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Development of a Platform for Lateral Flow Test Devices with the Capability of Using Multiple Fluids

Wilke Föllscher
University of Rhode Island, wfoellscher@my.uri.edu

DEVELOPMENT OF A PLATFORM FOR LATERAL FLOW TEST DEVICES WITH THE CAPABILITY OF USING MULTIPLE FLUIDS

BY

WILKE FÖLLSCHER

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WILKE FÖLLSCHER

APPROVED:

Thesis Committee:

Major Professor Mohammad Faghri

Constantine Anagnostopoulos

Keykavous Parang

Nasser H. Zawia
DEAN OF THE GRADUATE SCHOOL

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ABSTRACT

This study presents the development of a 3-fluid microfluidic device for the application in immunoassays. The test uses a microfluidic valve in order to sequentially load the reagents autonomously onto the detection area after adding the sample. The development of the multi-fluid circuit allows the application of an enzyme-linked assay in a lateral flow device as to provide with an improved sensitivity compared to strip tests available on the market. For fabricating the channels of the device, a wax printer was used. The layers were attached using double-sided tape. In order to advance the reliability of the device, new fabrication method were applied in this study. After optimizing parameters such as reagent concentrations, reagent volumes and the dimensions of the device, a calibration curve using rabbit IgG was created. The limit of detection was then obtained. Furthermore a housing for the device was developed. By compressing the microfluidic valve, an improved reliability of the valves was obtained. For the goal of an autonomously running device, a reagent storage was incorporated into the housing. The reagent storage provides with the ability to operate the test without additional interference after adding the sample. Based on the results of this study, an improved lateral flow test was obtained. The developed 3-fluid device using an enzyme-linked assay is able to detect rabbit IgG down to a concentration of 4.7 ng/ml. This results in a limit of detection better than in a conventional ELISA conducted on microtiter–plates (8.6 ng/ml). Advantages such as lower reagent volumes, lower time to result and biodegradability of materials used during the development of this device were achieved.

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CHAPTER 1 - INTRODUCTION

Over the last few decades, lateral flow immunoassays (LFIA) have become very important tools for a wide range of diagnostic applications. The reasons for their use include sensitivity, suitability for the detection of a variety of different substances, and a relative short time-to-result. As a result, these devices can be used in resource-poor settings, near-patient and non-laboratory environments.

In 2005, over-the-counter and professional lateral flow devices achieved global revenue of $1.88 billion [33]. In 2010, this number increased to $2.73 billion. As of today, lateral flow tests are used to detect pathogens, drugs, hormones and metabolites in humans, in animals, in food and in the environment. Because of their ability to detect a wide range of substances, these tests are not only used by people with certain disorders or food allergies, but they are also used in hospitals and police stations where a yes/no-result is needed. Nevertheless, an improved LFIA device is desirable to provide the detection of additional biomarkers, and give faster, more reliable, and quantitative results [33, 34, 42].

This study focuses on the design of a new paper-based lateral flow test device capable of using multiple fluids. The overall goal of the proposed study is to develop a portable multi-fluid system which autonomously runs the assay after adding the sample fluid. This study entails the integration of the paper-based microfluidic valve technology (PBMV) developed in Prof. Faghri's laboratory [7]. The study will advance the application of PBMV technology to develop strip tests with superior sensitivity and functionality compared to existing test strips available on the market.

The first part of the problem is to develop a lateral flow test device on paper. This device must be able to handle multiple fluids. For the application in Point-of-Care diagnostics, it is desired that only one reagent (the sample) is utilized to start the test. This means that the actual test is completed once a certain amount of sample is added on the device. Other reagents, needed for further reactions in the test, must have been previously incorporated in the device. The core of the system is a valve which holds the reagents at a certain position. Using this valve, a fluidic circuit can be designed on paper, which can control the flow of two or more fluids without an operator's intervention. Since every reagent in the test needs a certain amount of time for the reaction at the detection spot, a methodology to sequentially load the reagents at a specific point after starting the test has to be developed. The next step is to determine the right amount of reagents required to run the assay. Based upon the number of reagents implemented, the dimensions of the actual system will be determined. It is desirable to miniaturize the device to reduce the volume of sample and reagents needed and for reducing the unit manufacturing cost. At the end, the optimal size of the waste area of the system will also need to be determined. To be safe, this area must be able to hold all of the fluid volume which is passed over the detection spot.

The second aspect of the proposed research is the application of a newly developed method for the fabrication of the devices. Since every device consists of several layers of different materials that need to be prepared and assembled by hand, the assembly is a very time-consuming procedure. Therefore, a methodology to produce many chips at the same time will increase the output and will lower the price of the device. Additionally, a standardized procedure will enhance the reliability of the

chips. A limitation for this part of the problem is the minimum dimensions of the chip which influence the number of chips produced in the same batch. This study will focus on the improvement of the PBMV technology in the new fabrication method.

Another aspect of this study is the application of rabbit IgG in order to prove the suitability of the device for immunoassays. The model analyte rabbit IgG is tested on the device in different concentrations and a calibration curve will be developed as well as the limit of detection for this analyte. A colorimetric method is used in order to be able to see the signal without the need of specialized equipment. The signal will also be digitalized using ImageJ in order to compare the results precisely. Finally, it will be investigated whether the reagents used to fabricate the PBMV affect the sensitivity of the assay.

The last aspect of the proposed study is to develop a housing to hold the test strip and to store the reagents. Most commercially available lateral flow tests contain a housing designed for ease of handling. The incorporation of a housing reduces the risk of misuse by the operator since only the detection spot where the signal develops and the sample pad are accessible. The rest of the test is not of interest to the user and should be covered by the housing. In this case, application of the sample, for example, at the wrong end of the test is not possible. For the purposes of the multi-fluid circuit to be developed in this study, the housing needs to also provide storage capabilities for the reagents needed to run the assay. Moreover, it is desirable that the utilization of the device for the actual test involves little to no manipulation besides the application of the sample.

CHAPTER 2 – REVIEW OF LITERATURE

2.1 Review on Immunoassays

An immunoassay is a biochemical test used to detect the presence or absence of an analyte in a solution. This technique uses the interactions between the analyte itself and an antibody with a high affinity to bind to the analyte (antibody-antigen reaction). The antibody is labeled so that the presence of the desired analyte can be measured. To detect a certain analyte, appropriate reagents have to be chosen along with their reaction schemes. Next, a diagnostic method needs to be chosen in order to test.

In this chapter, an introduction of the most important diagnostic methods, the reagents needed and different kinds of reaction schemes of the analytes are given.

2.1.1 Diagnostic Methods

The current diagnostic market offers a wide variety of tests. The two most important methods are Lateral Flow Immunochromatographic Assays (LFIA) and Enzyme Linked Immunosorbent Assays (ELISA). LFIA can be used in near-patient environments, whereas a conventional ELISA has to be performed in a laboratory.

Lateral Flow Immunochromatographic Test Strips or Lateral Flow tests are simple devices used to detect the presence or absence of a certain analyte in a sample. They can be used without the need of expensive equipment. They are therefore, suitable for home testing, POC-testing and laboratory testing. Typically, LFIA strips consist of a sample pad, a conjugate pad, an absorbent pad and a detection area consisting of test lines. The sample pad is located at the beginning of the strip where

the sample with the analyte (for example hcG to test for pregnancy) is applied. The sample then wicks through the strip and passes through the conjugate pad. On this pad detection antibodies with a high affinity to the target analyte and tagged with a label (mostly colloidal gold- or latex beads) have been deposited and dried during device fabrication. While the sample passes through the conjugate pad, the labeled antibodies are able to bind to the analyte. The fluid then continues wicking through the strip to the detection area. The labeled antibodies and analytes are conjugated to capture antibodies that have been immobilized at the test line during the manufacturing process. At a certain concentration of the conjugate, the signal develops. The control line located after the test line is used to confirm that the test operated correctly. At the control line, antibodies with an affinity to the detection antibodies from the conjugate pad are immobilized. This line, therefore gives a color every time a sample is passing, even if the desired analyte is not contained in the sample fluid. A typical setup of a commercially available LFIA including a housing is shown in Figure 1.

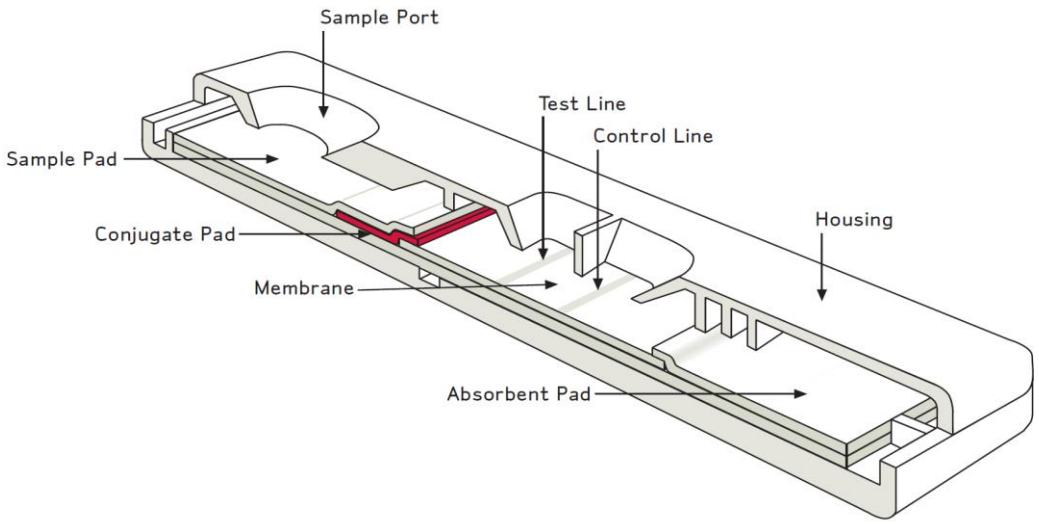

Figure 1: Setup of a commercially available LFIA [29]

The disadvantage of most LFIA on the market is that they only provide a qualitative result. These tests are not very helpful for quantitative assays. Another drawback is that if the sample is not a fluid (e.g. in food testing), a pretreatment is needed to accomplish a test. Additionally, by using an inaccurate sample volume, the precision of such a test can reduce rapidly. Another issue is the possibility of false positive signals, which can be caused by chemicals with a similar structure than the antibody. Also, false negative signals may be present and are caused by using the wrong extraction buffer or improper volumes. This problem also influences the specificity and sensitivity of the respective device. Finally, since most tests are detected visually by non-technical personnel, there is always a component of misinterpretation in the result [6, 34, 36, 42].

Besides LFIA, the current diagnostic market also offers the so called Enzyme Linked Immunosorbent Assays (ELISA). These tests are currently used in hospitals and laboratories. Some of the applications of these tests include the monitoring of antibody responses to vaccination, detection of proteins, hormones and pesticides. Since a traditional ELISA is performed on microtiter plates in a 96-well format, along with certain suitable equipment, these tests can be used to handle high number of samples in short time. Using this method, it is possible to provide not only a yes/no-response as in LFIA, but also a quantitative detection for biological agents since the signal intensity is directly correlated to the antigen concentration. Additionally, ELISA is more sensitive and more specific than a commercially available lateral flow test. A problem encountered with ELISA is the need of highly trained personnel and

expensive equipment. Furthermore, the test itself has to be done at a laboratory and needs at least a few hours to get the results [9, 17].

2.1.2 Biological Reagents in Immunoassays

To perform an immunoassay, a series of different reagents are needed. In this part, the reagents used in immunoassays will be introduced and discussed in detail.

An antigen in immunoassays is mainly the analyte being tested. Antigens are the substance that the immune system tries to eliminate, by mounting an immune response. This response consist of the production of antibodies by the immune system specific to the antigen [10].

Antibodies or immunoglobulins are multifunctional proteins. They are produced by the immune system of vertebrates. They are the essential molecules for fighting an infection caused by antigens (see above). Antibodies are very specific and possess a high affinity to a certain antigen. If an antigen (e.g. a virus) enters the system of a vertebrate, the immune system produces an antibody. This antibody is able to react specifically with the antigen (immune response) and renders it ineffective. This reaction is taken into account during immunoassays. Two kinds of antibodies are used for testing, the capture (or primary) antibody and the detection (or secondary) antibody. Both have a high affinity to bind to the analyte. The primary antibody is typically immobilized at the test line or the walls of the wells in the microtiter plate. Then the detection antibody, tagged with a label, is introduced. The detection antibody also binds specifically to the antigen and the label is used for visualization of the reaction [2, 19].

Antibodies can be produced by injecting an antigen into an animal for which an antibody is desired. Animals used in this procedure include mammals, such as mice, rabbits or goats. After the injection, an immune response (see above) takes place. The immune system automatically produces specific antibodies for the injected antigen. By extracting the blood from the animal, a serum rich in antibodies can be attained. These antibodies will be specific and affine to the antigen, previously injected. In the serum two different kinds of antibodies can be present: (1) Monoclonal antibodies are highly specific for a single epitope on a multivalent antigen (2) Polyclonal antibodies have the ability to bind to different epitopes on an antigen [10].

Labels are typically chemically linked or conjugated to the detection antibody or antigen. For labelling the desired molecule, a variety of different labels are available. The main ones are discussed below [11].

In lateral flow tests, the commonly used labels are nanoparticles. These are usually gold or carbon particles, but colored polymer particles can also be used. One of the main advantages in using nanoparticles is that they don't need additional steps to attain a signal. If enough labeled detection antibodies are present, a change in color can be seen by the naked eye. Also, the particles can be produced in many colors. This makes them suitable for various types of samples. If for example, whole blood is used, a particle, either black or dark blue would be chosen. Furthermore, most particles can be conjugated easily to the desired antibody. Additionally, the particles can be produced in a variety of sizes. This makes them suitable for a wide range of different materials with different pore sizes [29, 42].

One of the most popular labels is enzymes. Enzymes are being widely used in immunoassays, especially in microtiter ELISA. Enzymes include horseradish peroxidase (HRP), alkaline phosphatase (ALP) and glucose oxidase. Using an enzyme always introduces another step to the assay. Since the enzyme itself does not give a detectable signal, another reagent, the substrate, needs to be introduced into the system. When the substrate reacts with the enzyme, a signal is produced either as a change in color, an emission of light, or production of current. One advantage of an enzyme based immunoassay is that they provide superior sensitivity compared to nanoparticles. On the other hand, a disadvantage of an enzyme based immunoassay is that the enzyme conjugates cannot be stored at room temperature for a long time. They also require several washing steps to remove excess conjugates. These reasons make them less suitable for lateral flow immunoassays. But since they provide a higher sensitivity than nanoparticles, current research focuses on the use of enzymes in LFIA [41, 42].

