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FABRICATION AND CALIBRATION OF A MICROFLUIDIC DEVICE FOR PATTERNED SURFACE MODIFICATION

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ABSTRACT

In this work the feasibility of using a microfluidic device for the arraying of proteins has been tested. The major advantage of this concept consists in keeping the proteins hydrated during the experiments. The working principle of this arrayer is to activate spots by an activation stream and to do subsequent functionalisation by reagent streams flowing perpendicular to the first stream. The surface pattern was provided by a MAPL-chip (Molecular Assembly Patterning by Lift-off). It consists of well defined areas of PLL-*g*-PEG-biotin surrounded by a protein resistant surface of PLL-*g*-PEG. The PDMS flow cell was fabricated by soft lithography and sealed to the MAPL-chip by pressure. Initial testing of the microfluidic device was performed with food dyes and fibrinogen Alexa Fluor 488. The position and the width of the different streams were easily adjustable by using different flow rates in the channels. A second testing series included the testing of the microfluidic device with the streptavidin/biotin system, using streptavidin Alexa Fluor 633 for the activating and biotin 4-fluorescein/unlabelled biotin for the reagent streams. The result of the tests showed that the microfluidic device worked well and thus the concept of using a microfluidic device for arraying of protein should work as well. The fabricated microfluidic device shows possible applications for arraying of biotinylated vesicles , biotinylated proteins or histagged proteins for immobilisation on Ni^{2+} chelating surfaces.

1 INTRODUCTION

In the 1970s the first microfluidic device was constructed at Stanford University. The growth of interest in molecular biology, especially genomics, in the following years has stimulated the development of technology for the analysis of complex mixtures of macromolecules as for example DNA and proteins in aqueous solutions by capillary electrophoresis (CE). The benefits of microfluidic devices are diverse: They offer a decrease of costs in manufacture, use and disposal as well as a reduction in analysis time. By use of microfluidic devices the consumption of reagents and analytes is reduced and separation efficiency and portability are increased. These early systems were manufactured by technology derived from microelectronics as photolithography and etching in silicon and glass. The disadvantages of this technology consisting of the use of relatively expensive materials and the requirement of high temperature or voltage for the sealing led to a rapid development of new fabrication technologies with new materials. The advantage of these new materials, all polymers, are the low price, the possibility of fabrication by moulding or embossing and that they can be sealed thermally or by adhesives [1]. Among these polymers polydimethylsiloxane (PDMS) became most prominent. It is optically transparent, non-toxic, commercially available and its hydrophobic surface can easily be converted to hydrophilic. Furthermore it has a Young's modulus that makes it a moderately stiff elastomer [2]. Nevertheless PDMS shows the disadvantage of being incompatible with organic solutions [3]. Standard techniques for the fabrication of microfluidic devices include micromachining, soft lithography, embossing, in situ construction, injection molding and laser ablation [4-9]. Nowadays microfluidic devices are used in biology for DNA analysis [2], cell sorting [10], as biosensors [1], as devices for cell culturing [11] and as devices for cell and protein patterning [12, 13].

Protein patterning is performed on so called protein arrays where proteins are immobilised on well defined areas for quantification or functional analysis [14, 15]. One type of surfaces on which proteins can be immobilised are surfaces with a high inherent binding energy to proteins in general [16]. The most common of these substrates are hydrophobic plastics to which most proteins adsorb physically by van der Waals, hydrophobic and hydrogen-bonding interactions. The disadvantages of

these adsorbtion mechanisms are that the immobilised proteins build clusters on the surface and that most of them denaturate and thus lose their functionality. Therefore protein immobilisation is preferably performed on surfaces, which offer specific binding sites for certain proteins. Examples for such binding mechanisms are biotinylated proteins that bind to streptavidin-coated surfaces or His-tagged proteins binding to Ni^{2+}-chelating surfaces. This binding sites are situated on well defined areas surrounded by protein resistant surfaces to prevent non specific adsorption. The detection of immobilised proteins is mainly performed by fluorescence using charge-coupled device (CCD) cameras or laser scanners with confocal detection optics. Furthermore radioactivity, chemiluminescence or label-free plasmon-resonance based detection systems can be used [14].

The first method of printing proteins onto surfaces was using instruments designed for DNA spotting [16, 17]. Due to long printing times coupled with small volumes which are spotted onto the surfaces this method generally leads to drying of the protein spot. Therefore other printing methods as deposition by a hydrogel stamper inked with an aqueous protein solution [18], inkjet printing [19], electrospray through a dielectric grid mask [20] and direct application of protein solutions via microfluidic networks [12, 21] were developed, to keep the proteins hydrated during the experiment. But up to now these methods remain an area with considerable need for innovation and improvement.

The attraction of developing protein arrays is the amount of information that can be obtained as a function of research time and sample quantity. The enormous potential of this microspot technology was demonstrated by DNA chips leading to the decoding of the human genome in far less time than expected. Moreover, from a commercial perspective, protein chips appear to be a highly lucrative market estimated to grow to $500 million by 2006 [22].

The goal of the present work was to test the feasibility of a potential protein arraying method. The purpose of this method was to obtain local functionalisation of an array of nine spots with the help of a microfluidic device. Thereby functionalisation was generated in a two step process. In a first step the spots were activated with a laminar stream of labelled streptavidin. In the second step the activated spots were functionalised by labelled and unlabelled biotin streams perpendicular to the activating

stream. The prepatterned surface used for the experiment was provided by a MAPL-chip (Molecular Assembly Patterning by Lift-off) [23, 24]. It consists of well defined areas with specific binding sites for streptavidin surrounded by a protein resistant area. The protein resistant material is Poly(L-lysine)-*g*-Poly(ethylene glycol) (PLL-*g*-PEG), a polycationic copolymer that adsorbs spontaneously from solution onto negatively charged surfaces [25-27] and the protein binding areas are composed of the same polymer showing biotin functionalysed PEG-chains, PLL-*g*-PEG-biotin.

A major advantage provided by this arraying method is that the functionalisation of the different spots is performed only in solution. This fact makes it very attractive for the arraying of vesicles or proteins because denaturation of proteins and collapse of vesicles due to drying effects can be excluded.

2 PHYSICAL CONDITIONS IN MICROFLUIDICS

In microfluidic systems new effects become dominant due to the microscopic dimension of such systems. These effects are laminar flow, fluidic resistance, surface area to volume ratio, diffusion and surface tension [28]. For the work described in this report the effects of laminar flow and diffusion are most relevant and therefore explained below

2.1 Laminar Flow

In physics two different flow regimes are described:

1. Turbulent flow
2. Laminar flow

The turbulent flow regime is chaotic and unpredictable. The consequence of this is, that it is impossible to predict the position of a particle in the fluid stream as a function of time. In contrast to it, laminar flow describes a regime in which two or more streams flowing in contact with each other do not mix except by diffusion. To decide which of this flow regimes is present in a given system, the Reynolds number Re can be considered.

$$\mathbf{Re} = \frac{\rho \nu \mathbf{D_h}}{\mu} \tag{1}$$

with: ρ = fluid density
ν = characteristic velocity of the fluid
μ = kinematic viscosity
D_h = hydraulic diameter

For flow in circular pipes D_h defines the pipe diameter. Re < 2100 generally indicates a laminar flow. As Re approaches 2100, the fluid starts to show signs of turbulence and for Re > 4000 the flow is considered to be turbulent [29].

If flow is analysed in rectangular channels, D_h defines the channel width. For this geometry laminar flow is found for Re < 525, the transition region is given for 525 < Re < 1000 and turbulent flow is observed for Re > 1000 [29].

The calculation of the Reynolds number for the flow cell described in this work leads to a value for Re = 30. The values taken for ρ and μ were the one of pure water

and the velocity, calculated from a flowrate of 10 ml /h, had a value of 0.02 m/s. This result for Re shows that the microfluidic devices works in the regime of laminar flow.

2.2 Diffusion

Due to laminar flow conditions in most microfluidic systems diffusion becomes the only mechanism that leads to mixture of laminar flows. In one dimension diffusion can be described by the following equation:

$$d^2 = 2Dt \tag{2}$$

with: D : diffusion coefficient
t : time
d : average distance by diffusion

3 PURPOSE AND DESIGN OF THE MICROFLUIDIC DEVICE

3.1 Purpose

The purpose of the microfluidic device developed in this work is to generate local functionalisation of an array of 9 spots. Therefore a MAPL-chip (Molecular Assemply Patterning by Lift-off) provides the prepatterned surface. The principle how the local functionalisation is obtained is shown in Fig. 1.

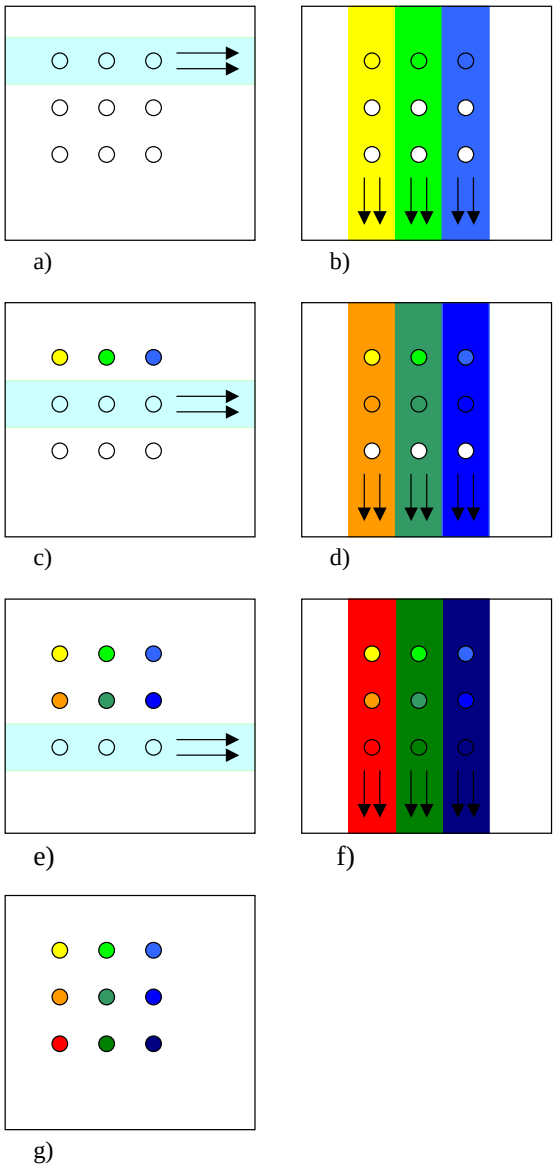


Figure 1: Function principle of local functionalisation of a prepatterned surface in a microfluidic device

The spots are arranged in a array of 3 x 3 spots. In a first step an activating laminar stream is led over the chip activating the first row of spots (Fig. 1 a). Thereafter these three activated spots are functionalised by three laminar streams, which flow perpendicular to the activating stream (Fig. 1 b).

These two steps are repeated for the second and third row of spots on the MAPL - chip (Fig. 1 c) - f). The only change consists of using different reagent for the reaction streams.

Thus a square of 9 differently labelled spots is obtained (Fig. 1 g).

