysis of this more robust species worked, then the original species in the sample is most likely destroyed, too. For qualification of the PCR, an internal control could be added which is detected with additional microarray spots as well.

An **integration of a microarray** into a lab-on-a-chip device would enhance the test integration level. The used material would need a sufficiently low auto fluorescent for a high signal-to-noise-ratio. Best results are achieved with glass as a carrier, but COC polymer for instance has a low fluorescence as well. Another task is to make sure that the detection probes stick well to the chamber walls. During the washing steps, a loss of probes is to be avoided. In this work the carrier plate was a surface modified glass slide. It was removable glued to the lab-on-a-chip device. After hybridization the glass slide was separated from the lab-on-a-chip device and read out in a separate laser read out equipment. An alternative would be the integration of microarray spotted on a glass die glued inside a hybridization chamber on chip.

µFLATLab provides a toolkit with a broad range of robust microfluidic operations like valving, pumping etc. which are integrated to one automatable diagnostic DNA based test.
The transportable processing instrument can be used with different disposable lab-on-a-chip devices with changing design to address a broad range of possible diagnostic and analytical applications. At this point the processing of the test needs manual handling steps for connecting tubes and applying a sample and reagents. A standardized interface regarding micro-to-macro world connection pattern, pressure levels, temperature connection and software implementation is the next logical step to a user friendly and fully automated test. The concept has proven to be fully automatable, if the way towards further integration and improvement of interfaces is preceded consequently.
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