Besides nanoparticles and enzymes, radioactive isotopes are used. The signal is attained by measuring the radioactivity emitted by the conjugate. Because of the need of radioactive material, and due to the improvement in sensitivity of other techniques, the use of this radioactive isotopes method has been reduced in recent years. Other labels used are fluorescent labels, such as quantum dots. This type of label is an emerging class especially for lateral flow immunoassay [11, 42]. To attain even higher sensitivities, current research is focusing on signal amplifying systems. A simple and often used amplification system is the biotin-streptavidin system using enzyme labels.

One advantage of this method is the high resistance against extreme pH values, temperatures, organic solvents and other denaturing agents [11].

2.1.3 Reaction Schemes

In immunoassays the two predominant reaction schemes used are the non-competitive and the competitive format. Both methods are mainly utilized in solid-phase immunoassays. This means, that the primary or capture antibodies are attached or immobilized to a solid phase surface, such as the walls of the dimples in a microtiter-plate [1, 41].

The non-competitive principle for immunoassays is used for analytes with a high molecular weight (e.g. serum proteins or allergens) and multiple antigenic sites such as hcG or HIV. This method is considered to offer the highest specificity and sensitivity of all assay formats. This format can be divided into two subcategories, the one-site and the two-site non-competitive assay. The two-site or "sandwich" immunoassay contains a capture (or primary) antibody immobilized at the desired surface. The sample containing the analyte to be tested is applied on this surface. An immune reaction between the primary antibody and the analyte then takes place, i.e. the two molecules bind. In the next step, a detection (or secondary) antibody is added into the setup. This antibody is tagged with a label (e.g. a colored nanoparticle). Once added, the secondary antibody binds to the analyte forming a sandwich consisting of the primary antibody, the analyte and the secondary antibody with a label. After removing unbound reagents, the amount of labeled antibodies can be measured. Since the secondary antibody will not bind in absence of the analyte, the amount of labeled antibodies is proportional to the concentration of analyte. A simple schematic of the

principle of the two-site "sandwich" immunoassay is shown in Figure 2. The one-site non-competitive immunoassay only needs one antibody, and the detection antibody equipped with a label. Other than in the two-site non-competitive assay, the analyte is immobilized rather than the capture antibody. After introducing the labeled detection antibody into the setup, an antibody-antigen reaction takes place. In the end, the unbound detection antibodies are washed away and the bound labeled antibodies can be measured [1, 4, 10].

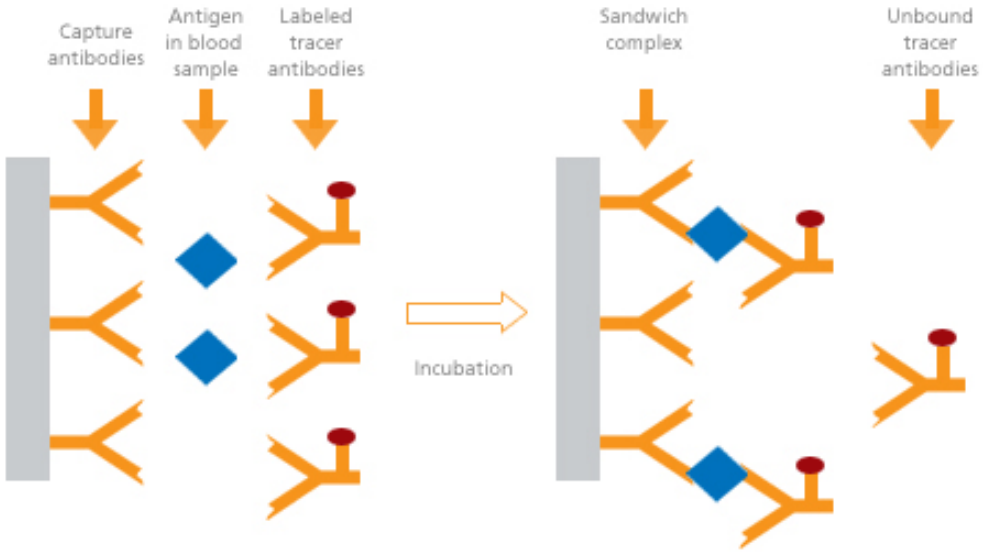

Figure 2: Schematic of principle of non-competitive "sandwich" immunoassays [35]

Since sandwich immunoassays are limited to large molecules, a different method to detect low molecular weight analytes, like drugs or environmental pollutants, is needed. Small molecules, which cannot bind to two antibodies simultaneously, are tested using the competitive format. This method is based on analytes from the sample and labeled analytes competing to bind to immobilized specific antibodies. It is important to know that, in this format, not the detection antibody but the analyte itself is labeled. Detection antibodies are not needed in a competitive immunoassay. The

competitive format can be divided into two categories: (1) the one-step competitive assay and (2) the two-step competitive assay. In the one-step assay the first stage of the reaction is to incubate the sample containing analytes and labeled analytes. Both are added in defined quantities to the setup with the immobilized capture antibodies. In this setup, the labeled and unlabeled analytes are competing for a defined, limited quantity of primary antibodies. After washing away all unbound analytes, the signal intensity can be measured. The difference to the non-competitive format is that the signal intensity is inversely related to the analyte concentration. If no analyte is present in the sample, the labeled analytes are binding to the primary antibodies, giving a high signal. This means, the higher the unlabeled analyte concentration, the lower the developed signal [1, 4, 10]. A schematic of the competitive one-step immunoassay format is shown in Figure 3. The top shows an example of a low analyte concentration, compared to the labeled analyte (high signal). On the bottom an example of a high analyte concentration (no signal) is shown.

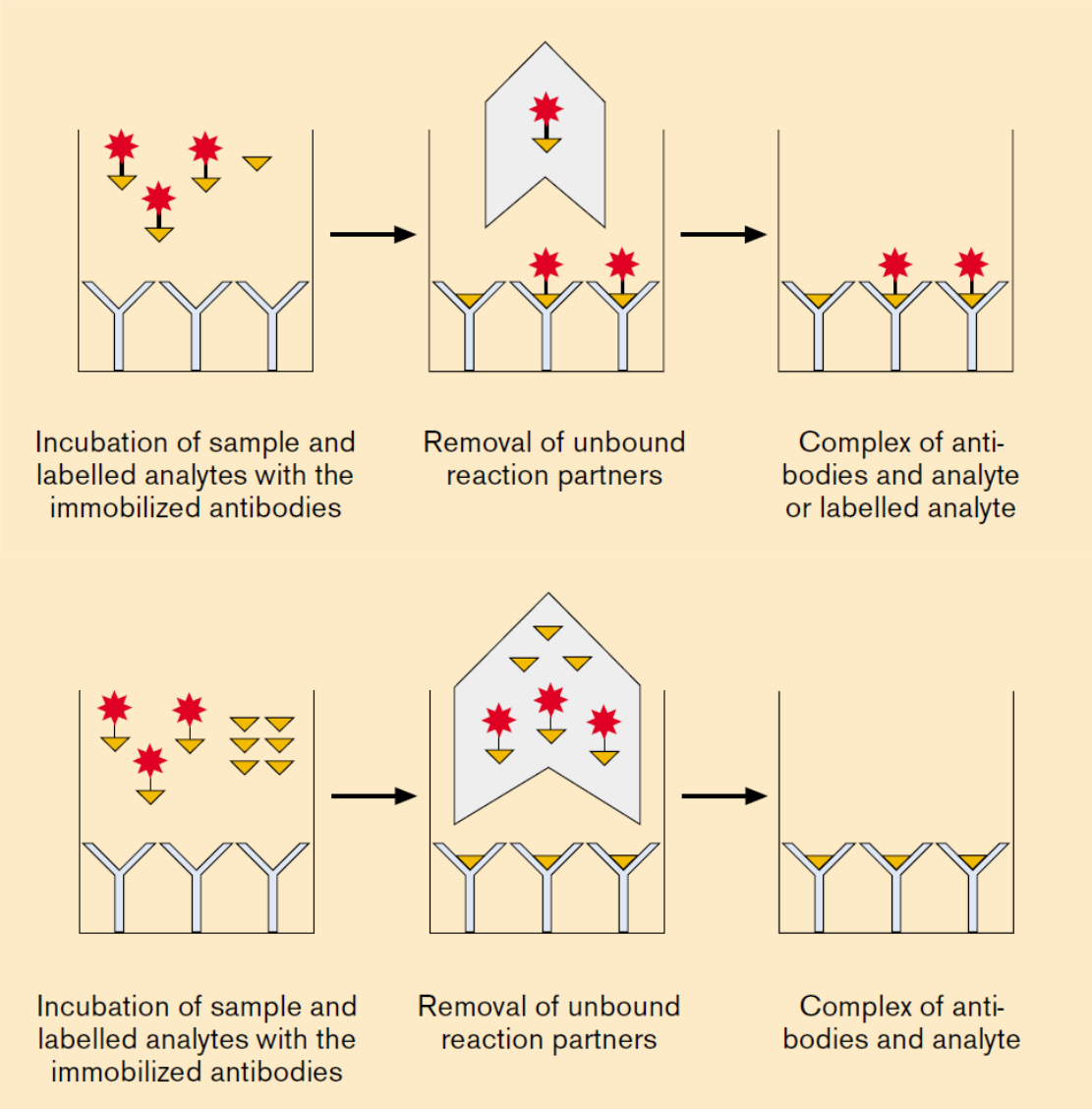

Figure 3: Schematic of principle of one-step competitive immunoassays (Top: example with low analyte concentration; Bottom: example with high analyte concentration) [1]

The two-step method introduces another step but provides improved assay sensitivity. In the two-step method, the unlabeled analyte of interest is first incubated with the antibodies. Note that there is an excess concentration of antibodies presence compared to the analyte present. In a second step, a labeled analyte is added. After removing all unbound reagents, a measurement of the immune complex can be performed similar to the one-step competitive assay [1, 4, 10].

2.2 Current State of the Art in Lateral Flow Test Development

In recent years, the development of lateral flow immunoassays has been focused on exploiting the technique to achieve higher sensitivities and quantitative results on commercially available devices. To improve the sensitivity, some companies have replaced the commonly used colloidal gold beads with fluorescence dyes and paramagnetic particles. For an analysis of these tests, reading devices are needed since these substances cannot be detected by the naked eye. This is not desirable for an easy-to-use POC device, especially in environments such as third world countries or home applications. Besides the need for the extra step of using a reader of some type to see the test result, there are also issues with portability, cost, simplicity of operation and speed of such a test [42].

Because of the desired quantitative results in LFIA, current research focuses on the development of enzyme-based lateral flow immunoassays. At the Hong Kong University of Science and Technology, scientists are working on semi-quantitative enzyme-based LFIA using a bar code like system which is based on the different reaction time on successive lines. Shortly after the start of the test, these lines develop depending on the concentration of the analyte in the sample. At the end, a concentration can be attained by counting the test lines, which are built in a ladder bar format. The more lines that are visible, the higher is the concentration of the desired analyte in the sample. This semi-quantitative way of LFIA is a first step to fully quantitative devices. However, since this test needs a detection area with several test lines each having different reagent concentration, new problems will be introduced regarding manufacturability and cost [13, 14].

2.3 Paper-based Microfluidic Devices

In 2007, Microfluidic Paper-Based Analytical Devices (μPADs) were introduced as a new field in microfluidics. In the past few years, many research groups have focused on finding new ways to fabricate microfluidic devices on paper. μPADs offer simple, fast and low cost fabrication processes. They are also easy to use and require only minimal infrastructure. Used as the main material, paper offers great advantages. Paper by itself is biocompatible and biodegradable and it has good mechanical properties. Moreover, paper is cheap and it is available all over the world. Taking all these advantages into account, μPADs can have a huge impact in the diagnostic market and not only in the developing world [30, 43].

The basic idea of μPads is to pattern hydrophilic-hydrophobic patterns on paper leading to the creation of microfluidic channels. Several techniques have been developed including photolithography, plotting with an analogue plotter, ink jet etching, plasma treating, wax printing, ink jet printing, flexography printing, screen printing and laser treatment. The purpose of all these techniques is to render the channels hydrophilic, whereas the rest of the paper is hydrophobic. The fabrication mentioned above can be categorized into two approaches. The first approach is the selective hydrophobization or one-step fabrication. This category includes wax printing and inkjet printing. This approach deposits hydrophobic agents on selected areas on the paper and the untreated areas remain hydrophilic. A second category is to entirely hydrophobize the paper and then dehydrophobize the selected areas, the channels. The second category needs two steps and therefore requires more time and effort to create the channels. Most of the techniques classified into this category need

chemical modification of the fiber surface for which a chemical reaction between the cellulose fibers and the applied agent takes place. The advantages and disadvantages of the methods named above are shown in Table 1 [20].

Table 1: Advantages and disadvantages for different fabrication methods for µPADs [20]

Fabrication technique	Advantages	Disadvantages
Photolithography	High resolution of microfluidic channels (channel width of 200 µm; sharp barrier)	Requires expensive equipment; requires washing step; devices are vulnerable to bending
Plotting	Patterning agent (PDMS) is cheap; devices are flexible	Poor barrier definition; cannot be readily applied to high throughput production
Ink jet etching	Single printing apparatus to create microfluidic channels by etching required	Complicated process; need of customized printing apparatus; not suitable for mass fabrication
Plasma treatment	Very cheap patterning agent (AKD)	Different masks for different microfluidic patterns on paper required
Wax printing	Produces massive devices with simple and fast (5-10 min) fabrication process	Requires expensive wax printers; heating step after wax deposition required
Ink jet printing	Very cheap AKD; produces massive devices fast and simple; requires only a desktop printer	Requires an extra heating step after AKD deposition; requires modified ink jet printers
Flexography printing	Allows direct production in existing printing houses; no heat treatment needed	Requires two prints of polystyrene solution; requires different printing plates
Screen printing	Produces devices with simple process	Low resolution of microfluidic channels; requires different printing screens for creating different patterns
Laser treatment	High resolution (minimum pattern size of about 62 µm)	Microfluidic channels do not allow lateral flow of fluids; requires extra coating for liquid flow

To select a proper technique, a range of factors need to be considered. These include equipment availability, cost of materials, simplicity of fabrication and the desired application of the device. Presently, ink jet printing and wax printing tend to be the two most promising techniques. They offer low cost patterning agents and a large number of devices can be produced on one single sheet of paper in a short time. The method using wax in order to form the channels was introduced in 2009 by Lu et al. [26]. At first, different approaches were tried by using a wax pen and simply drawing the patterns. Later, a wax printer was used and improvement in resolution was achieved. In order to use this method for printing channels on paper, another step has to be made. Since the wax is only printed on one side of the surface, heat has to be applied to diffuse the wax through the paper. Only after the wax has wicked through the paper, the channels are formed and can be used. Different temperatures for wax diffusion through paper have been evaluated by Zhong et al. [43]. It was found that the temperature should be between 75 and 125 °C. Zhong et al. observed, that a temperature below 75 °C is not sufficient for diffusion of the wax throughout the depth of the paper. They also discovered that a temperature of over 125 °C leads to curling of the paper. Zhong et al. used different kinds of paper that included printing paper, laboratory towels and paper napkins. Phillips et al. [18] used Whatman Chromatography Paper or Whatman Filter Paper for printing the channels. Their method for wicking the wax through the paper is to use a Hotplate running at 150 °C. The paper is placed on the hotplate with the printed side up. To ensure that the printed paper has even contact with the surface, a weight is applied onto the paper. This procedure runs for about 1 minute and 45 seconds to approximately 2 min. After this

time, the wax has wicked through the complete depth of the paper, forming the hydrophobic channels. When the wax printing technique is used, it has to be considered, that the wax will not only wick downwards in the paper, but also spreads to the sides. The channels become smaller (approx. 0.5 mm), which has to be taken into account while designing the channels. A disadvantage of using wax for printing the channels is the relatively poor resolution of the channels compared to photolithography. However, the resolution obtained using wax-printing, is acceptable for the majority of applications [3, 7, 20, 43].