3.2 Design

The function principle of the microfluidic device demands a certain design. First of all the flow cell must have two perpendicular crossing channels to enable the laminar streams as shown in Fig. 1. Secondly each of these channels must have three different inlets. Two different designs were considered. The first design has three different inlets at the ends of the crossing channels (Fig. 2). The second design shows two crossing channels for the baseflow in which an inlet system disembogues [30](Fig. 4 - 6). This inlet system consists of three channels (see Fig. 7) injecting three streams from above into the baseflow.

1st design:

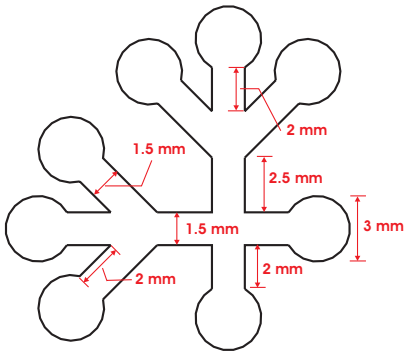


Figure 2: Design of the flow cell

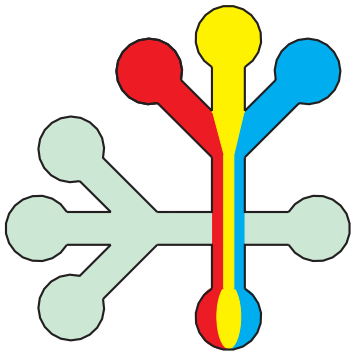


Figure 3: Microfluidic device showing laminar streams

2nd design:

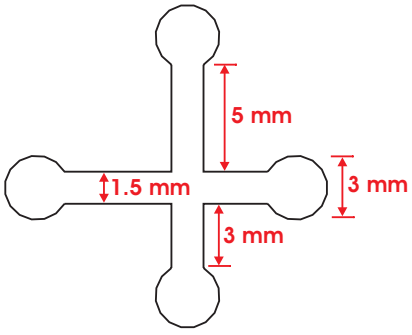


Figure 4: Design of the flow cell

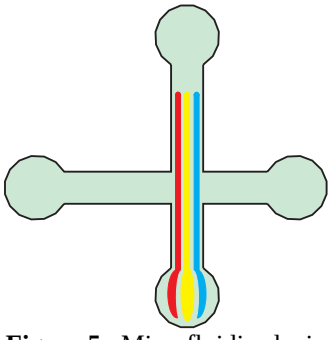


Figure 5: Microfluidic device showing laminar streams

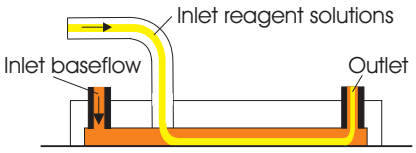


Figure 6: Cross section through the flow cell

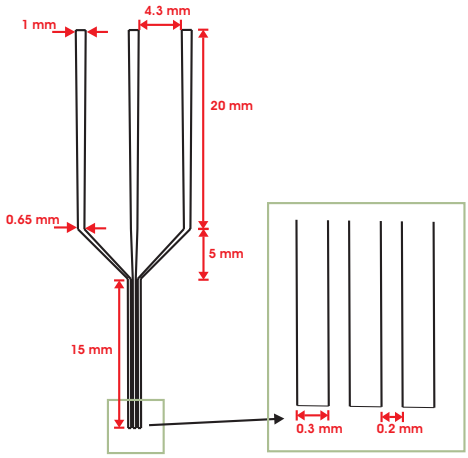


Figure 7: Inlet system for the reagent solution

In the following figure the design of the MAPL-chip is shown:

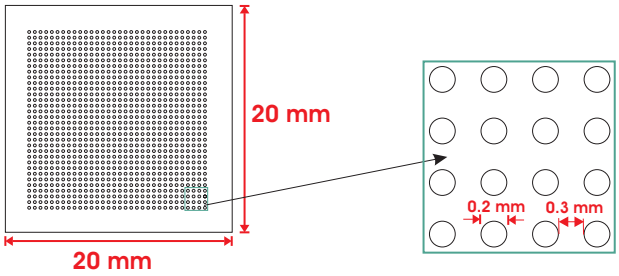


Figure 8: Design of the MAPL-chip

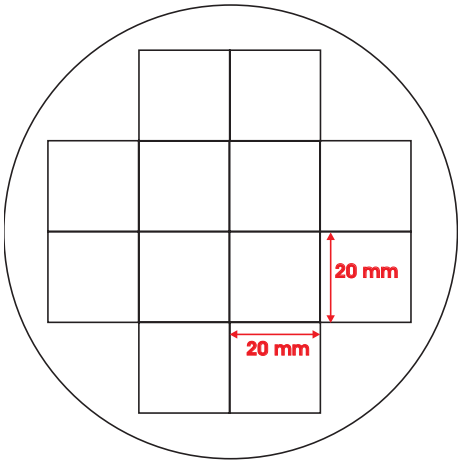


Figure 9: Assembly of MAPL-chips on a 4-inch wafer

4 MATERIALS AND METHODS

4.1 Materials

4.1.1 Material used for the fabrication of the flow cell

The flow cell is made of polydimethylsiloxane (PDMS) as mentioned in chapter 4.2.1. The structure of PDMS is shown in the following figure:

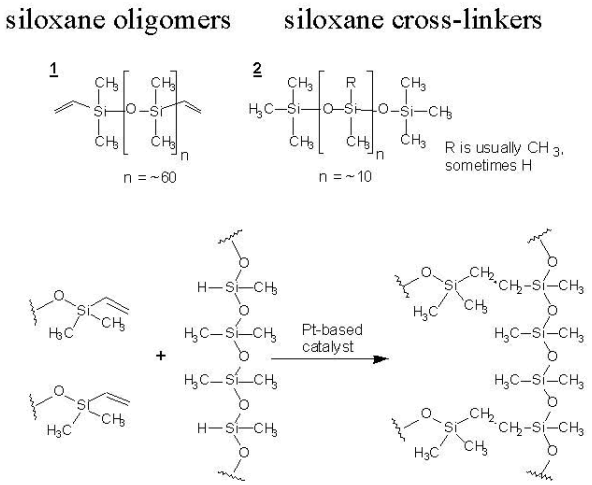


Figure 10: Structure of PDMS

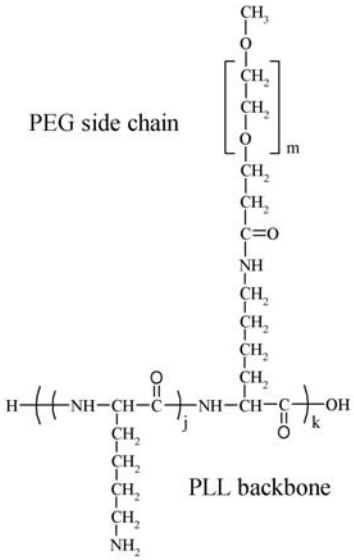
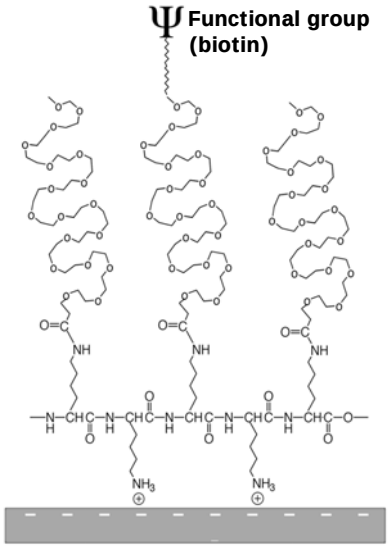
The repeating -O-Si$(CH_3)_2$-groups lead to a hydrophobic surface. By exposing the surface to an oxygen or air plasma it is rendered hydrophilic due to the introduction of silanol groups (Si-OH) and the destruction of methyl groups (Si-CH_3) by the plasma [3, 31].

PDMS seals irreversibly to a range of materials as PDMS, silicon oxide, silicon nitride, polyethylene, polystyrene and glassy carbon [1, 32]. Therefore both surfaces have to be cleaned and exposed to an oxygen plasma. The reason for this irreversible sealing is the formation of covalent -O-Si-O- bonds by a condensation reaction of the silanol groups on the PDMS and the -OH-containing functional groups on the other materials produced during plasma exposure.

4.1.2 Materials used for the fabrication of the MAPL-Chip

The materials in the following tables are used to produce the MAPL-chip:

Table 1: Materials used for the fabrication of the MAPL-chip

PLL-*g*-PEG: Poly(L-lysine)-*g*-Poly(ethylene glycol) Polycationic protein resistant copolymer, that adsorbs spontaneously from aqueous solutions onto negatively charged surfaces such as oxides of niobium, titanium, silicon and indium tin oxide [25-27] Used polymer: PLL(20)-*g*(3.4)-PEG(2), FD09, produced by Firat Durmaz, LSST, ETHZ	
PLL-*g*-PEG-biotin: Used polymer: PLL(20)-*g*(3.5)-PEG(2)/PEG(3.4) biotin 42%, SP-15-03 produced by Stéphanie Pasche, BIG, ETHZ	
Hepes 2: Hepes 2 was used to disolve PLL-*g*-PEG and PLL-*g*-PEG-biotin. It is a 150 mM solution of ultra pure water and HEPES powder (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid from Fluka) with an adjusted pH of 7.4.	
Wafer: 4-inch Pyrex 7740 wafer from SensorPrep, Nb_2O_5-coated with a dc-magnetron 7600 from Leybold at the PSI in Villigen, CH	
Photoresist: S1818 from Shipley, USA	

4.1.3 Materials for the assembly of the microfluidic device

In the following table the materials used for the assembly of the microfluidic device are shown:

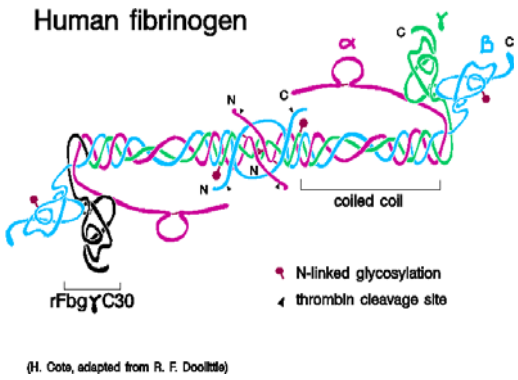
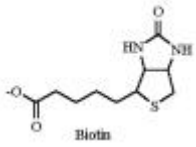
Table 2: Materials used for the assembly of the microfluidic device

Material	Manufacturer
Tubes: - black-black: inner diameter:0.76 mm - green-green: inner diameter:1.85 mm	 Bioblock Scientific, F Bioblock Scientific, F
Steel hose coupling: Length: 16 mm Inner diameter: 0.84 mm	Ismatec SA, CH
Valves: - Small rotary valve, OMF-1111 - Discofix	 Milian SA, CH Braun, D
Coverslip: Size: 24 x 50 mm Thickness: 0.13 - 0.16 mm	Menzel Gläser, D

4.1.4 Material used for the function tests

The materials used for the testing of the microfluidic device are shown in Table 3:

Table 3: Materials used for the testing of the microfluidic device

Fibrinogen: Fibrinogen, Alexa Fluor 488 conjugate Concentration: 25 µg/ml in Hepes 1	Molecular Probes	
Streptavidin: Streptavidin, Alexa Fluor 633 conjugate Conc.: 20 µg/ml in Hepes 1	Sigma Aldrich Sigma Aldrich	
Biotin: -Biotin-4-fluorescein Conc.: 30 µg/ml in Hepes 1 -Anti-Rabbit IgG, biotin conjugate Conc.: 25 µg/ml in Hepes 1	Molecular Probes Sigma Aldrich	
Food dye	Migros, Zurich	
Hepes 1: Hepes 1 was used to dilute the proteins. It is a 10mM solution of ultra pure water and HEPES powder (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid from Fluka) with a pH adjusted to 7.4.		