For designing the channels, graphical layout software is needed. For instance, Phillips et al. [18] used the CleWin Layout Editor. But also the Adobe Illustrator or Inkscape can be used. It is important that the software used is vector-based; otherwise, the scaling of the patterns would lead to a lack in resolution. As a consequence, the quality of the printed channels would be poor.

Using the above described technique, it is possible to produce 2D devices in paper. By introducing more layers of patterned paper, it is possible to attain a 3D microfluidic device. 3D μPADs offer several advantages, like the potential to use different materials for each layer. Also, very complex shapes can be incorporated into those devices. Two different approaches to this have been made. One is using a folding technique. The other one introduces layers of double-sided tape to attach the functionalized papers to each other [18, 24, 25].

In the first approach, the channels are printed on a single sheet of chromatography paper using photolithography. The device is then simply folded by hand, following a certain pattern, similar to Origami. Since no glue or adhesive layers are introduced to

the device, an aluminum housing was produced to support the microfluidic system. Figure 4 shows the sheet of paper before the folding steps (a), the top- and bottom-layer of the chip (b and c) as well as the housing (d). In (e), the unfolded layers of the chip after running the test are shown. A total of nine layers are printed on one sheet of paper. Every layer consists of several channels which result in a working 3D device after assembly. One advantage of this technique is that the folding process takes only a short amount of time. No tools or special alignment are needed for assembly. Furthermore the device can easily be disassembled after use for parallel analysis of all layers [18, 25].

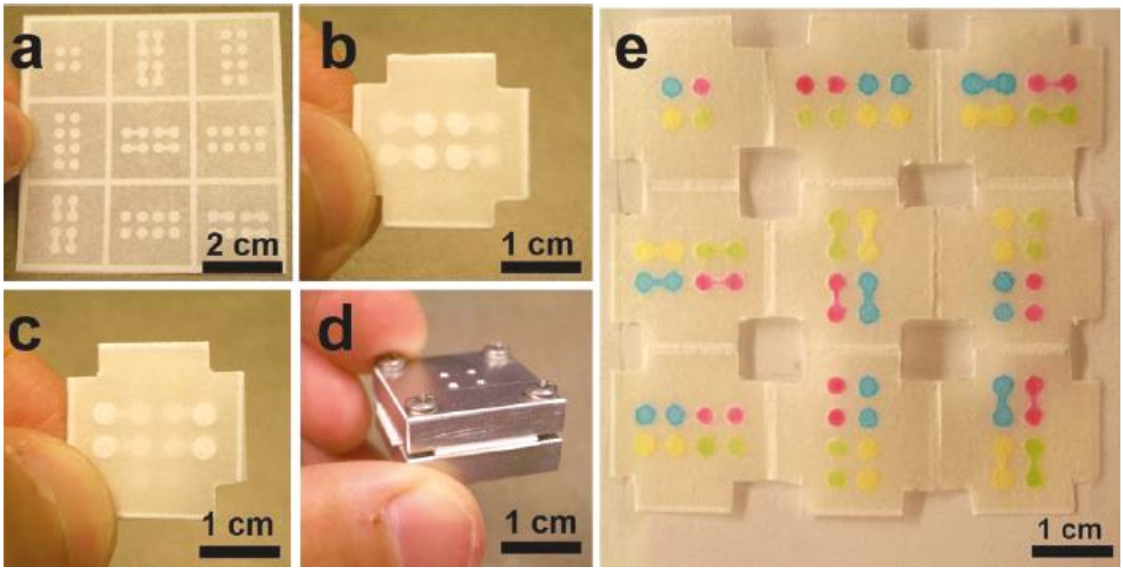

Figure 4: Several pictures showing a 3D microfluidic device on paper assembled using the technique of Origami [25]

The second approach to build a 3D microfluidic device on paper is using double-sided tape to attach layers patterned with channels to each other. This technique was first introduced by the Whiteside group of Harvard University [28]. In their first trial, several layers of patterned paper were used. These were attached to each other with double-sided adhesive tape. The tape contained positioned holes, which were either

punched out using a biopsy punch or a laser cutter. The holes in the tape were used to allow fluid to flow through the tape to another layer with hydrophilic channels. To fill the cavities introduced by the tape, cellulose powder was used. The now bridged gap between the respective layers allowed the fluid to wick into a different layer. With this method, devices can be fabricated suitable for multiplexed analyses [24, 28].

A very important factor in paper-based devices is that fluid flow is entirely controlled by capillarity. The movement of a fluid front in a dry membrane of constant width depends on the resistance that the already wetted paper introduces. This resistance increases the further the liquid moves into the strip. This means, that the further the fluid flows into the membrane, the slower the flow gets. This can be described by the Washburn equation [39],

$$L = \sqrt{\frac{\gamma D t}{4\mu}} \qquad [1]$$

where L = the distance moved by the fluid front, D = the average poresize of the paper, t = time, μ = the viscosity of the liquid and γ = the effective surface tension of the liquid. Using this equation, the time a fluid needs to travel to a certain distance or the length it moves in a specific amount of time can be calculated. In order to apply this equation for the flow in a membrane, it needs to be assumed that a non-limited source of fluid and a constant cross-sectional area is given [39].

The above described equation is only applicable for dry channels. By introducing a fluid into a pre-wetted channel, the flow characteristics can be described by using Darcy's law [43]:

$$Q = -\frac{\kappa WH}{\mu L}\Delta P \quad [2]$$

In this equation Q = the volumetric flow rate, WH = the area of the channel perpendicular to the flow, L = the length of the channel, κ = the permeability of the paper, ΔP = the pressure difference along the paper channel and μ = the viscosity of the fluid. By assuming a constant cross-sectional area throughout the whole length of the channel, this equation can be simplified to:

$$q = -\frac{\kappa}{\mu L}\Delta P \quad [3]$$

with q being the flow rate of the fluid. The above equation can be helpful for designing multi-fluid paper-based devices, in which more than one fluid passes over a certain region [43].

By knowing the volume V of the fluid, the timing for a second fluid to flow across a pre-wetted channel can be calculated using equation 2. The equation then becomes:

$$t = \frac{V}{Q} = \frac{\mu L^2}{\kappa \Delta P} \quad [4]$$

The above equations can be helpful in determining the initial channel lengths or timing in paper-based microfluidic devices.

For further control on timing, Fu et al. [12] introduced several techniques. Fu et al. investigated the transport time of fluids in channels of different width and length. Also, a method to create a delay in channels was introduced which included a simple trehalose solution applied on the channel. After drying, the trehalose formed a barrier for the liquid. Hence, the fluid flow was significantly slowed down at the point of the barrier. By treating a larger area with trehalose solution, a longer delay could be attained. Fu et al. [12] also introduced a method to sequentially deliver several reagents to a certain point. Three staggered inlets were used in the device. An example of the principle is shown in Figure 5.

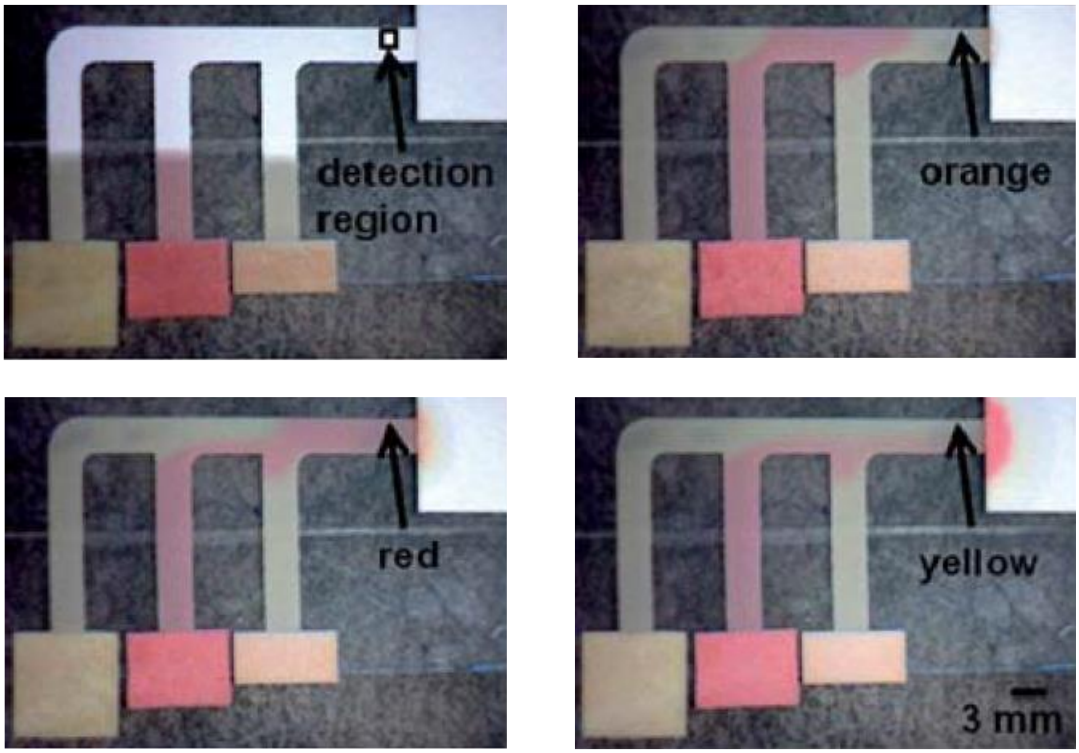

Figure 5: Technique to sequentially load reagents on a certain area introduced by Fu et al. [12]. Pictures taken after 10 s, 5 min, 15 min and 35 min.

In this device, the three fluids are applied at the same time. By using different channels lengths from each source, the fluids can sequentially pass through the detection region [12].

Besides using channels of different length to control the fluid flow, several other approaches have been made. A simple approach developed by Noh et al. [32] is a wax-based metering system for 3D µPads. With this simple approach, wax solutions of various concentrations between the connections of the different layers are applied. The solutions disperse into the layers and the fibers in different layers are coated with different amounts of wax. Hence, a control of the fluid flow in the pathways of the device could be attained. Furthermore, the control of the fluid flow could easily be altered by simply using different wax concentrations [3, 32].

Another approach was introduced by Li et al. [21] in which mechanical switches on paper-based devices were created. These switches were produced by cutting flaps across flow channels. The flow channels itself are not connected, until the flap is pushed down. After bridging the channel, the fluid is able to flow from one side to the other. Using this technique, a precise control of reagents and even the introduction of multiple fluids on one device are possible.

Whiteside's group at Harvard University developed 3D programmable devices on paper. By using perforated double-sided tape, but not filling the cavities, barriers originated. These gaps prevented the fluid from passing into other layers. Hence, a manipulation after fabrication is made possible. By applying force to the gap, the channels are being connected and liquid is able to flow [27]. A simple schematic is shown in Figure 6. Another technique to control the fluid flow and to sequentially load reagents on the detection area is a microfluidic valve in paper developed by Hong Chen et al. at [7] the University of Rhode Island. This method will be introduced in more detail in the next section.

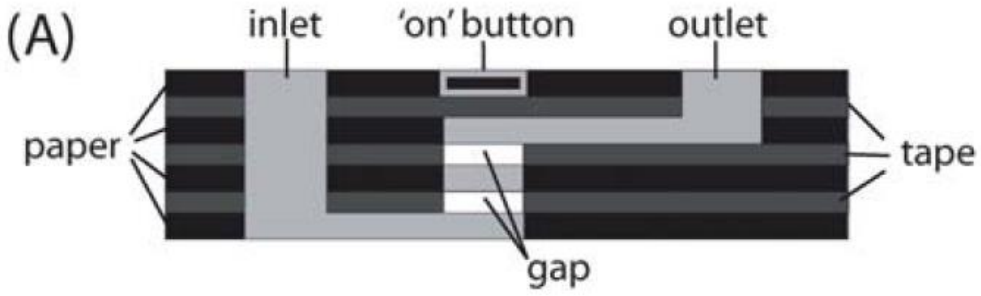

Figure 6: Schematic of programmable device developed by the Whiteside group [27]

Paper-based devices are an emerging field in microfluidics. Nevertheless, a lot of research has been done resulting in a variety of different possible applications. The

main application for µPADs cannot only be seen in biochemical analysis but also for medical and forensic diagnostics. µPADs have also proved to work for urinalysis, determination of the human blood type, the detection of neurotoxins or even ELISA tests. µPADs can be used as on-demand devices as well as for ready-to-use devices. On-demand devices contain no reagents. The user is adding these depending on what analyte needs to be tested. Ready-to-use devices are designed as complete sensors for which the indication reagents are already integrated in the system. Detection of various analytes, like glucose, lactate and uric acid in a urine sample has been successfully proven with these paper-based devices. Also, the quantification of nitrite in an unknown sample has been investigated. Cheng et al. from Harvard University [8] developed an approach to use paper-based microarray plates for ELISA. In general, four detection methods have been proven to work on µPADs: Colorimetric, electrochemical, chemiluminescence and electrochemiluminescence. Most research has been focusing on the colorimetric detection since it is a readout that can be accomplished fast and can even be done by the naked eye. Besides the use for immunoassays to detect biomarkers, µPADs have been developed for use in temperature and humidity sensors as well as for the detection of microorganisms, metals, gases and even drugs [20, 20, 22, 24, 30]. Examples of detection methods and analytes applied to µPADs are shown in Table 2.

Table 2: Examples of detection methods and analytes on paper-based devices [20]

Device type	Detection method	Used analyte	Application
Paper-based microfluidic devices	Colorimetric detection	Glucose, nitrite, ketones, human IgG, pathogenic bacteria	Health diagnostics (e.g. urinalysis, saliva analysis, pregnancy test, blood typing)
		Alkaline phosphatase	Biochemical analysis (e.g. enzyme activity)
		Fe(III)	Environment monitoring
	Electrochemical detection	Glucose, cholesterol, lactate	Health diagnostics
		Pb(II), Au(III)	Environment monitoring
		Ethanol	Food quality control
	Chemiluminescence detection	Glucose, uric acid	Health diagnostics
	Electrochemiluminescence detection	Nicotinamide adenine dinucleotide (NADH)	Biomedical analysis
Paper-based microarray plates	Colorimetric detection	Rabbit IgG, HIV-1 antigen (gp41)	Biochemical analysis (e.g. ELISA)
	Chemiluminescence detection	Iron in the hemoglobin	Forensic (e.g. detection of blood)

Though low cost, easy to produce and simple to use, µPADs have limitations. Due to sample retention and evaporation during use, the efficiency in regard to fluid consumption is low. Another disadvantage is that some hydrophobic agents are not capable of holding fluids with a low surface tension in the channels- the fluid can pass

the hydrophobic barriers. µPADs fabricated using the wax-printed channels are one example. They don't block the pores of the paper, but lower the free surface energy of it. Also, the limit of detection in µPADs is usually high compared to other methods. Therefore samples of very low concentrations might not be detectable using paper [20].

More research has to be done in order to achieve reliable and high quality µPADs. Future research will focus on the mass production of these devices as well as improving their reliability and sensitivity. New fabrication techniques may need to be developed as well as the improvement of existing methods. To gain more precise control of the fluid flow and the amounts of reagents needed, further research on the capillary wicking in paper is needed. Today, most devices are produced using filter paper or chromatography paper. New papers with certain unique properties could help improve the performance of these devices as well extended them to many other applications [20, 24].

2.5 Review on Detection Limits for Several Analytes in Immunoassays

In this section various immunoassays available on the market are reviewed in regard to their limit of detection (LOD) and the analytes tested.

One very important analyte to be tested these days is the Human chorionic Gonadotropin (hcG). It is a hormone produced during pregnancy. Lateral flow tests to test for pregnancy are available on the market since the 1970s. In order to detect a possible pregnancy early, the LOD is very important for these tests. Today, test strips using nanoparticles as labels can detect concentrations down to 10 mIU/mL of hcG in urine, which is around 0.6 ng/ml (1 IU of hcG equals 6 x 10^{-8} grams). This

concentration of hcG in urine is usually detectable 3-4 weeks after the last menstrual period [40].

Another analyte which can be detected using a LFIA is gluten. Gluten is a protein found in wheat and other related grain species. People having gluten sensitivity (around 6 to 7 % of population) need to be able to determine whether food contains gluten or not, since already small doses can be life-threatening [37]. Biomedal is one company offering several different devices to test for gluten in food. One device consists of a simple test strip and can be used in home applications. The device offers a sensitivity of 5 ppm, which calculates to a concentration of 5 µg/ml [5].

A widely used model analyte for proving the function of new developed devices is rabbit IgG. Rabbit IgG will be used in this study in order to show the ability of running an immunoassay on the developed multi-fluid circuit. This analyte is cheap and therefore commonly used in research. The limit of detection for rabbit IgG in a conventional ELISA using microtiter-plates is 8.6 ng/ml [38]. This method needs expensive equipment, high amounts of reagents and has a long time-to-result. In the previous section, a paper-based ELISA was introduced, developed by Whiteside's Group at Harvard University. The LOD determined for rabbit IgG using a colorimetric readout is 2.7 µg/ml [8]. It is less sensitive than the traditional ELISA conducted on microtiter-plates, but needs less reagent volumes and the results can be attained faster. Li et al. [23] were using an electrochemical readout on paper with electrodes made from graphite ink. The signal is attained by measuring the change in current during the redox reaction on the detection area. The LOD for this method with the analyte being rabbit IgG is 146.3 ng/ml [23]. A disadvantage compared to the colorimetric readout is

the need of equipment to measure the developing signal. In Table 3 detection limits for various analytes such as veterinary drugs, pesticides, mycotoxins, infectious viruses and bacteria on LFIA are shown.

Table 3: Overview of the limit of detection for several analytes on lateral flow devices [31]

Analytes	Assay format	Label	Sample	Sensitivity
E. coli recombinat protein	Sandwich	Colloidal gold	Sera and stools	4 ng/ml
Botulism neurotoxin D	Sandwich	Colloidal gold	Faecal	50 pg/ml
Botulism neurotoxin A	Sandwich	Colloidal gold	PBS	1-50 ng/ml
Staphylococcus aureus enterotoxin B	Sandwich	Colloidal gold	PBS, human urine and serum	10 pg/ml
Hepatite B antigen	Sandwich	Europium chelate-loaded silica	Sera	0.03 mg/ml
Progesterone	Competitive	Colloidal gold	Bovine milk	0.6 mg/ml
Clenbuterol	Competitive	Colloidal gold	Swine urine	3 ng/ml
Ochratoxin	Competitive	Colloidal gold	Coffee	5 ng/ml
N-methylcarbamate	Competitive	Colloidal gold	Water	0.25 mg/ml
Aflatoxin B1	Competitive	Colloidal gold	Rice, corn, wheat	2.5 ng/ml

2.5 Paper-based Microfluidic Valve Technology (PBMV)

In the previous section, several techniques to control the fluid flow and to sequentially load reagents on a certain area were discussed. Another method developed by Hong Chen et al. [7] includes a microfluidic valve which is similar to an electronic diode. This valve is a two-terminal component that allows the fluid to pass in one direction, but stops it if coming from the other direction. This is attained by using the click chemistry. At first, a 2D device was introduced. A simple schematic of the 2D device containing a valve is shown in Figure 7.

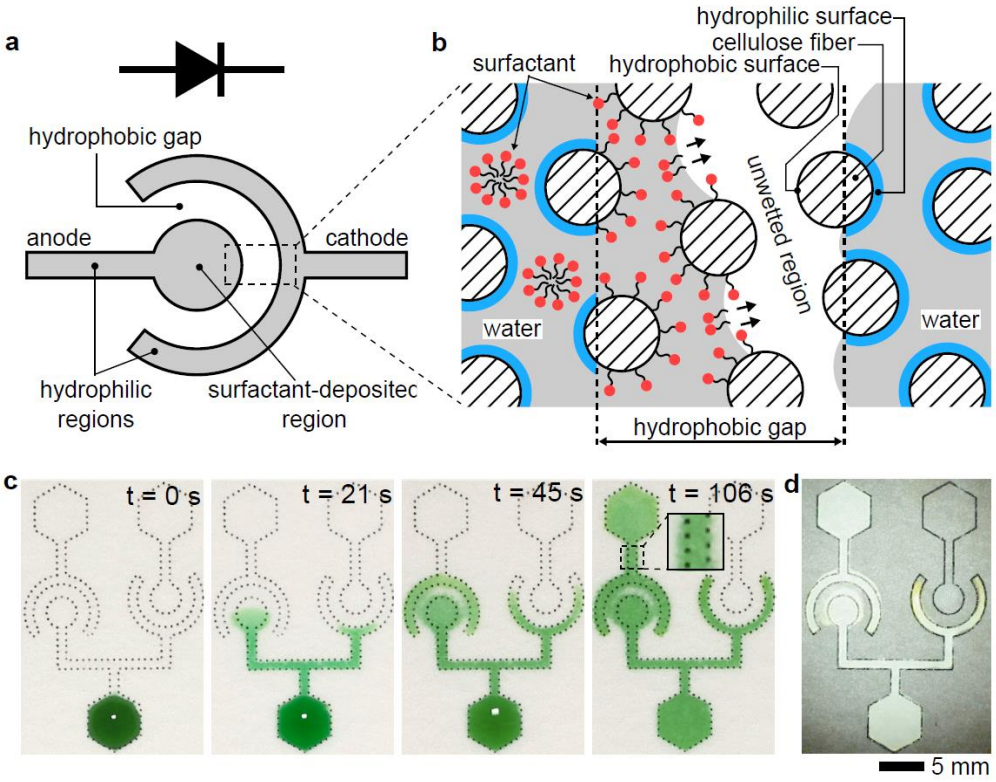

Figure 7: (a) Shows the symbol and a schematic of the fluidic diode. (b) Illustrates the working mechanism of the diode. (c) Shows Photographs of a simple 2D device with two oppositely-configured diodes [7]

The diode consists of a hydrophobic area on one side (cathode) and an area where surfactant is dried (anode) on the opposite side. If a fluid reaches the hydrophobic area of the diode, it is not able to pass. By introducing another fluid from the other side, the surfactant dissolves in the fluid. The surfactant then reduces surface tensions of the interfaces of the diode. This opens the diode and the other fluid is now able to flow. After attaining a 2D device containing a diode, a 3D paper-based microfluidic circuit was developed. Simultaneously the diode was adapted to work in a 3D system. Double-sided tape and several layers of paper equipped with channels were used. The double-sided tape was patterned with holes, using a biopsy punch or a laser cutter. In these, the valve is placed now consisting of disks with the hydrophobic disk on the

top. After assembly, a fluid can be introduced on top of the valve and is not able to pass through to the lower channels. Hence, it has to be triggered by a fluid reaching the diode from the bottom channels. A precise control and sequential loading of multiple fluids is thus possible. An example of a 3D circuit including a diode is shown in Figure 8.

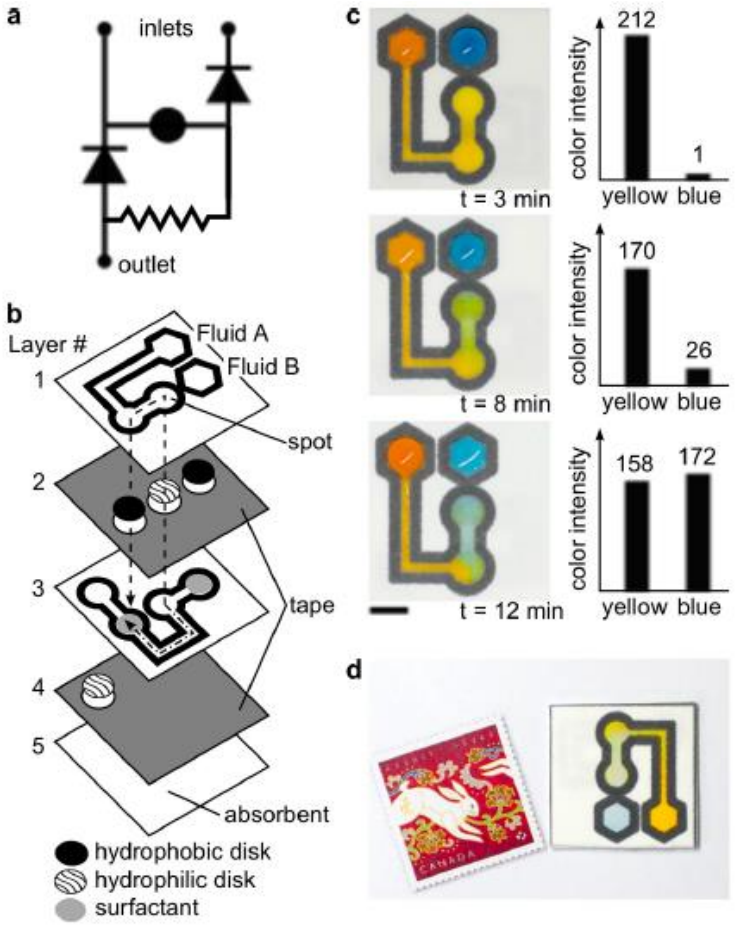

Figure 8: (a) Symbolic representation of the sequentially-loading circuit. (b) Schematic of the layers. (c) Photographs showing the opening of the valves using food coloring [7]

Several different reagents to functionalize the cathode hydrophobic have been tested. Allyltrichlorosilane dissolved in perfluorocarbon oil as carrier fluid was found to be the best reagent. For producing the anode of the diode, Tween 20 is dissolved in Ethanol and applied on the desired area. Precise techniques to treat the paper were

investigated by Hong Chen [7]. After further development, both parts of the diode are now fabricated as disks. This is achieved by treating whole sheets of filter paper either hydrophobic or with surfactant. The disks can then be produced by using a laser cutter.

By changing the length of the trigger channel, a precise timing of the second fluid is possible. No mechanical buttons or switches are needed. Using this method, multi-fluidic circuits running autonomously have been developed [7].

CHAPTER 3 - METHODOLOGY

3.1 Fabrication of Paper-based Microfluidic Circuits

In this section, the fabrication of microfluidic circuits is discussed. The materials used for the device as well as the equipment needed are introduced in detail. The fabrication of the paper-based microfluidic valve is also shown. By the end of this chapter, an explanation along with an illustration of the assembly of the devices can be seen. Note that the fabrication of the 3D-circuits in this thesis is based on creating channels on paper by using a wax-printer. Several layers with channels are assembled using double-sided tape. This method has been explained in the preceding chapter. This method was chosen because of the equipment and materials available in the laboratory. Furthermore, as mentioned earlier, this method is considered to be one of the most promising techniques in terms of material costs, simplicity of fabrication and the possibility to produce a large amount of devices on a single sheet of paper.

3.1.1 Selection of Materials and Equipment

The selection of appropriate materials is a crucial step in the development of paper-based microfluidic devices. Choosing the incorrect materials can result in poor sensitivity and specificity of the device. A LFIA consists of a conjugate pad, sample pad, a test membrane and the absorption area for holding the used reagents.

The conjugate pad of the device is fabricated using glass fiber (SterliTech GA-55 Membrane Filters). On the conjugate pad, the labeled detection antibody is applied. These antibodies must be released when the sample passes the conjugate pad, since the

amount of labeled detection antibodies released is limiting how much analyte can be measured. For this, a material which provides with a good release rate and low non-specific binding is needed. Glass fiber is commonly used in lateral flow test since it provides with these properties. Two different glass fiber materials are available in the laboratory, with and without binder. The binder is added to the glass fiber so that the structure of the material is maintained when liquid passes. Tests for selecting the glass fiber for the multi-fluid circuit were conducted in a parallel study in Prof. Faghri's laboratory by Roman Gerbers [15]. It was found, that the glass fiber with binder provides with a better conjugate release and is therefore used in the device.