4.2 Methods

4.2.1 Fabrication of the Flow Cell

The first step to manufacture the microfluidic device is the fabrication of the (PDMS) flow cell. This process is composed of the following two subprocesses:

1. Mould fabrication by photolithography
1. Replica moulding with PDMS

Mould Fabrication

For the fabrication of the mould photolithographic technique was used (Fig. 11). Photolithography is a highly developed technique for producing accurate patterns which requires clean-room facilities and expensive equipment [4, 11, 33].

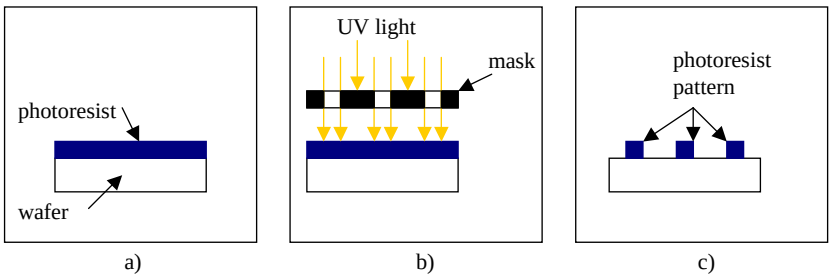


Figure 11: Main steps of photolithography: a) spincoating of the potoresitst, b) illumination of the photoresist with UV light and c) developing of the photoresist pattern

A 4-inch <110> silicon wafer from Wafernet GmbH was cleaned and dried for 10 min in a plasma cleaner (300E from TePla). On this dry wafer 3 ml of photoresist (SU-8 50, MicroChem Company) was homogeneously applied by spincoating (max. speed: 1500 rpm, spincoater: RC 5 GYRSET from Karl Süss) (Fig. 11 a). Then the prebake was performed. Therefore the wafer was placed on a 50°C warm hot plate for 5 min. The hot plate was then heated up to 100°C and held at this temperature for 60 min. In the next step the wafer and the photomask (foil showing the design of the flow cell, 64’000 dpi, from jdphoto glued to a glass plate) were installed in a mask aligner (MA6/BA6 from Karl Süss), where the photoresist was exposed to UV light through

the mask for 44.4 sec to apply 400 J/cm^2 (Fig. 11 b). This exposure to UV light starts a crosslinking reaction in the photoresist, which is finished during a postbake (5 min at 60°C, then heated up to 95°C, held at 95°C for 45 min). The pattern was obtained by developing the wafer in different solutions (Fig. 11 c). Thereby the photoresist not exposed to UV light was solubilised first in a GBA developer for 2 min, in a PGMEA developer for 2.5 min and finally in a second and purer PGMEA developer bath for 1 min. By rinsing the wafer afterwards in isopropanol and drying it with nitrogen the final mould was obtained. The height of the mould was found to be around 90 μm as measured it with a proflilometer (P10 from Tencor).

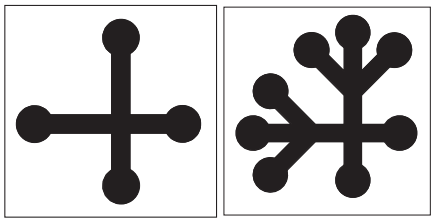


Figure 12: The black structures represent the photoresist patterns of the 1st and the 2nd design.

After fabrication the moulds were controlled with a light microscope. It was found that they show a lot of cracks (Fig. 13). Due to this observation the process parameters of the pre- and postbake and the developing were varied but without any significant improvement. In a second step the illumination time was changed. It has been observed that a reduction of illumination time results in a higher density of cracs and vice versa (Fig. 13 - 15).

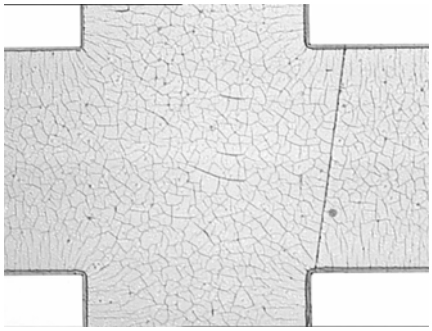


Figure 13: Light microscope image of the final mould in the cross section of the channels. Illumination time: 44.4 sec (400 J/cm^2)

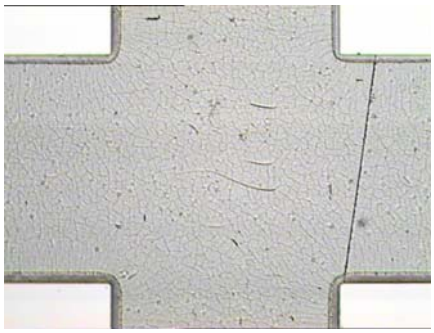


Figure 14: Light microscope image of the final mould in the cross section of the channels. Illumination time: 33.3 sec (300 J/cm^2**)**

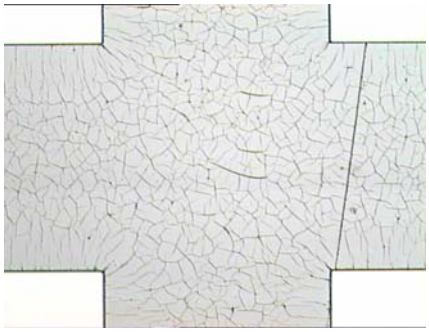


Figure 15: Light microscope image of the final mould in the cross section of the channels. Illumination time: 55.5 sec (500 J/cm^2)

To decide if these cracks are situated on the surfaces of the photoresist and therefore influence the quality of the flow cell, the moulds were controlled with a SEM (LEO 1530 from Leo Electron Microscopy Ltd.). It was discovered that the cracks are not situated on the surface and therefore they shouldn't affect the quality of the microfluidic device. Thus the fabrication parameters were not further investigated. The final fabrication parameters can be found in Appendix C.

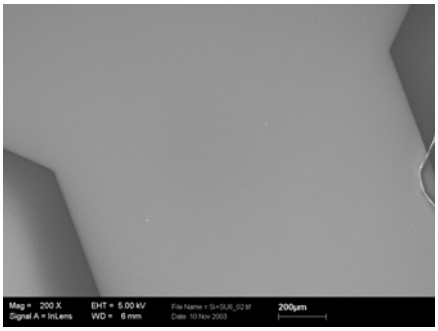


Figure 16: SEM images of the mould

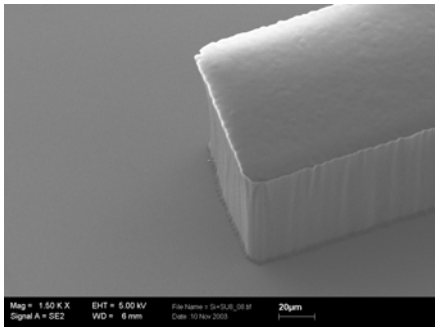


Figure 17: SEM images of the mould

Replica Moulding

Replica moulding is the process of producing a polydimethylsiloxane (PDMS) replica from a structured master. This was carried out by mixing PDMS (Sylgar 184, Dow Corning) precursor and curing agent at a ratio of 10 : 1. Thereafter the mixture was degassed in vacuum to get rid of the air bubbles generated during mixing, then cast over the mould and finally cured at 80°C for 24 hours. Undercuring is known to affect the release of small molecular weight oligomers and mechanical stability of PDMS [34]. Therefore the PDMS was cured much longer then proposed in the data sheet from Dow Corning [35]. After curing the PDMS replica could easily be peeled off the mould.

For the second design the replica moulding includes one step more. Before the flow cell mould is cast a PDMS replica from the inlet system (see Fig. 7) must be produced and irreversible sealed to a flat piece of PDMS as described in chapter 4.1.1. Thereafter the inlet system is glued to the channel of the mould with a soluble glue. This fixation makes the handling easier and also prevents the PDMS from filling up the channels of the inlet system. Due to problems with the fixation of the inlet system on the mould this design was not further investigated.

4.2.2 Fabrication of the MAPL-Chip

The function of the MAPL-chip in the microfluidic device is on one hand the provision of a surface patterning on which protein binding takes place on well defined areas and on the other hand to seal the channels of the PDMS flow cell. The fabrication relies on a combination of an initial top-down photolithographic step and a following bottom up molecular assembly step. The first step (prepatterning) defines the pattern geometry and the second step (MAPL-patterning) introduces the biochemical function (Fig. 18) [23].

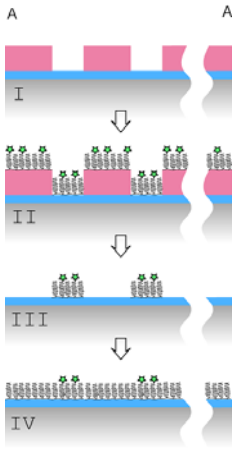


Figure 18: Schema of the MAPL-patterning technique

Prepatterning

The process of the prepatterning is also a photolitographic process as the fabrication of the mould described in chapter 4.2.1. A Nb_2O_5-coated wafer was dried on a hot plate (Goller Reinraumtechnik) for 2 min at 115°C. Then 1.8 ml of photoresist (S1818, Shipley) was applied on the wafer and spincoated for 40 sec. (speed: 4000 rpm, acceleration: 4000 rpm/s). The spincoating was followed by a soft bake (temperature: 115°C for 2 min). An illumination step was then carried out on a maskaligner (MA6 from Karl Süss, lamp power: 500 Watt, illumination time: 7-10 sec). For this positive photoresist the illumination doesn't lead to a crosslinking of the photoresist but a destruction of the chemical bonds of the exposed parts. The adjacent developer bath removes then the photoresist exposed to UV light. For the developer

bath a mixture of water and Microposit 315 developer at a ratio of 5 : 1 was used. The developing lasted 45 sec and was followed by an additional water bath to removed the developer from the mould. The resulting prepatterning of the MAPL-chip is shown in the following figure:

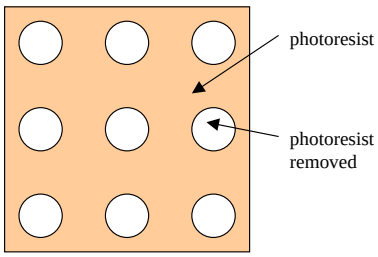


Figure 19: MAPL-chip after prepatterning

Before the MAPL-patterning was performed the wafer was cut in 2 x 2 cm pieces with a wafer-dicing machine (ESEC, Switzerland).