The test area where the capture antibody is applied is made from nitrocellulose (Whatman AE 100 Membrane filters with a poresize of 12 µm). Nitrocellulose provides with a high internal surface area and a high protein binding capacity. These properties make this material suitable for immobilizing the primary antibody. The nitrocellulose binds proteins electrostatically which refers to the interaction of the dipole of the nitrate ester with the dipole of the peptide bonds of the protein. During this interaction, a very strong bond is formed, making it impossible for the primary antibody to move. Hence, it is immobilized.

The material used to fabricate the channels of the device is Whatman Filter paper No. 41 (203 mm x 254 mm sheets). As explained earlier, filter paper is a relatively low cost material and provides with a good fluid flow rate.

For the absorption area, glass fiber (SterliTech GA-55 Membrane Filters, binder-free) is used. As for the extension of the absorption area, blotting paper (Gel Blot Paper GB003 20 x 20 cm) is used being placed at the bottom of the chip. Glass fiber

provides with a good fluid holding capacity and a fast absorption rate which makes it suitable for its use in the absorption area. The blotting paper has a slower flow rate but a large capacity. Therefore, this material is used as an extension of the absorption area. Additionally, due to its thickness, blotting paper functions as supportive backing of the device.

Double-sided Ace Plastic Carpet Tape (Item No. 50106) is used in order to connect the layers of patterned paper. To prevent the reagents from evaporating, the top of the device is covered using transparent single-sided tape. The reagents are then forced to flow through the paper by capillary flow and impedes them from flowing on top of the paper. This is very important for the conjugate pad and the test membrane. If the fluid is not forced through those materials, the result will lack in sensitivity.

As previously mentioned, the material containing the microfluidic channels is Filter paper. To pattern the paper with channels, a wax printer (Xerox ColorQube 8570) is used. For this the respective layers have to be designed in Inkscape. The areas on which wax is applied are designed using black. On the other hand the channels are left blank. A simple example of a channel designed in Inkscape is shown in Figure 9. After printing the channels, the wax has to melt through the paper in order to form the hydrophobic barriers. This procedure is accomplished using an oven (FisherScientific Vacuum Oven, Model 280A) running at 140 °C. The melting procedure is performed for 1 minute.

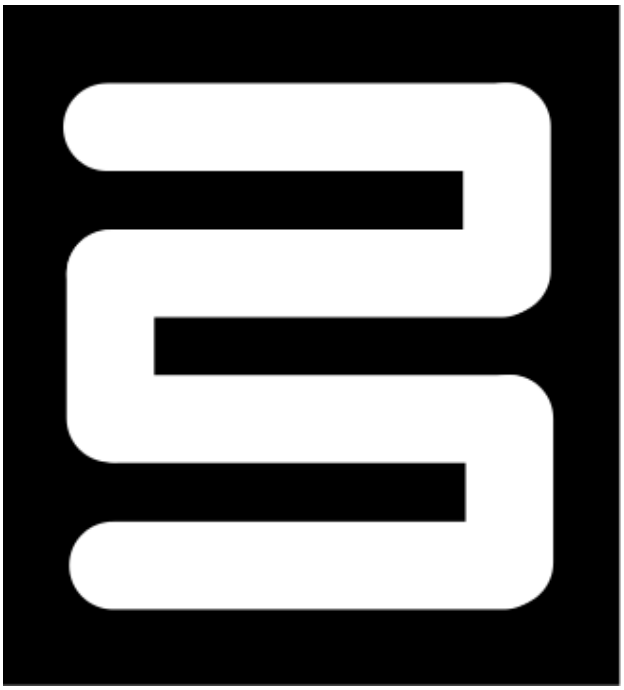

Figure 9: Example of a microfluidic channel designed in Inkscape; the printer prints the black areas, whereas the channels are left blank

In order to pattern all layers of the chip and cut the conjugate pads and the test membrane into the right size, a laser cutter is used (Epilog Mini 40 Watt CO_2 laser cutter). The laser cutter can be accessed like a printer. The patterns are designed in Inkscape. Using this software, the laser cutter cuts the desired shapes into the material. For every material, the laser cutter needs different cutting speeds and power settings. The settings used for the respective materials are listed in Table 4.

Table 4: Power settings of the laser cutter used to cut the materials for the device

Material	Laser Power	Vector Speed	Vector Power	Frequency
Blotting Paper	40 %	40 %	17 %	5000
Glass fiber (conjugate pads)	40 %	85 %	7 %	3025
Double-sided tape	40 %	50 %	25 %	5000
Filter paper	40 %	55 %	15 %	5000
Nitrocellulose	40 %	40 %	2 %	4250
Glass fiber (absorption area)	40 %	45 %	13 %	5000
Single-sided tape	40 %	50 %	20 %	5000

3.1.2 Fabrication of Paper-based Microfluidic Valve (PBMV)

The paper-based microfluidic valve technology allows precise control and sequential loading of multiple fluids in one device. This section focuses on the amount of reagents used to fabricate the respective disks of the valve.

For fabricating the hydrophobic disks, filter paper is used as material. To treat this material hydrophobic, a solution of 200 µl allyltrichlorosilane mixed with 12 ml of perfluoro-compound FC-72 is used. The allyltrichlorosilane is the reagent, rendering the paper hydrophobic, and the FC-72 functions as a carrier fluid. Small sheets of filter paper (4 in x 5 in) are soaked with this solution. After treating one sheet, another sheet is placed on top and treated the same way. A total of 8 sheets can be fabricated. The stack of soaked filter paper is then dried at 60 °C on a hotplate.

The surfactant disks are prepared in a similar pattern. The reagents used are Tween 20 and the carrier fluid is Ethanol. A solution containing 0.4 g of Tween 20 and 10 ml of Ethanol is prepared. This solution is applied on filter paper and allowed

to dry. Two sheets of filter paper (8 in x 10 in) can be treated with this amount of solution.

Besides the surfactant and hydrophobic disks, the valve also contains a hydrophilic disc. This disk is needed to connect the layers containing the microfluidic channels and is placed in the junction of the trigger channels. After the paper is dry, the disks are cut out with the laser cutter. The masks used to cut the disks is designed in Inkscape. An example is shown in Figure 10.

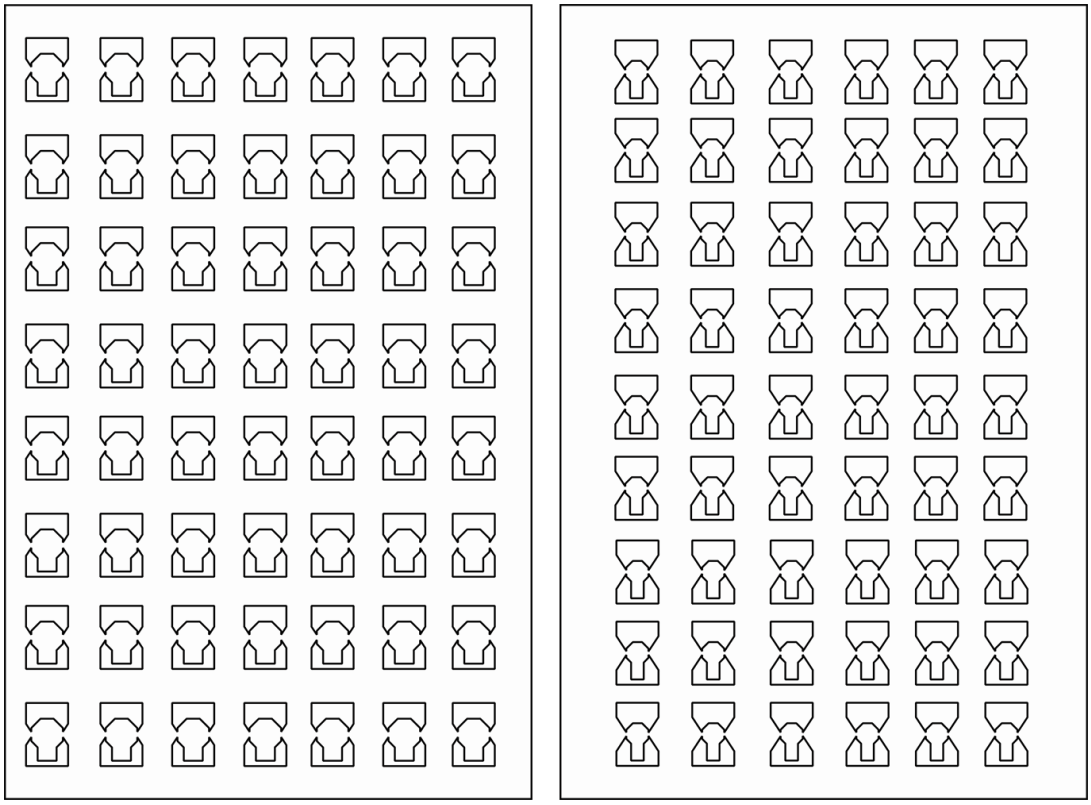

Figure 10: Example of mask used to cut the disks of the valve (left: mask for hydrophobic disks; right: mask to cut the surfactant disks and the hydrophilic disks)

For assembly reasons, the hydrophilic and surfactant disks are smaller than the hydrophobic disk. They have to be placed in the respective hole in the double-sided tape. However, the hydrophobic disk is not supposed to fit in the hole. It is designed larger to seal the hole, so the valve does not leak.

3.1.3 Assembly of a Multi-fluid Microfluidic Device

After designing the layers and preparing the materials, the device can be assembled. The assembly is a crucial step since it is made by hand. Alignment errors can occur and thus they have to be avoided. The device is fabricated from bottom to top. The bottom layer is the blotting paper, which is part of the absorption area and serves as supportive backing. On this, a layer of double-sided tape is attached. Then the first layer of filter paper, which is patterned with the trigger channels to open the valves, is applied. The next step is to place the surfactant and hydrophilic disks in the respective holes on the tape. The tape is then attached on top of the other layers, so that the discs are positioned between the tape and the filter paper with trigger channels. Now, the protective layer is peeled off of the tape and the hydrophobic disks can be placed onto the holes. It is very important that the disks are placed in a precisely manner. Even the smallest misalignment can cause leakage and can lead to the failure of the whole chip. After attaching the disks onto the tape, the test membrane and the conjugate pads, as well as the glass fiber for the absorption area are placed at the desired position. The top layer, containing the main channel and the inlets of the device, can be attached on top of the stack of the already assembled layers. In order to force the fluid to flow through the conjugate pad and the test membrane, a small area of the main channel is cut out with the laser cutter. These areas are located where the conjugate pad and the nitrocellulose have been placed. To finalize the assembly and to prevent the evaporation of the reagents, the transparent top tape is placed on top of the device. In Figure 11 – 14, the steps of the assembly are shown in detail.

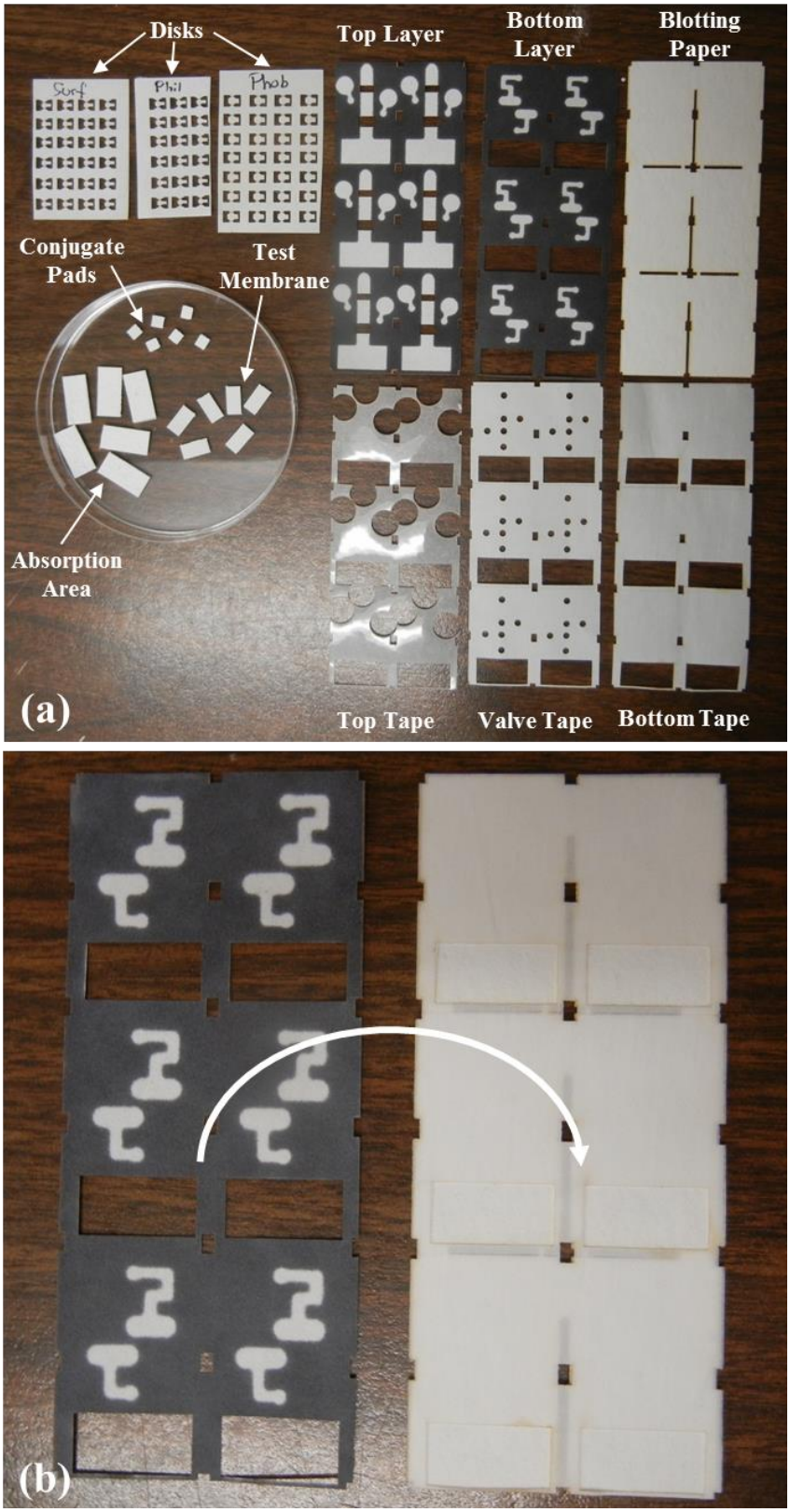

Figure 11: Photos showing the fabrication of the multi-fluid device: (a) All materials and layers for assembly. (b) Assembly of bottom layer and blotting paper with bottom tape

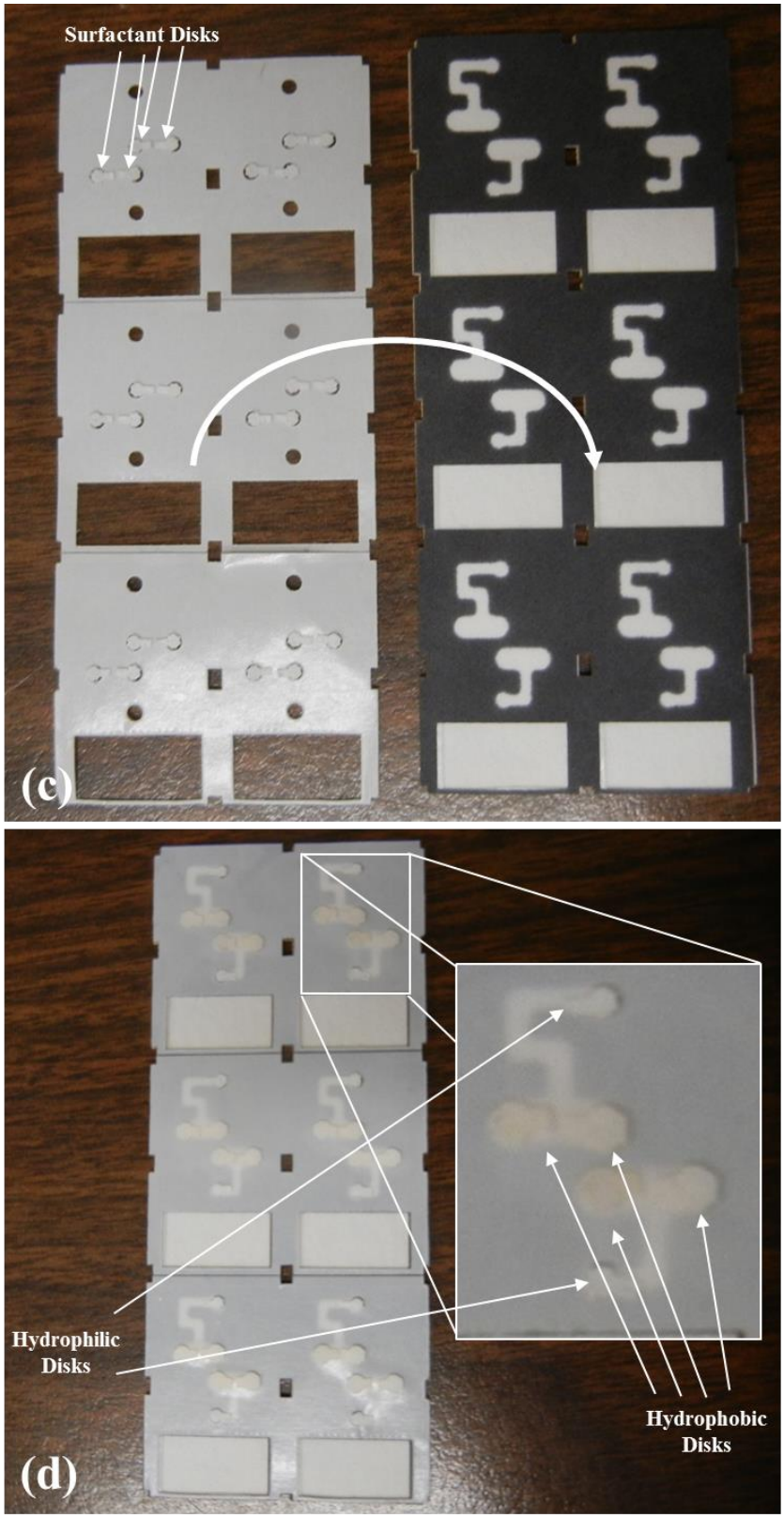

Figure 12: Photos showing the fabrication of the multi-fluid device: (c) Insertion of surfactant disks into respective holes in valve tape and then attachment onto already assembled stack. (d) Insertion of hydrophilic disks and hydrophobic disks from the other side of valve tape

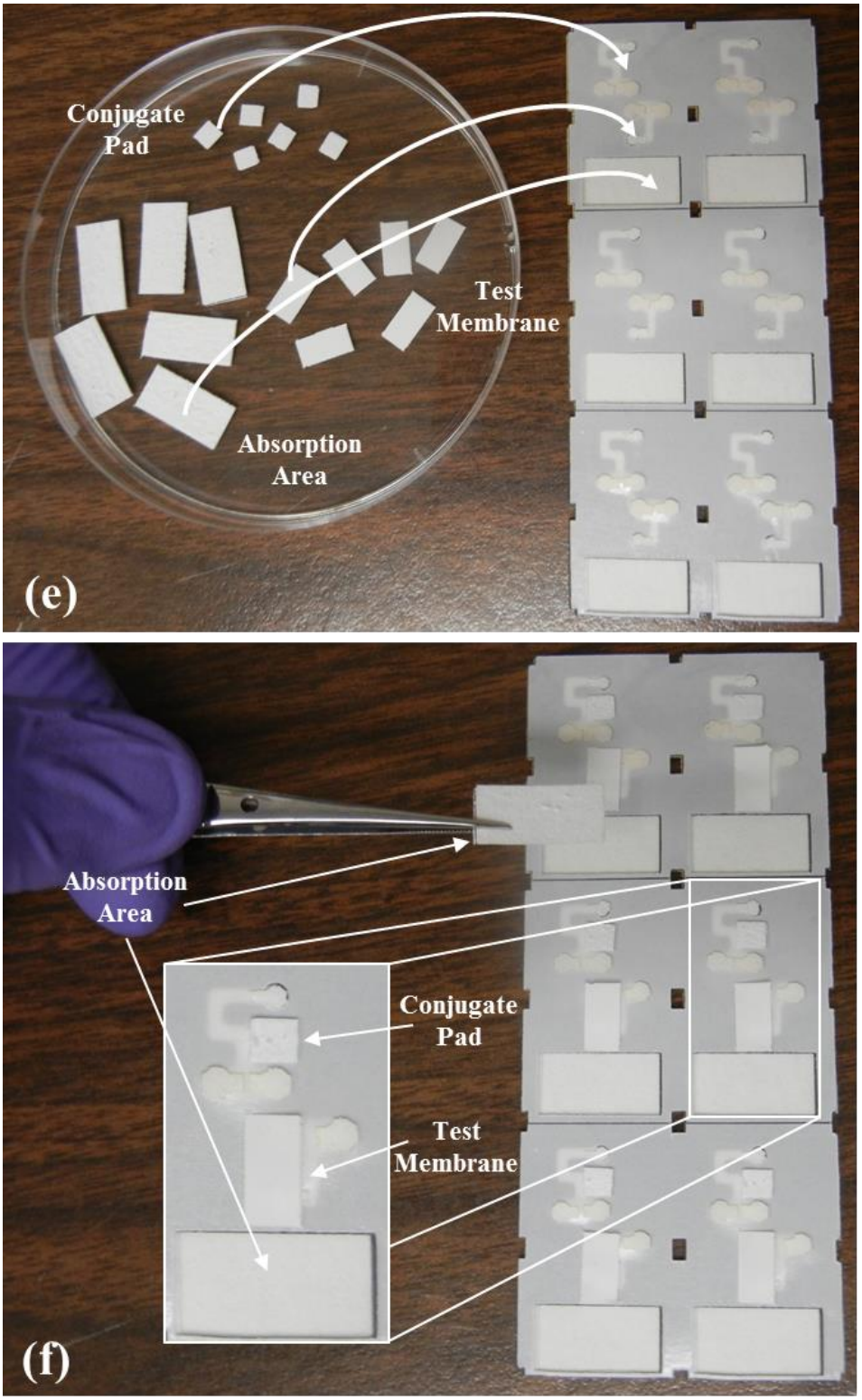

Figure 13: Photos showing the fabrication of the multi-fluid device: (e) Placing of conjugate pads, test membrane and absorption area on the device. (f) Photo showing positioned materials

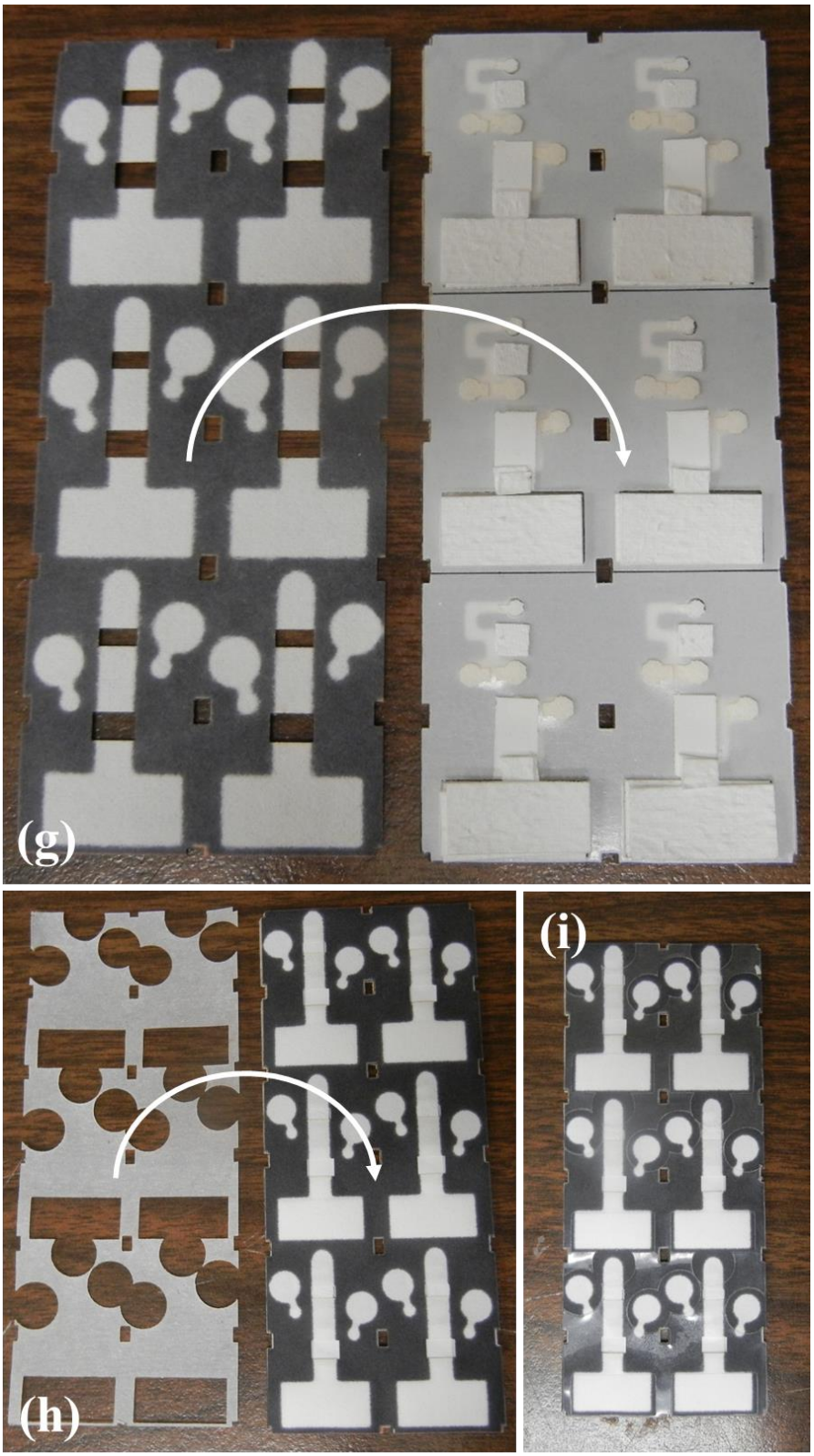

Figure 14: Photos showing the fabrication of the multi-fluid device: (g) Attachment of top layer onto the stack. ((h) Attachment of top tape on top of the device. (i) Photo showing the finished assembly of a batch of 6 devices

3.2 Development of the 3-fluid Microfluidic Circuit

In this study, a 3-fluid circuit is developed on paper. This work is based on previous experiments conducted by Jeremy Cogswell and Roman Gerbers at Prof. Faghri's laboratory. J. Cogswell developed a 2-fluid circuit using one valve. A schematic of this device is shown in Figure 15.

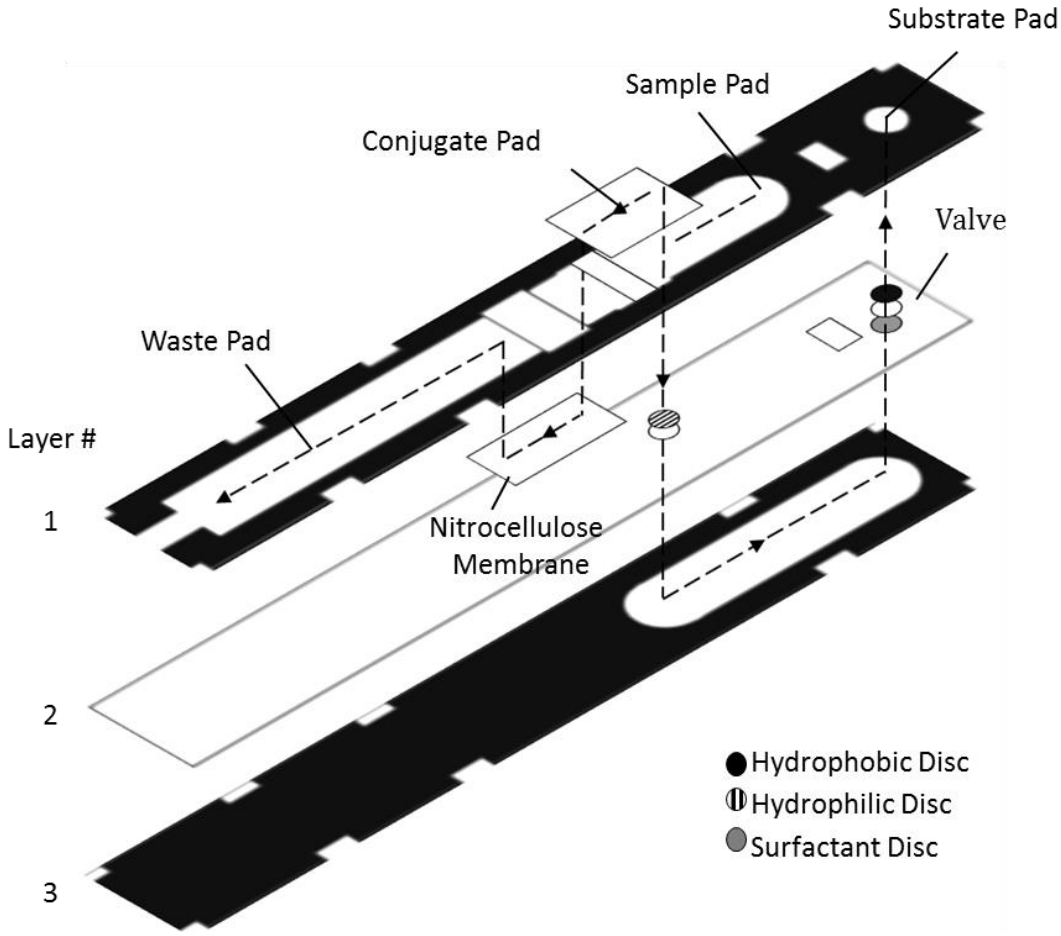

Figure 15: Schematic of 2-fluid circuit using PBMV technology developed by J. Cogswell

This device works like a conventional LFIA. However this device introduces a second fluid (substrate) in order to conduct an ELISA. The layers 1 and 3 are made from filter paper with printed wax channels. The two layers are connected using double-sided tape. The tape is perforated so that the fluids can go from layer 1 to layer

3 and back. In this perforation, the valve is placed. After the sample is added, it wicks through the channel. At the junction to the trigger channel, the fluid divides. One part of the fluid passes through the conjugate pad and the test membrane forming the sandwich of antigen and antibodies at the detection spot. A small amount of fluid goes down to layer 3 moving towards the valve. When the sample reaches the bottom of the valve, the surfactant in the lower disc dissolves in the fluid and neutralizes the effect of the hydrophobic disc. The substrate stored on top of the valve is then released into the channel and flows over the detection area where the signal develops.