MAPL-Patterning

In a first step the 2 x 2 cm chips were cleaned. Therefore they were placed vertically in a glass beaker which was previously cleaned with Piranha solution. The beaker was filled with ultra pure water (Millipore) and placed in the ultrasound bath for 5 min. Thereafter the chips were dried under a nitrogen stream and then cleaned in an oxygen plasma (PDC-23G from Harrick Scientific Corporation) for 10 sec to remove residual organic contaminants. After the plasma treatment the samples are placed on a parafilm in the flow box and a drop of PLL-*g*-PEG-biotin (0.1 mg/ml in Hepes 2) was applied onto the samples for 40 min. The samples were then rinsed with ultra pure water and dried under a nitrogen stream.

Subsequently the photoresist was removed from the samples. Therefore NMP (N-methyl-pyrrolidone for peptide synthesis from Fluka) was flushed over them for 5 sec. The samples were then vertically placed in a Piranha solution-cleaned beaker filled with NMP and subsequently ultrasonicated for 2 min. The NMP in the beaker was

replaced by fresh NMP and the samples were ultrasonicated for another 2 min. Then they were transferred to a new beaker filled with a mixture of NMP and ultra pure water at a ration of 1:1 and were sonicated for 1 min. Subsequently they were rinsed in an agitated bath of ultra pure water for 5 min. After a final rinse with ultra pure water they were dried under the nitrogen stream.

In the last step of the MAPL-patterning the bare surface of the samples were backfilled with PLL-*g*-PEG. Therefore the samples were placed on a parafilm in the flow box and a drop of PLL-*g*-PEG solution (0.1 mg/ml in Hepes2) was applied on them for 40 min. After a final rinse with ultra pure water and subsequent drying under a nitrogen stream the final MAPL-chips were obtained.

4.2.3 Assembly of the Microfluidic Device

To obtain a working microfluidic device the PDMS replica must be sealed to a flat object, for example a cover slip or a MAPL-chip. Due to a small contacting surface and a lot of mechanical stress from the weight of the tubes the reversible sealing by Van der Waals forces was insufficient. Therefore irreversible sealing was tried to obtain according to the process described in chapter 4.1.1. But in most of the cases it occurred that the PDMS replica did not seal at all to the cover slip or just partially. Even changing the exposure time in the oxygen plasma and applying pressure during connecting did not improve the sealing. Thus another method was tested: Sealing of the PDMS replica to a coverslip by pressure. For this reason a special device was constructed, which is shown in Figure 20. The method of sealing by pressure worked very well and was therefore used to carry out all adjacent described experiments.

Figure 20: Device to seal flow cell by pressure

Figure 21: Pressure device with installed flow cell

The preparation process preceding the experiments and the execution of the experiments are described in the following paragraphs:

1. Preparation of the flow cell:

At the ends of the channels of the PDMS replica holes (diameter: 3 mm) were punched. The flow cell was then cleaned with ultra pure water, dried with nitrogen and exposed to air plasma for 30 sec to render the surface hydrophilic. Subsequently the flow cell was placed on a cover slip or MAPL-chip and then installed in the pressure device so that the hose couplings lay above the punched holes (Figure 21).

1. Connecting of the flow cell to the surrounding device:

The flow cell was connected to the surrounding device, which consists of all the tubings and valves (see Fig. 22 - 23).

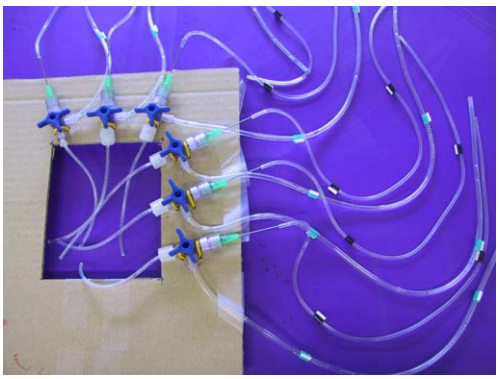


Figure 22: Surrounding device

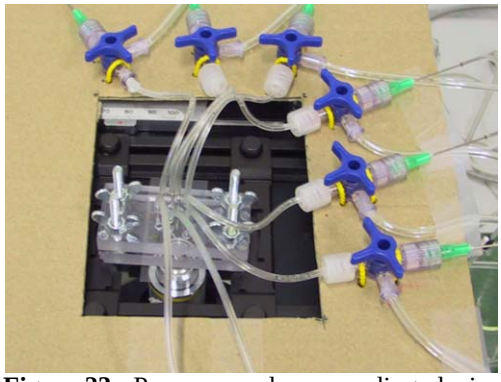


Figure 23: Pressure and surrounding device placed on the microscope

1. Filling of flow cell:

The flow cell and the surrounding device were both placed on the microscope (Fig. 24). The whole microfluidic device was then filled up with water or buffer from the outlet side with a syringe. Then the filling is checked with the microscope to ensure that no air bubbles remain in the channels.

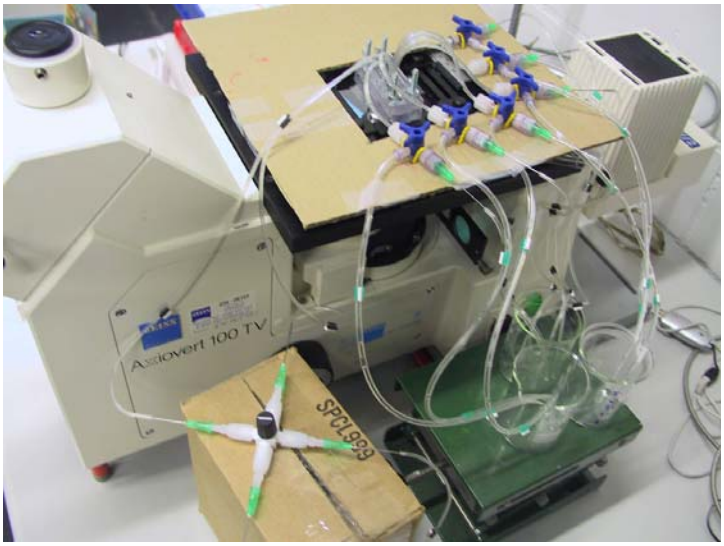


Figure 24: Final setup of the microfluidic device

1. Execution of the experiments:

The experiments were performed on the final setup by using gravitational flow. In a first step the big green marked tubes are filled with 400 ml of the required protein solution. Then the tubes filled with protein were placed in a beaker filled with Hepes 1. The difference in height (see Fig. 25, ΔX) of the buffer level of the beaker generating the protein flow and the outlet was 12.5 cm. The buffer level of the beakers generating the buffer flow was a bit heightened compared to the buffer level of the protein flow generating beaker (ΔY = 0.5 cm, see Fig. 25). This was done to prevent an overlapping of the different stripes of adsorbed proteins in one channel. After the adjustment of the buffer level in the beakers the flows through the flow cell were started by opening the valves of the surrounding device. After the whole amount of reagent run through the flow cell the following buffer flow was continued to rinse the device. After swithching to the perpendicular channel the procedure was repeated.

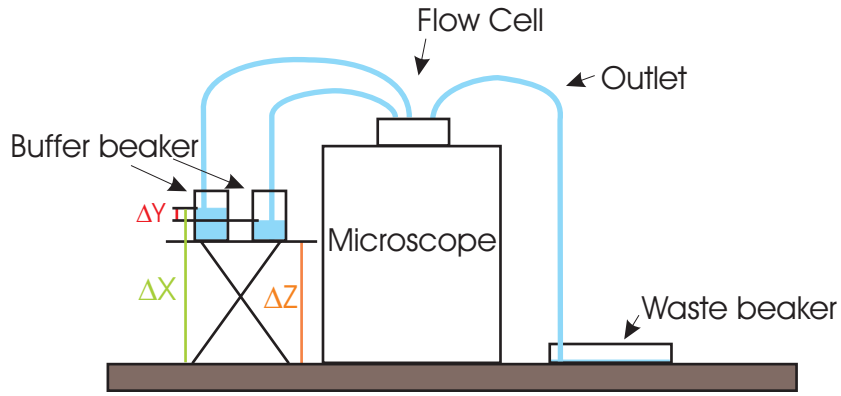


Figure 25: Schema of the final design

4.2.4 Testing of the Microfluidic Device

The following tests have been performed on the microfluidic device:

1. Flow rate measurements
1. Function test with food colours
1. Function test with fluorescently labelled proteins

Flow Rate Measurements

The microfluidic device was calibrated by measuring the flow rate in function of the height difference between the water level in the beaker and the outlet (ΔX, see Fig. 25). Therefore the height of the water level in the beaker was maintained at 2 cm and the height of the table (ΔZ) was varied. The weight of water flowing through the microfluidic device during 120 sec was measured and the flow rate in ml/h was calculated.

Function Test with Food Coloring

In the first function tests the width of the middle laminar stream as a function of ΔY (in Fig. 25) was determined. Therefore the water level in the beaker generating the external streams was maintained at 2 cm/ 8 cm and the water level generating the middle streams was varied in height by adding or

removing water. This experiment was observed through a light microscope
In a second experiment the switching from one channel to the other was tested and a movie from this tests was recorded.

Function Test with Fluorescent Labelled Proteins

In a first experimental series simple structures as for example two perpendicular stripes of adsorbed fibrinogen were tried to obtain. The geometries were analysed using a fluorescent microscope.

The goal of the second experimental series was to produce a chessboard pattern with streptavidin Alexa Fluor 633, biotin 4-fluorescein and biotin IgG on a PLL-*g*-PEG-biotin coated cover slip or a MAPL-chip (see Fig. 26 and 27). By attempting this result the feasibility of the main purpose, namely to functionalize nine spots on a MAPL-chip differently, is proved.

The red stripes on the surfaces in Fig. 26 shows, where the fluorescent streptavidin has bound to the surface and the brown colour indicates the areas where fluorescent biotin (green) bound to the streptavidin. After the tests were performed the samples were analysed with a confocal laser scanning microscope (LSM 510 from Zeiss).

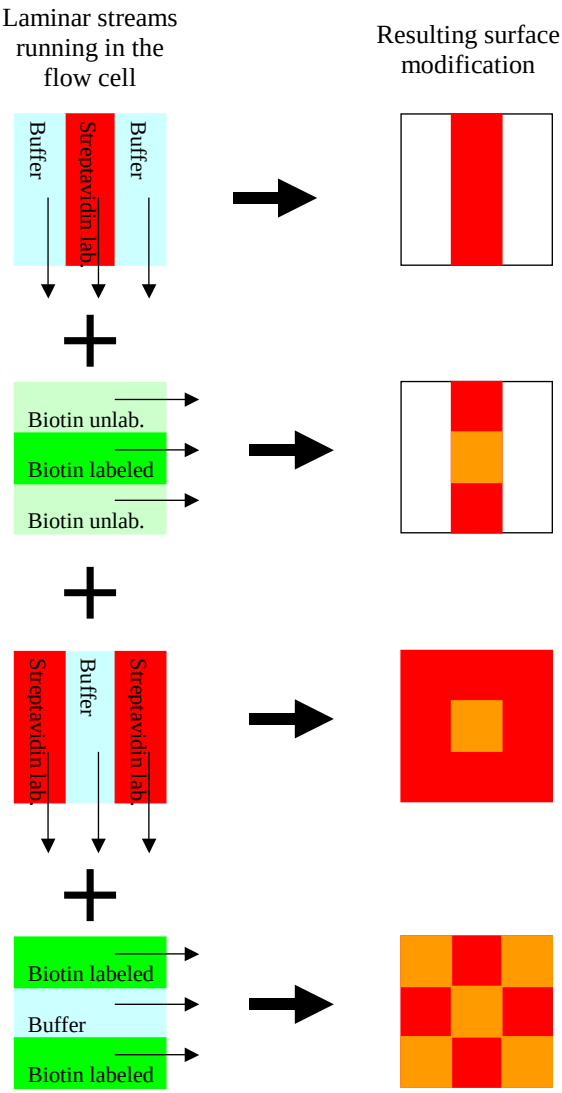


Figure 26: Procedure to manufacture a chessboard pattern

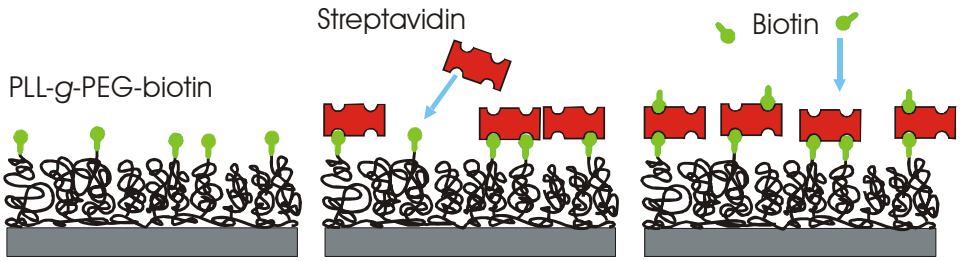


Figure 27: Binding mechanism of streptavidn and biotin

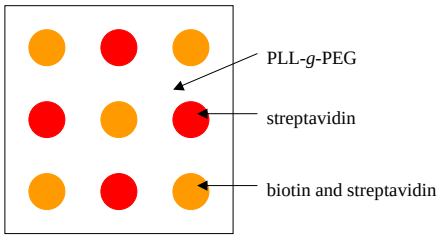


Figure 28: Chessboard pattern of nine spots on a MAPL-chip

5 RESULTS AND DISCUSSION

5.1 Flow Rate Measurements

The results of the flow rate measurements are shown in the following graph.

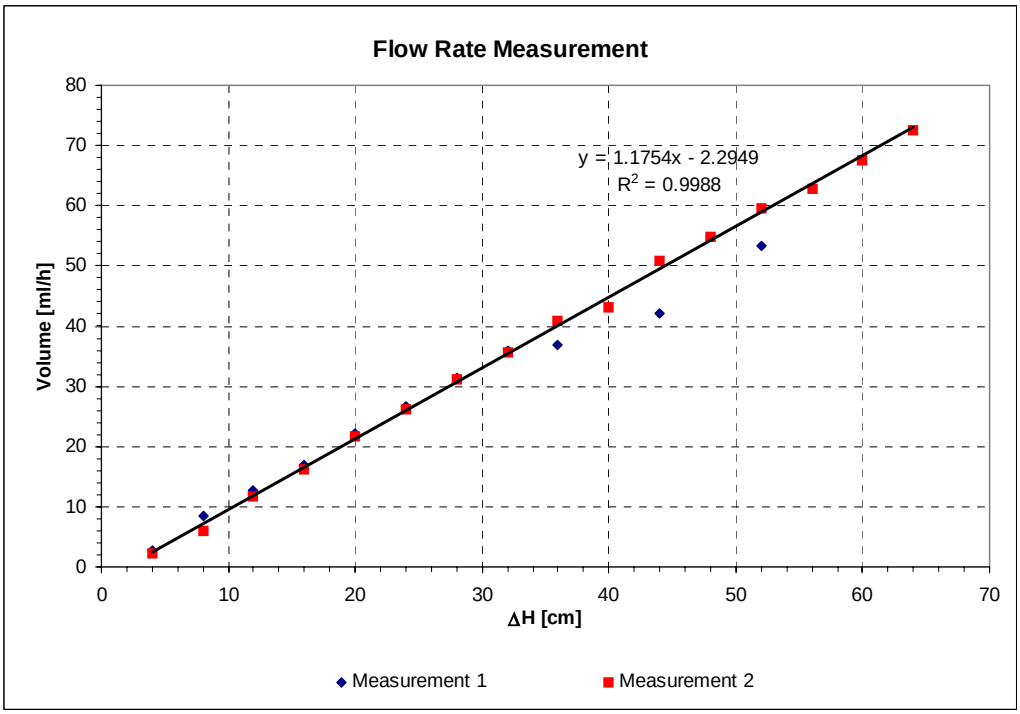


Figure 29: Graph of flow rate measurements

The obtained curve is linear as expected. The question came up if these results are reproducible. Probably the reproducibility is not really given because it is difficult to apply always the same force with the pressure device thus influencing the height of the channels and consequently the flow rate.

5.2 Function Test with Food Coloring

Figure 30 shows the variation of the width of the middle stream in dependence of the difference of the water level in the middle and the external streams generating beakers. In this first experiment the water level of the external flow was maintained at a height of 2 cm. Due to the effect of extension of the flow in the cross section of the channels the stream width was always determined in the channel.

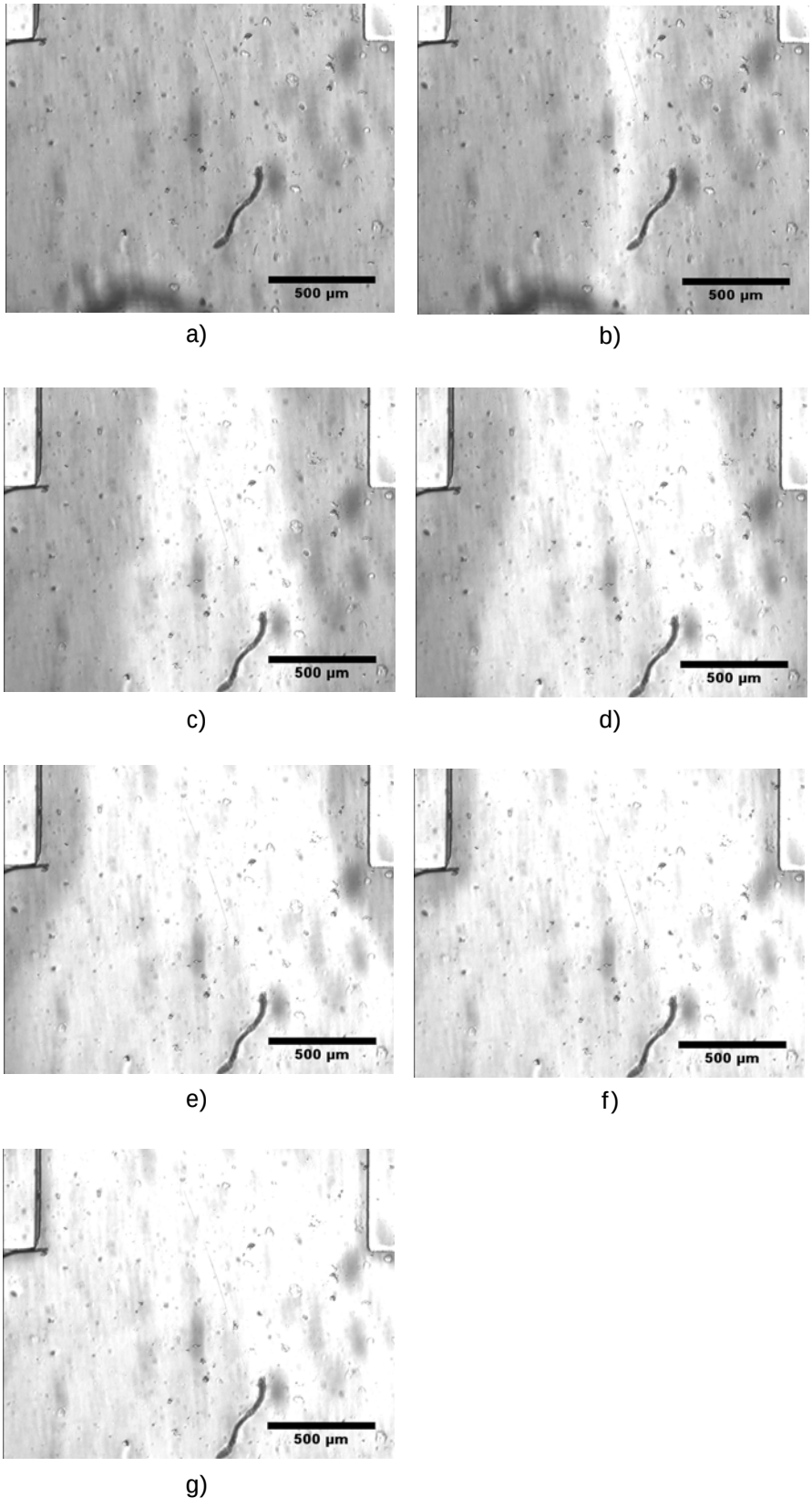


Figure 30: Variation of the width of the middle stream is shown in the cross section area of the microfluidic device. The water levels of the external streams were maintained at 2 cm above the outlet while varying the water level of the middle flow from a) 1cm b) 1.5 cm c) 2 cm d) 2.5 cm e) 3 cm f) 3.5 cm to g) 4cm.

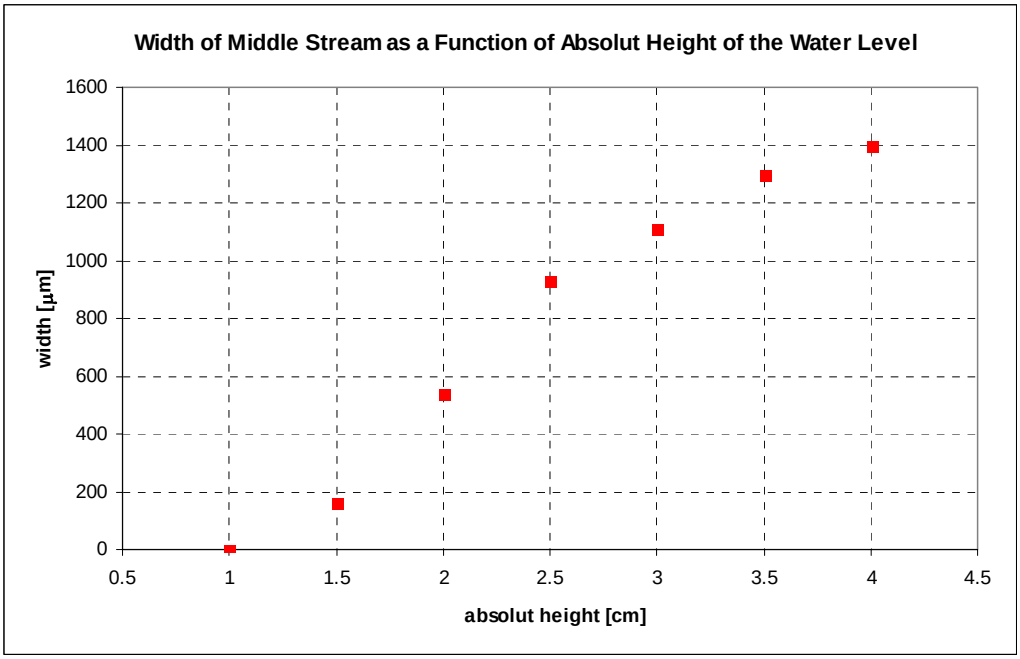


Figure 31: Width of the middle stream as a function of height difference of the water level in the middle and the external streams generating beakers. Water level hight of external streams: 2 cm.