This device is using one valve to store the second fluid. The main disadvantage of using this device is the time to run the assay. For triggering the valve, the sample has to flow into layer 3. When the valve is opened, the substrate has to flow back in the same channel. Since this channel is already wet, the flow rate is very slow which increases the time to finish the assay. Another problem, in this device, is that the substrate passes the conjugate pad while flowing to the detection area. In the case, that still some enzyme-labeled detection antibodies are present in the conjugate pad, the enzyme reacts with the substrate, before it reaches the detection area. This renders the substrate ineffectively which lowers the amount of active substrate reaching the detection spot. Also, a high background noise develops, leading to a high limit of detection.

In order to reduce the time to run an assay and reduce the background, a new design was introduced by Roman Gerbers [15]. A schematic of this design is shown in Figure 16.

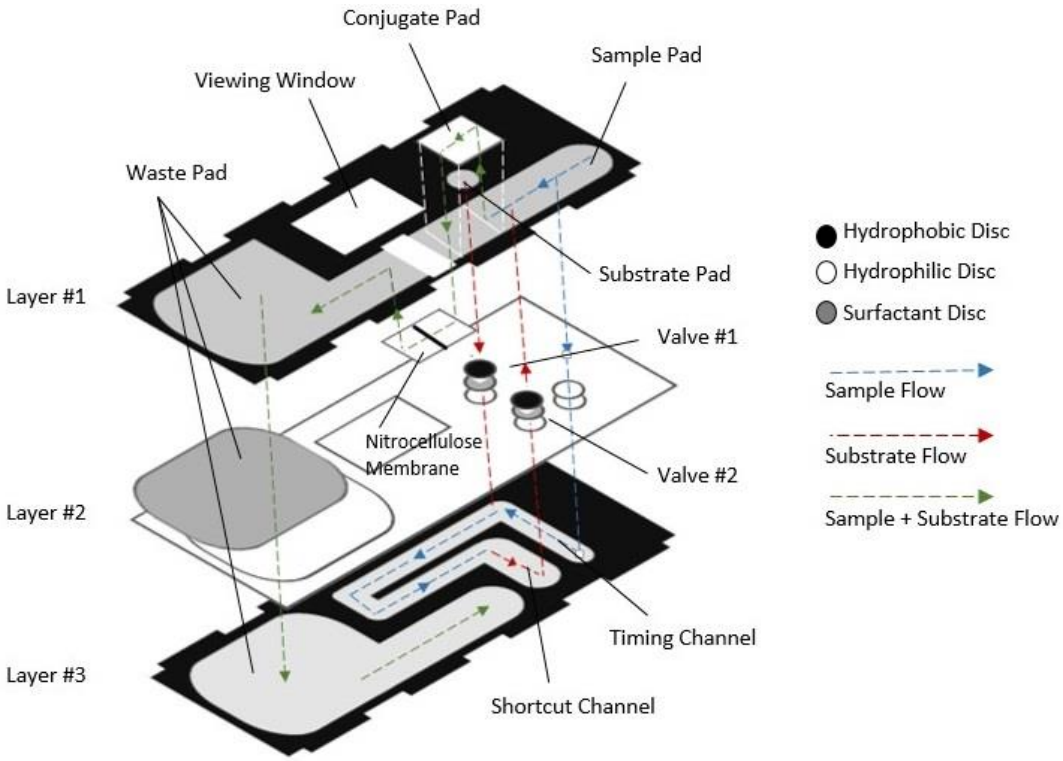

Figure 16: Schematic of 2-fluid microfluidic circuit using 2 valves, developed by Roman Gerbers [15]

The design is for the most part similar to the previous device. The difference is that two valves are used to introduce the second fluid into the system. They are placed next to each other, with one holding the second reagent and the other one placed below the main channel of the device. If the sample is applied, the fluid divides as in the previous design. Most of the fluid wicks through the main channel and a little amount flows into the trigger channel to open the valves. The trigger channel leads the fluid to the oval-shaped end, where both valves are positioned. The fluid then dissolves both surfactant discs at the same time, so the two valves open simultaneously. The fluid stored on top of one valve flows down into the trigger channel. Since the trigger channel introduces a high resistance due to being already wet, the fluid flows through the other valve and directly into the main channel. The

fluid does not have to wick back through the trigger channel as in the previous design. Hence, the time to finish the assay is reduced. Also, the outlet of the second fluid is able to be placed behind the conjugate pad. This technique prevents the substrate from passing over the conjugate pad, leading to a lower background noise and a better sensitivity.

Using this technique, a device with the capability to incorporate 3 fluids can be developed. In the 3-fluid device, an additional wash step is introduced in order to wash away all unbound reagents in the channels. Then, the substrate is triggered and thus, the enzymatic reaction takes place. For the purpose of a 3-fluid device, a second trigger channel has to be introduced. The junction from the main channel to the second trigger channel is placed between the detection area and the absorption area. The third fluid is therefore triggered by the fluid, which already passed the detection spot. This means that no reagents are wasted in the second trigger channel. For both, the second and the third fluid, the technique using two valves is applied. In order to reduce the time to run the assay, both outlets for the fluids are placed as close to the detection area as possible. A schematic of the 3-fluid design using four valves is shown in Figure 17.

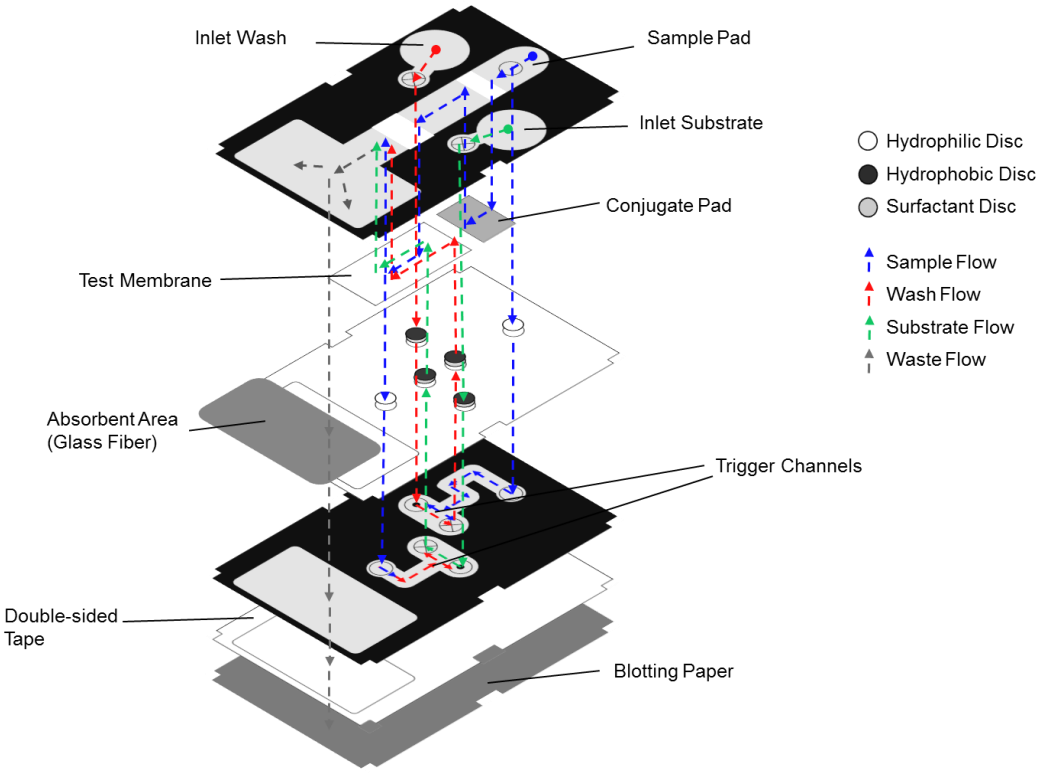

Figure 17: Schematic of the 3-fluid device developed in this study

For the purpose of a reliable device giving repeatable results in respect to timing and functionality, the following methods to attain the right channel lengths, reagent volumes and the size of the absorption area are used:

(1) To determine the lengths of the first trigger channel, the Washburn equation, introduced in section 2.3, is applied. The length of the main channel is determined based upon the shortest length in which all junctions, outlets as well as the conjugate pad and detection area can be inserted. Based upon preliminary results, the width of the trigger channels is fixed to 2 mm. The main channel has a width of 5 mm. The length of the first trigger channel is calculated based on the timing of the 2-fluid circuit developed by Roman Gerbers [15]. It is desired, that the second fluid is introduced just as the sample is completely wicked into the channels. 130 µl of sample were used in the 2-fluid design in order to flow an appropriate amount of analyte over

the detection area. This amount of sample needs around 80 seconds to completely wick into the channels. The second fluid therefore needs to be released into the system after 80 seconds. Preliminary results have shown that the valves need at least 15 seconds to open, after fluid reaches the surfactant disc. The opening time for the valves is deducted from the time it takes for the sample to wick into the channel in order to improve the timing of the device. The calculated trigger channel length using t=65 seconds is used for the first trigger channel. For applying the Washburn equation the effective pore diameter of the filter paper as well as the viscosity and the effective surface tension of the fluid are needed. For water the effective surface tension at room temperature is 0.0728 N/m and the viscosity is 0.001002 Ns/m^2. The effective pore diameter is determined experimentally by measuring the time it takes for the fluid to move 5 cm in a strip of filter paper with a constant width. 5 Strips with a width of 1 cm and a length of 5 cm are dipped into water. By measuring the time it takes for the fluid to travel 5 cm in those strips, the effective pore diameter of the filter paper can be calculated using the Washburn equation. For this the average time for all 5 strips is used. Having all these parameters and t=65 seconds, the length of the first trigger channel can be calculated.

(2) The second trigger channel used to introduce the third fluid, the substrate, cannot be calculated using the Washburn equation. The reason is, that the junction to this channel is located very close to the absorption area. The majority of the fluid flows into the absorption area and not into the trigger channel. A non-limited source cannot be assumed and the Washburn equation is not applicable to calculate the length of the second trigger channel. The length of this channel is therefore determined

experimentally by testing different lengths. Channels with a length of 14 mm, 15.5 mm and 17 mm are tested. The amount of wash applied in the device is 60 µl. This amount is desired in order to replace the fluid in the channel between the first outlet and the end of the detection area 10 times. The test is performed using colored fluids representing the sample and the wash. 130 µl of sample and 60 µl of wash are applied and tested for every channel length. The length of the second trigger channel giving the best results in terms of opening right when the wash is wicked into the channel, is used. The procedure is performed several times for every length tested.

Based on preliminary results, 80 µl of substrate are being used in the biological experiments. This amount of substrate is sufficient in order to provide with good results for rabbit IgG.

(3) The absorption area of the test can be seen in Figure 17. It consists of the glass fiber placed in the dedicated area inside the device and blotting paper. The absorption area has to hold the 270 µl of reagents (130 µl of sample, 60 µl of wash and 80 µl of substrate) applied during testing. Only the amount remaining in the channels, can be deducted. For determining how much liquid is remaining in the channels, small amounts of fluid are applied into the main channel, the trigger channels, the inlets and the conjugate pad. The amount which can be held by those channels is the amount deducted from the total amount of fluid. The resulting value has to be absorbed by the absorption area. In order to determine the size of the absorption areas it is tested, how much fluid binder-free glass fiber and blotting paper can hold per square centimeter. It is desired, that the absorption area can hold 2.5 times the amount of liquid used in the test in order to maintain a high and relatively

constant fluid flow rate. Additionally the glass fiber needs to hold 75 % of the fluid, since it provides with a better absorption rate, than blotting paper.

3.3 Improvement of Fabrication

The developed 3-fluid microfluidic circuit using two valves per fluid provides with a short assay time, less background noise and therefore a higher sensitivity compared to previous methods. An issue which has to be faced is the reliability of the valves and the time to fabricate the devices. Four hydrophobic discs, four surfactant discs as well as two hydrophilic connection discs are needed per chip. The assembly of six chip using the method described in section 3.1.3 takes about fifty minutes. Every discs has to be placed manually and with caution to prevent misalignment. A technique developed by Roman Gerbers [15] adapts the way the channels of the device are printed. The disks are designed so that they are surrounded by wax. The disks are printed on filter paper, the same material used for the single disks. An example is shown in Figure 18. The white spots are located exactly where the disks need to be placed in the device. Since these layers have the same size like the other layers of the device, they can therefore be placed easily on the desired location.