As Figure 30 shows the width of the middle stream can easily be varied by increasing or decreasing the water level in the beaker generating the middle flow. Furthermore the images point out that the middle flow widens up in the cross section of the channels. This effect indicates pressure loss in the closed perpendicular channel which is possibly due to expansion of the tubes.

The images of the same experiment performed at a water level of the external streams of 8 cm can be found in Appendix A. Figure 32 presents the resultant channel width.

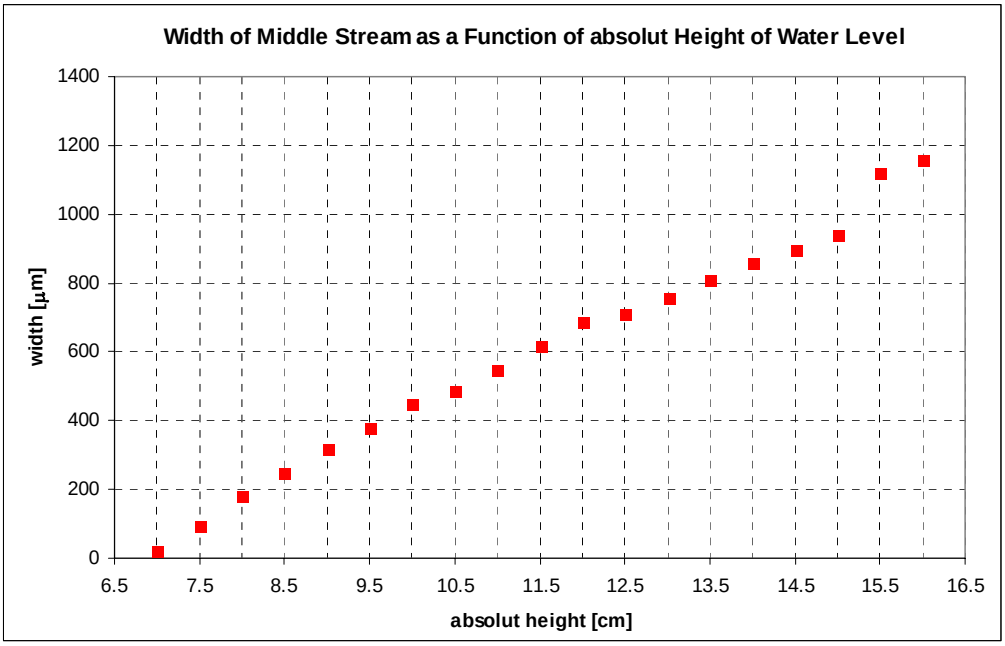


Figure 32: Width of the middle stream as a function of height difference of the water level in the middle and the external streams generating beakers. Water level hight of external streams: 8 cm.

5.3 Function Test with Fluorescent Labelled Proteins

5.3.1 Tests with fibrinogen Alexa Fluor 488

In the following figures the images of the function tests with fibrinogen Alexa Fluor 488 are shown. It must be pointed out that all images were taken after a buffer rinse and such the structures in the images are no longer laminar streams but stripes of adsorbed proteins.

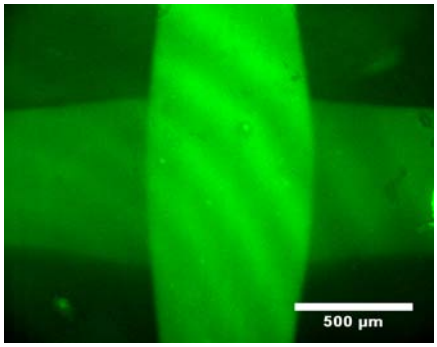


Figure 33: Fluorescence microscope image of two stripes of adsorbed fibrinogen Alexa Fluor 488

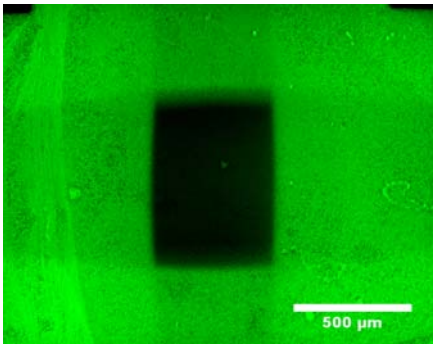


Figure 34: Fluorescence microscope image of stripes of adsorbed fibrinogen Alexa Fluor 488 obtained by conducting two external streams from both direction through the flow cell

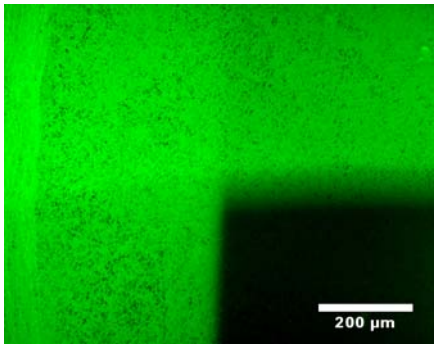


Figure 35: Fluorescence microscope image of the upper left corner of the black square in Figure 34.

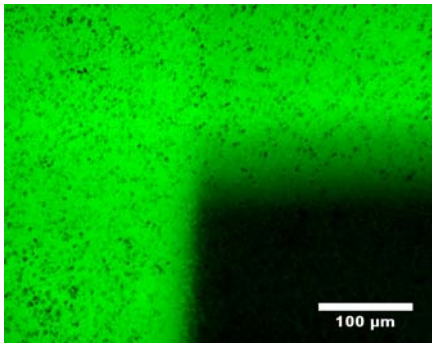


Figure 36: Fluorescence microscope image of the upper left corner of the black square in Figure 34

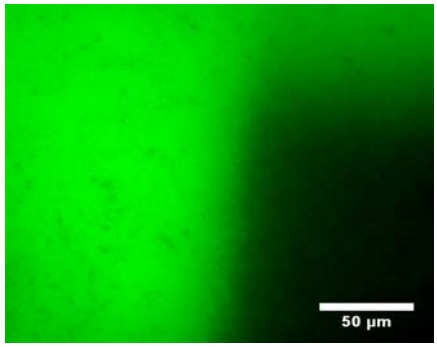


Figure 37: Fluorescence microscope image of the upper left corner of the black square in Figure 34

The images prove that with the constructed microfluidic devices stable streams can be obtained for the period of time of the experiment. It can also be seen that the diffusion area shows an extension of about 10 - 20 mm.

5.3.2 Chessboard pattern performed on PLL-*g*-PEG-biotin coated cover slips

In the following image series the results of the chessboard tests are shown. The red image of the particular image series shows the signal obtained from the streptavidin Alexa Fluor 633, the green gives the signal from the biotin fluorescein and the yellowish brown picture presents the sum of both signals.

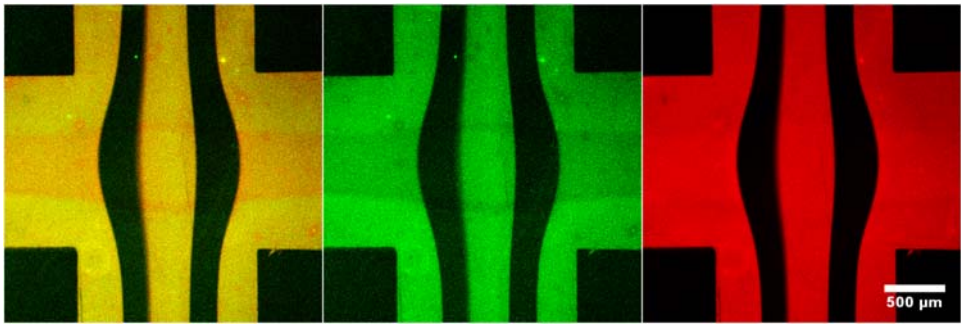


Figure 38: CLSM image in dry state of PLL-*g*-PEG-biotin coated coverslip after chessboard test. Red signal: streptavidin Alexa Fluor 633, green signal: biotin 4-fluorescein and yellow signal: sum of both the red and the green signal.

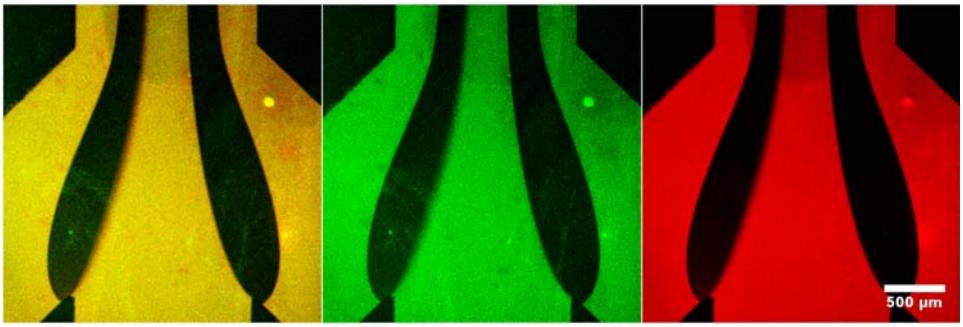


Figure 39: CLSM image in dry state of the area where the three streams come together of the same sample as shown in Fig. 38. Red signal: streptavidin Alexa Fluor 633, green signal: biotin 4-fluorescein and yellow signal: sum of both the red and the green signal.

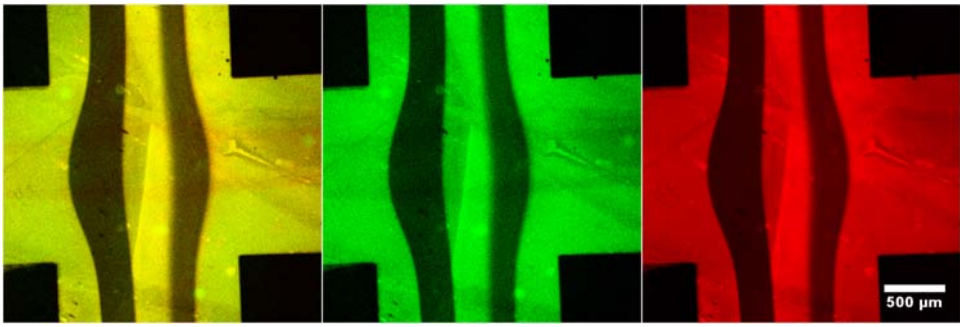


Figure 40: CLSM image in dry state of PLL-*g*-PEG-biotin coated coverslip after chessboard test. Red signal: streptavidin Alexa Fluor 633, green signal: biotin 4-fluorescein and yellow signal: sum of both the red and the green signal.

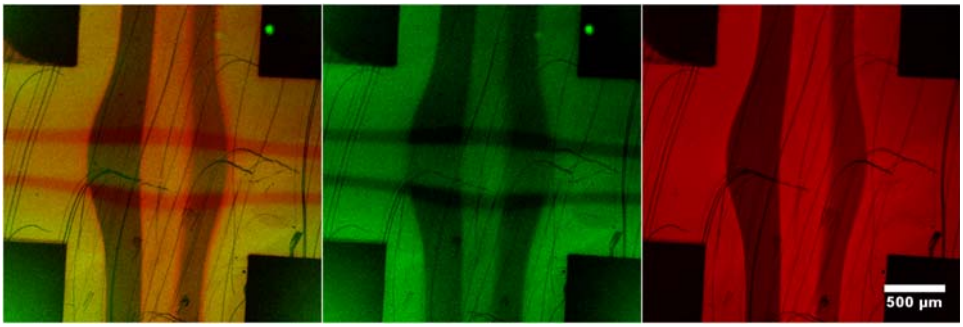


Figure 41: CLSM image in wet state (buffer solution) of the same sample as shown in Fig. 40. Red signal: streptavidin Alexa Fluor 633, green signal: biotin 4-fluorescein and yellow signal: sum of both the red and the green signal.

Figure 38 - 41 confirm that the microfluidic device works because in the red channel the different stripes of bound streptavidin can be clearly distinguished. The black stripes between the streptavidin stripes is the area, where streptavidin never came into contact with the surface. The reason for this black stripes is the difference in height of the buffer level between the beaker generating the buffer stream and the one generating the labelled streptavidin stream.

To the green biotin 4-fluorescein signal different things have to be mentioned. Firstly the bandpass filter did not work for the 5x objective with which the images were taken. For this reason the green images do not only show the signal of the fluorescently labelled biotin but also a signal generated from the streptavidin Alexa Fluor 633. Therefore it is very difficult to draw quantitative conclusions from these images. For example it is difficult to conclude if the blocking of the binding sides with unlabelled biotin did work. A second point to mention is that the obtained signal was found to be very weak. One explanation of this effect is, that the emission of fluorescein is pH dependent as shown in the Fig. 42. This signifies that images always have to be made under wet conditions. This lead to great improvement of the biotin signal as it is visible in Fig. 41. Other effects reducing the biotin signal are the quenching and the fact, that the PLL-*g*-PEG-biotin chains are flexible and therefore can bind to more than two of the binding sites of the streptavidin

molecule resulting in a considerable loss of free binding sides for the fluorescently labelled biotin [36] (figure 44).

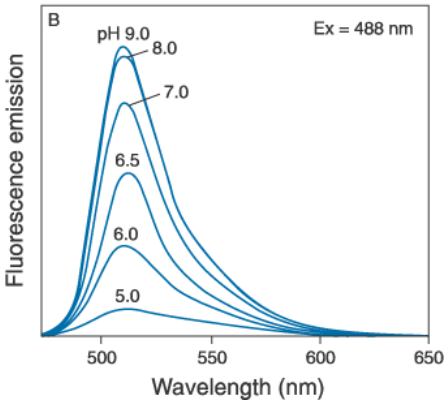


Figure 42: pH dependence of the fluorescein label

Figure 43: Effect that leads to a considerable reduction of binding sides for the labelled biotin

5.3.3 Chessboard tests performed on a MAPL-chip

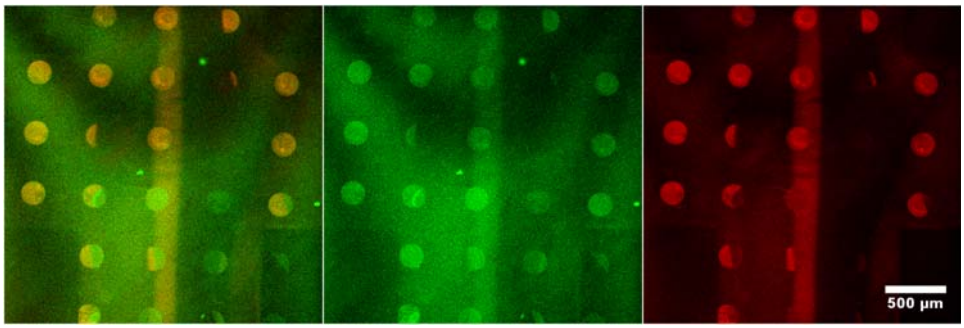


Figure 44: CLSM image under dry conditions of a MAPL-chip after chessboard test. Red signal: streptavidin Alexa Fluor 633, green signal: biotin 4-fluorescein and yellow signal: sum of both the red and the green signal.

Fig. 44 shows that the chessboard test on the MAPL-chip did not work. Nevertheless the images point out that unspecific adsorption of streptavidin on PLL-*g*-PEG took place indicating a need for an opimized patterning protocol.

6 CONCLUSION AND OUTLOOK

In this work it has been shown that soft lithography is an appropriate technique for the fabrication of flow cells. It has been pointed out that the sealing of PDMS to glass causes problems although it does not emanate from literature. Furthermore it has been demonstrated that the width of the streams and their position can easily be adjusted by varying the height of the water level in the stream generating beaker. It has also been shown that the concept of doing local functionalisation on a prepatterned surfaces with microfluidic devices works. Moreover this work has been called attention to problems with the biotin/streptavidin system and to difficulties (fluorophor crosstalk) occurring when working with two different fluorescent labels in one experiment.

Further work should include more chessboard tests performed on MAPL-chip with unlabelled streptavidin and fluorescently labelled biotin. Then the microfluidic device could be applied to other biochemical systems for example biotinylated vesicles, biotinylated antibodies or to the Ni^{2+} histag system on PLL-*g*-PEG NTA. An application of this system is the purification of proteins by fusing tags to proteins and arraying them on Ni^{2+} chelating surfaces [16].

Vesicles are used for drug delivery studies [37] and can contain membrane proteins. Thus a microfluidic device has a great potential to create vesicle arrays without the danger of drying.

A possible application of a micro array of biotinylated antibodies is the micro-immunoassay in which an array of different capture antibodies is produced and subsequently exposed to a biological sample. Analyte proteins bind to the immobilized capture agents and are then detected by fluorescence, luminescence etc. [16].

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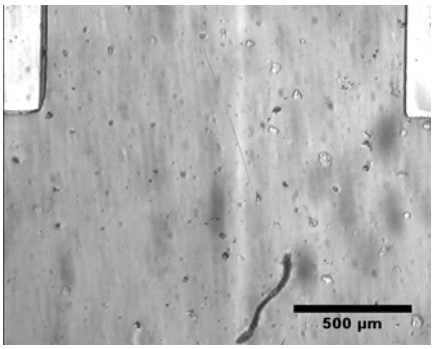
9 APPENDIX

A Results of function test with food colours



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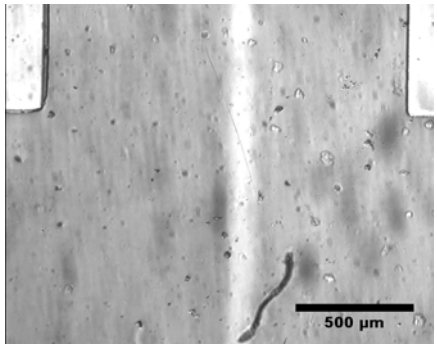

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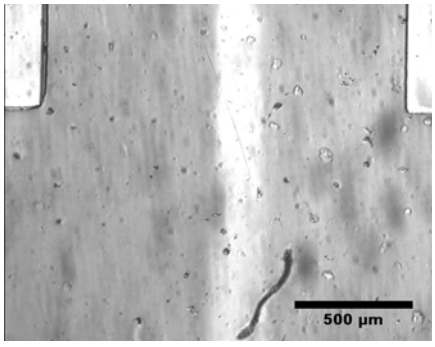

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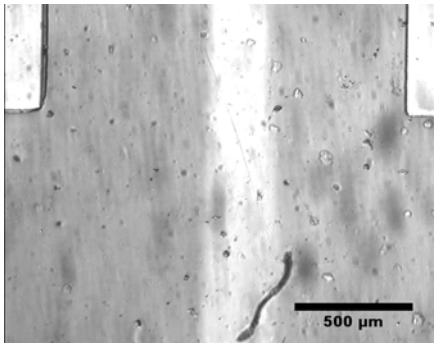

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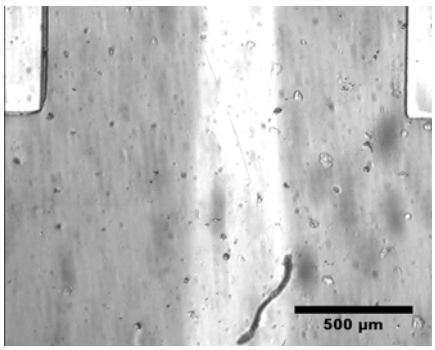

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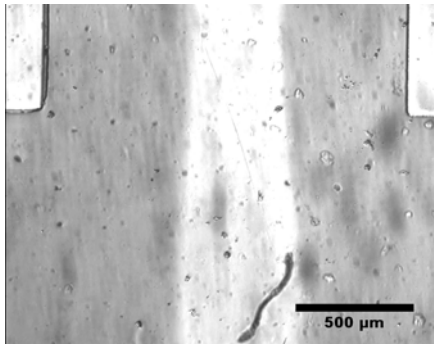

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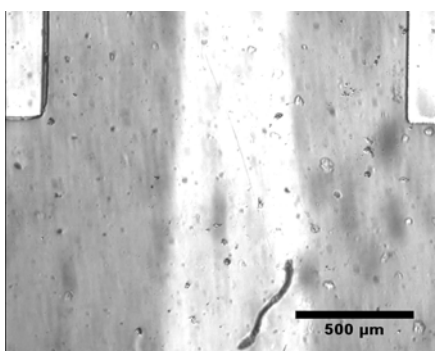

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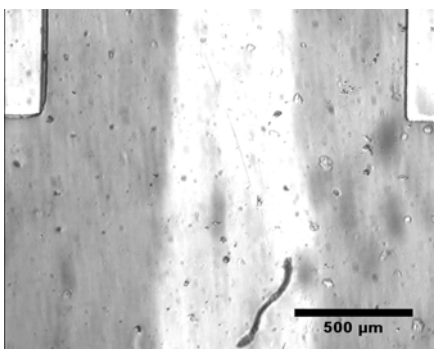

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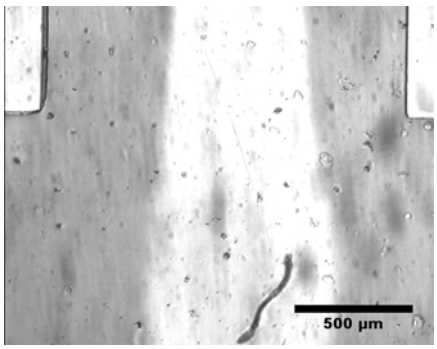

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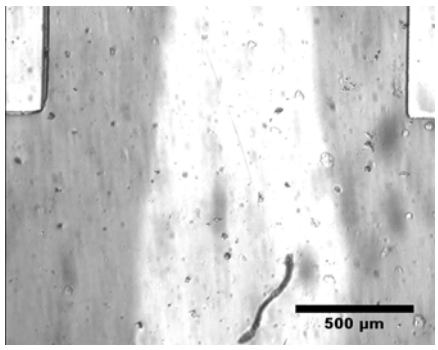

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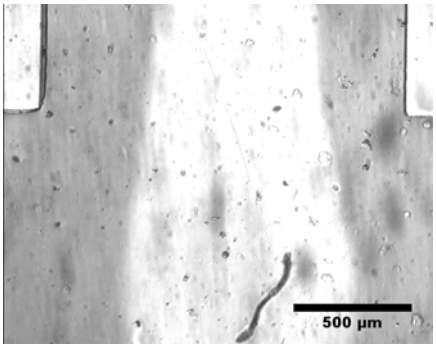

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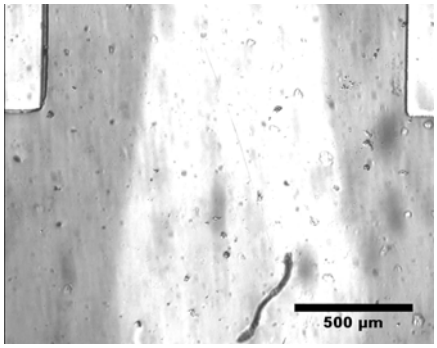

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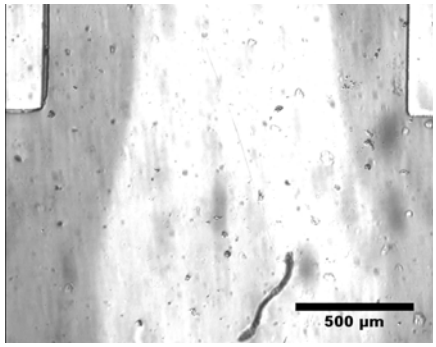

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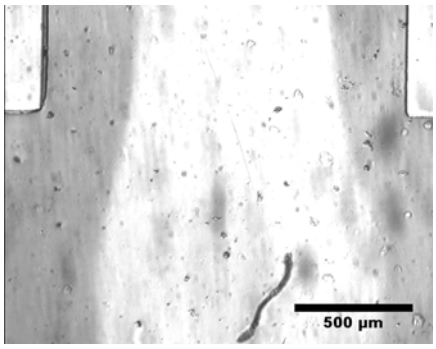

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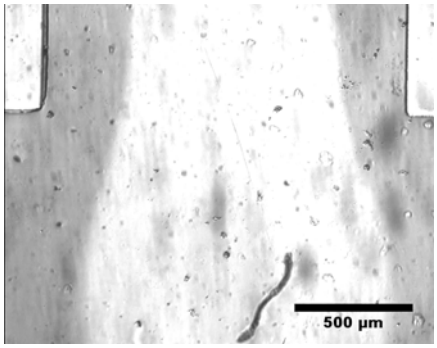
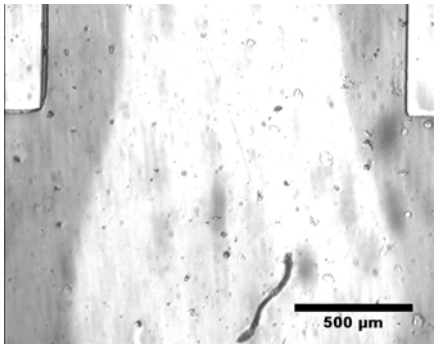


o)



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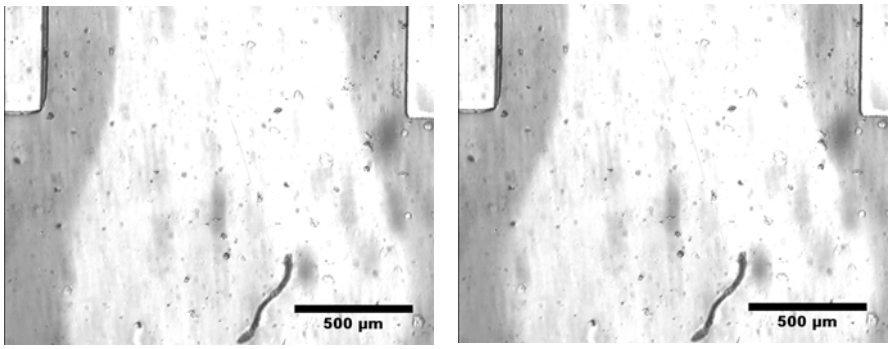

q) r)

Figure 45: Variation of the width of the middle stream is shown in the cross section area of the microfluidic device. The water levels of the external flows were maintained at 8 cm above the outlet while varying the water level of the middle flow from a) 7 cm b) 7.5 cm c) 8 cm d) 8.5 cm e) 9 cm f) 9.5 cm g) 10 cm h) 10.5 cm i) 11 cm j) 11.5 cm k) 12 cm l) 12.5 cm m) 13 cm n) 13.5 cm o) 14 cm p) 14.5 cm q) 15 cm to r) 15.5 cm

B Photomask

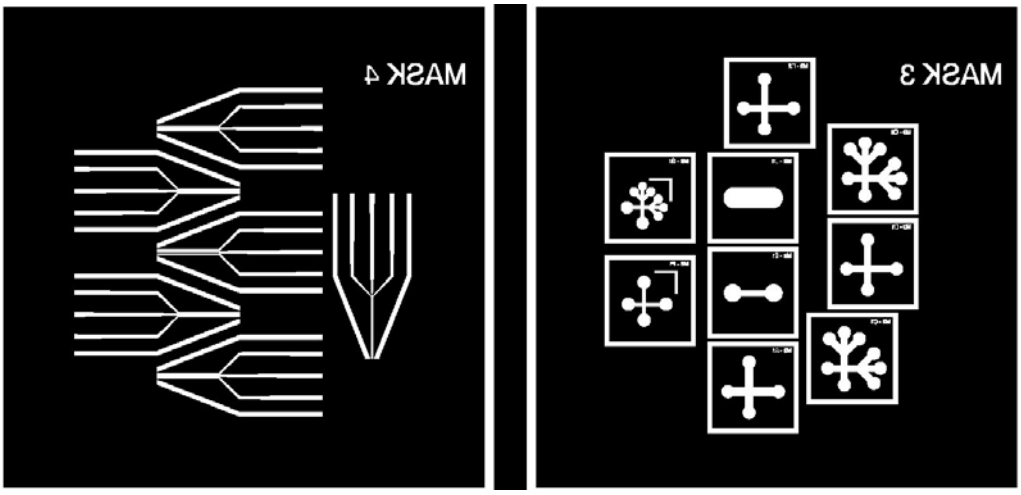

Figure 46: Photomasks used for the fabrication of the flow cell

C Fabrications protocols

i) Protocol of the fabrication of the mould

1. Cleaning: Put silicon wafer in the plasma cleaner
   Time: 10 min, power: 600 Watt
   Plasma cleaner: P10 from Tencor
2. Spincoating: Place wafer on the spincoater, apply photoresist, start spincoating
   Amount of photoresist: 3 ml
   Spincoating: With 200 rpm/s to 400 rpm, hold for 15 sec, then with 200 rpm/s to 1500 rpm, hold for 25 sec. Work with closed lid.
   Spincoater: RC 5 GYRSET from Karl Süss
3. Prebrake: Put wafer on hot plate
   Starting temperature: 50°C for 5 min
   Ramping: 37%
   End temperature: 100°C for 60 min
4. Illumination: Install mask (foil containing structures clued on a glass plate) in the maskaliner
   Place wafer in the maskaliner
   Required energy: 400 J/cm^2
   Power: 275 Watt,
   Soft contact
   Maskaligner: MA6/BA6 from Karl Süss
5. Postbake: Put wafer on hotplate
   Start temperature: 60°C for 5 min
   Ramping: 37%
   End temperature: 95°C for 45 min
6. Developing: Develop wafer in different developing baths:
   GBA developer: 2 min
   PGMEA developer: 21/2 min
   PGMEA rinser: 1 min
7. Final cleaning: Isopropanol, drying with nitrogen

ii) Protocol of the MAPL-chip fabrication:

Prepatterning:

1. Drying: Put Nb_2O_5 coated glass waver on hot plate
   Time: 2 min
   Temperature: 115°C
   Hot plate: Goller Reinraumtechnik

2. Spincoating: Place dried waver on spincoater, add photoresist, start spincoating
   Photoresist: S1818 from Shipley
   Amount: 1.8 ml
   Time:40 sec
   Velocity: 4000 rpm
   Acceleration: 4000 rpm/s
   Spincoating: Goller Reinraumtechnik

3. Soft bake: Put spincoated waver on hot plate
   Time:2 min
   Temperature: 115°C

4.Illumination: Install mask in maskaliner
   Place waver on maskaliner
   Time: 10 sec.
   Lamp power: 500 W
   Maskaligner: AL62 from Electronic Vision Company

5. Developing: Put waver in developing bath
   Developer: It is mixed with water at a ratio of 1 : 5.
   Time: 45 sec
   Developer: 315 from Microposit
   Rinsing: In ultra pure water for 5 min

6. Drying: On spincoater for 40 sec.

MAPL-Patterning:

PLL-g-PEG - biotin Adsorption:

1. Cleaning: Place sample in piranha solution cleaned beaker, fill it up with ultra pure water, put it in ultrasound bath for 5 min. Dry sample under nitrogen stream, put it in O_2-plasma for 10 sec.
   Plasma cleaner: PDC-23G from Harrick

2. PLL-g-PEG-biotin adsorption:
   Put sample on parafilm in flow box, add PLL-PEG biotin, let it adsorb.
   Amount: Enough to cover the whole sample
   Adsorption time: 40 min

PLL-*g*-PEG biotin 42%: 0.1 mg/ml Hepes2
Flow box: VSA 180 from Skan

3. Cleaning: Rinse sample under ultra pure water flow

Photoresist Lift-Off:

1. Lift-off: Fill beaker with NMP (N-methyl pyrrolidone for peptide synthesis)

Hold sample on edge, flush NMP homogeneously on it.
Time: 5 sec

Place sample in beaker, put beaker in ultrasound bath
Time: 2 min
Ultrasound bath: 5210 from Branson

Fill new beaker with NMP, transfer sample to new beaker, put beaker in ultrasound bath.
Time: 2 min

Fill new beaker with NMP and ultra pure water at a ratio of 1:1, transfer sample to new beaker, put beaker in ultrasound bath.
Time: 1 min

2. Cleaning: Put sample in ultra pore water bath
Time: 5 min
Rinse sample under ultra pure water flow, dry it with N_2 stream

Backfill, Second PLL-g-PEG Adsorption

1. PLL-g-PEG adsorption:
Put sample on parafilm in the flow box, add PLL-PEG, let it adsorb.
Amount: Enough to cover the whole sample
PLL-g-PEG: PLL(20)-g(3.4)-PEG(2), 0.1mg/ml Hepes 2
Time: 40 min

2. Cleaning: Rinse sample under ultra pure water flow, dry it with N_2 stream