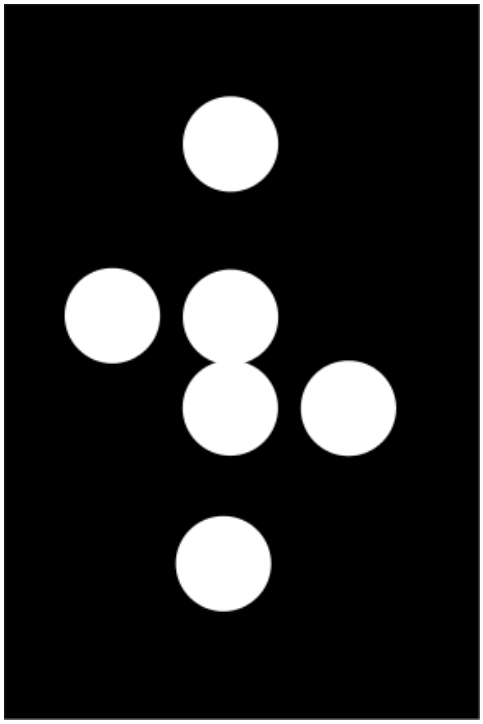

Figure 18: Schematic of printer layer for the hydrophobic and surfactant discs

Two layers need to be designed for the new fabrication method. One layer for the hydrophobic and one layer for the surfactant disks are required. By spotting the reagents on every spot, the disks can be functionalized.

In the previous method, the amount and concentrations of reagents needed to functionalize the disks was found by Hong Chen [7]. By applying the same concentrations, used before, it can be seen that the disks are not functionalized. To find the right amount and concentration of reagents needed for the test, spots with the size of the disks are printed in wax. By applying heat, the wax melts through the paper, forming hydrophobic barriers. On the blank spots the reagents are pipetted. An illustration of the used mask is shown in Figure 19.

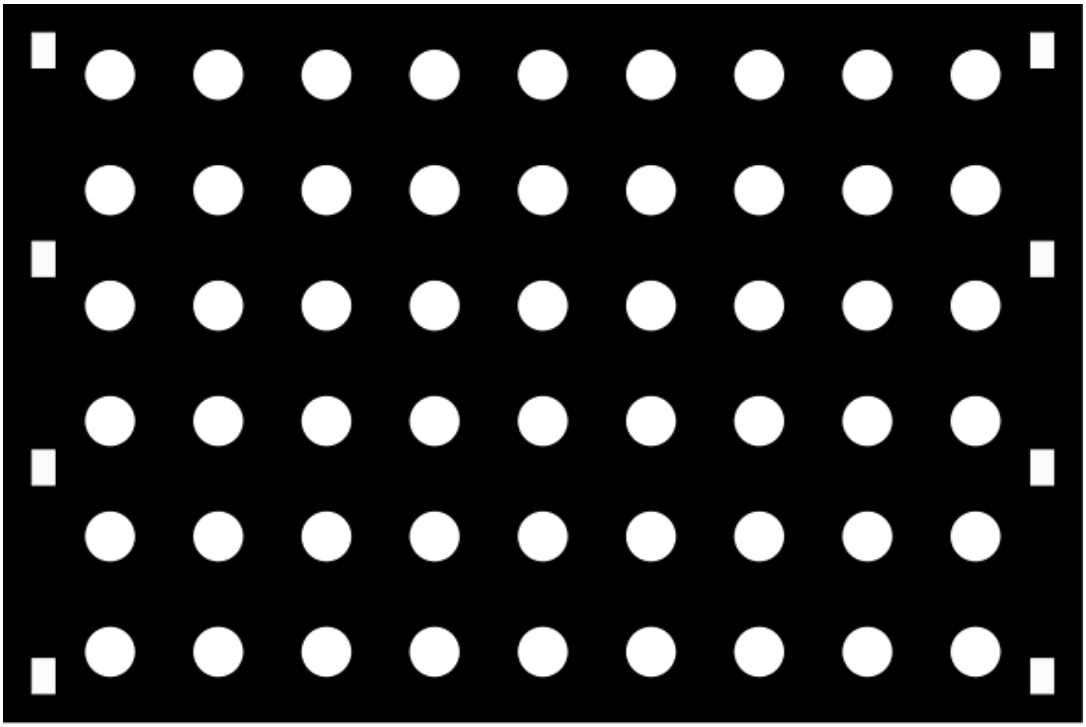

Figure 19: Schematic of mask used for finding the allyltrichlorosilane concentration

At first, the amount needed to functionalize the disks to be hydrophobic is determined. A series of different concentrations of allyltrichlorosilane in perfluoro-compound FC-72 is tested on the spots. The concentrations of allyltrichlorosilane tested are:

- 0.53 Vol.-%
- 1.06 Vol.-%
- 1.59 Vol.-%
- 2.12 Vol.-%
- 2.65 Vol.-%
- 3.18 Vol.-%
- 3.71 Vol.-%
- 4.24 Vol.-%
- 4.76 Vol.-%

2 µl are spotted on each spot for four times. In total, 8 µl are applied on each spot. This procedure is used since a single application of the solution would need very high

concentrations of allyltrichlorosilane. Concentrations higher than 4.76 % cannot be used, since the allyltrichlorosilane is evaporating too fast and the vapor is very toxic. At the end of the procedure, the spots are dried. Then small droplets (3 µl) of water are pipetted on the spots. A total of six spots per concentration is tested. The lowest concentration, which permanently stays hydrophobic, is the one used for the hydrophobic disks in the new fabrication method.

For the concentration of allyltrichlorosilane required to render the discs hydrophobic, the amount of surfactant needed to open the new valves reliable has to be examined. Experiments for testing the concentration of surfactant per disk were performed by Roman Gerbers. The amount of surfactant needed to open the valves was determined based upon the findings of the experimentation for the lowest concentration of allyltrichlorosilane. It was found, that a 35 weight percentage solution of Tween 20 dissolved in Ethanol gives the best results in terms of consistent opening and reliability. 2 µl of solution have to be applied per spot and allowed to dry [15].

Using the new developed method, the assembly time for the devices can be reduced dramatically. Six chips can be produced in less than 20 minutes. Also, since layers of discs are used, the risk of misalignment has been reduced. For more detailed explanations of the fabrication method, see [15].

3.4 Biological Testing

In this section, the methods used to test biological reagents in the developed 3-fluid device are introduced. At first, the reagents used in the device are defined. Next, methods to determine the right material for the conjugate pad and the concentrations of the reagents are explained. In the next section, the preparation of the materials and

reagents is discussed. Then the device is tested using the reagents introduced. A calibration curve is be created based upon the dose response of the device. Using the calibration curve, the limit of detection (LOD) is determined. In the end, a method to determine the effects of the valve reagents allyltrichlorosilane and Tween 20 is introduced.

3.4.1 Reagents Used for Testing the 3-fluid Circuit

In this section, the reagents needed for testing the 3-fluid device are introduced. It is desired to integrate the ELISA technique into the paper-based device. Rabbit IgG, a cheap model analyte, is chosen in order to prove that immunoassays can be performed in the 3-fluid circuit. The primary antibody is a monoclonal mouse anti-rabbit IgG with a concentration of 0.5 mg/ml. The secondary antibody is monoclonal from mouse labeled with alkaline phosphatase. For the secondary antibody, the concentration giving the best Signal-to-Background ratio is chosen (see next section). Monoclonal antibodies are chosen, since experiments performed using polyclonal antibodies resulted in false positive signals on the negative control. These experiments were conducted by Roman Gerbers [15]. A colorimetric readout is desired so that the results can be interpreted without the need for a specialized detector. As substrate, BCIP/NBT ((5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium) is used. Together with the enzyme, BCIP/NBT produces a color change from light yellow to dark purple which provides with an excellent contrast on the white paper.

For diluting the rabbit IgG and the secondary antibody, SuperBlocker Blocking Buffer is used. It provides with excellent stabilizing properties and prevents the reagents from denaturing. SuperBlocker is also used as a wash step in the device and

to block the channels, the test membrane and the conjugate pad in order to prevent non-specific binding. For completely releasing the labeled detection antibody from the conjugate pad, a sugar matrix is used. Trehalose and sucrose are available and is tested in order to attain the best conjugate release (see next section).

3.4.2 Determination of Reagent Concentrations

In order to determine the amount of sugar needed to attain the best release of the detection antibodies, a test is performed. Small pieces of glass fiber in the size of the conjugate pad are prepared (4 mm x 5 mm). The glass fiber is first blocked in order to prevent non-specific binding using SuperBlocker and allowed to dry at 37 °C. Sucrose and trehalose are dissolved in SuperBlocker in five different concentrations (1 %, 2 %, 5 %, 10 % and 20 %). Additionally a Mix of trehalose and sucrose in concentrations of 5 %, 10 % and 20 % are tested. These solutions are used to dilute the detection antibodies. The concentration of detection antibody is kept constant at 20 µg/ml in order to attain comparable results. Then 8 µl of the solution containing the sucrose and the detection antibody is applied onto the glass fiber and allowed to dry at 37 °C. After the glass fiber is dry, it is placed onto the blotting paper. In order to simulate the release of the detection antibody in the microfluidic device, 120 µl of SuperBlocker is applied on top of the glass fiber in steps of 10 µl. Then, the glass fiber is removed and 30 µl of substrate is applied onto each wet spot in the blotting paper. Since the detection antibodies are labeled, a color change is seen. The strength of the signal is directly correlated to the amount of labeled detection antibodies released in the procedure. The color intensity is then measured using ImageJ in order to determine the

amount of sucrose with the best release rate for the detection antibodies. Two pieces of glass fiber are tested per concentration.

The type of sugar and the concentration giving the best results in terms of the release of the detection antibodies is used in the biological experiments.

The next critical parameter in the sensitivity of the assay is the concentration of detection antibody. A very high concentration gives a strong signal. However, it can also cause a high background noise in the channels, whereas a low concentration can lower the sensitivity. A balance between signal and background noise is required in order to get good test results. To determine the optimal concentration of detection antibody, different concentrations are tested in the 3-fluid device. Conjugate pads are prepared similar to the procedure for finding the right amount of sugar. The type and amount of sugar giving the best results is used to dilute the detection antibodies. The test membrane is prepared as described in section 3.4.3. Preliminary experiments have shown that the optimal concentration of detection antibody can be found in the range from 20 µg/ml and 50 µg/ml. Four different concentrations are tested: 20 µg/ml, 30 µg/ml, 40 µg/ml and 50 µg/ml. Three iterations are performed for each concentration of detection antibodies using a constant concentration of analyte (rabbit IgG) of 500 ng/ml. Additionally, one negative control is conducted for each concentration of detection antibodies in order to eliminate the possibility of false positive signals. The intensity of the signal compared to the background noise is measured and evaluated. The concentration of detection antibody giving the best signal-to-background ratio is used in the biological tests.

3.4.3 Preparation for Testing

In this section the preparation of the materials and reagents for the biological tests is discussed. The materials used were introduced in section 3.1.1. In order to be able to use these for testing, they have to be prepared.

Before assembling the test, the channels printed on filter paper have to be blocked. For blocking these, SuperBlocker is used. It prevents the loss of reagents in the channels before they get to the detection area, by occupying the binding sites of the paper. SuperBlocker is applied onto the paper and allowed to dry at 37 °C. After drying, the procedure is repeated in order to attain the best blocking efficiency.

The test membrane (nitrocellulose) holds the capture antibodies. The membrane with a size of 5 mm x 10 mm. 0.3 µl of capture antibody at a concentration of 0.5µg/ml is spotted onto the center of the material. The capture antibodies immobilize on the nitrocellulose. The rest of the test membrane has to be blocked in order to prevent non-specific binding. The blocking is performed using SuperBlocker. The membrane is submerged into SuperBlocker for 10 minutes and then allowed to dry at 37 °C.

The conjugate pads also have to be blocked using SuperBlocker. 10 µl of blocker are applied on each pad and allowed to dry at 37 °C. Then a mixture of sugar and detection antibodies is applied with the concentrations found in the previous section. 8 µl are spotted onto each conjugate pad. Again, it is allowed to dry at 37 °C.

Now the device can be assembled and the biological tests can be performed.

3.4.4 Calibration Curve and Detection Limit of the 3-fluid Device

For evaluation of the behavior of the 3-fluid circuit in biological testing, different concentrations of rabbit IgG are tested. Ten different concentrations are used in order to be able to create a calibration curve. The concentrations tested are: 5 µg/ml, 1 µg/ml, 500 ng/ml, 100 ng/ml, 50 ng/ml, 10 ng/ml, 8 ng/ml, 6 ng/ml, 2.5 ng/ml, 1 ng/ml and a negative control. Every concentration is tested three times. The color intensities are measured using ImageJ. The ratio between signal and background is calculated and the mean value as well as the standard deviation are determined for every concentration tested. The negative control is handled differently, since a Signal-To-Background ratio cannot be attained. The overall background intensity of every negative control is measured and the mean is calculated. The ratio between every respective intensity to the mean intensity is used to determine the standard deviation. This value then indicates how much the background of the negative control varies in terms of color intensity. The mean ratio and error bars using the standard deviation for every concentration and the negative control are plotted over the concentration in a logarithmic graph. In order to attain the calibration curve, the data points of the different concentrations are approximated using the software CurveExpert. A sigmoidal model is used for approximation.

The detection limit is determined in two different ways: (1) The first approach is a visual approach. The lowest concentration of analyte giving a visibly detectable signal is considered to be the limit of detection in this method. (2) The second approach is the so called blank determination method. In this, the signal intensity resulting from the mean color intensity plus three times the standard deviation of the

blanks is considered to be the lowest concentration detectable. The equation is shown below:

$$LOD = I_{MB} + 3SD_B \quad [5]$$

with I_{MB} being the mean color intensity of the negative controls and SD_B being the standard deviation from the blanks. This method is used in order to attain a calculated value for the detection limit using the background noise of the negative controls [16].

3.4.5 Effects of Valve Reagents in the Immunoassay

In this section, a method to determine the effect of the valve reagents in the immunoassay is introduced. Tests are performed in order to establish if traces of allyltrichlorosilane and Tween 20 affect the results on the immunoassay. A device is fabricated containing no valves and therefore, none of the reagents mentioned above. All other parameters are kept constant for comparison. The reagents are then applied manually after each other in the same manner, as if valves are integrated in the system. A series of 2 different antigen concentrations with 3 iterative steps per concentration is tested. The concentrations tested are: 1 µg/ml and 100 ng/ml. These concentrations have also been used for determining the calibration curve. The mean signal intensity is then measured and compared to the results from section 3.4.4, where the PBMV technology was used. The differences in the resulting signals with and without valves are evaluated in order to estimate the effects of allyltrichlorosilane and Tween 20 in the system.

3.5 Development of a Housing for the 3-fluid Circuit

In this section the development, production and methods for testing of a housing for the 3-fluid microfluidic device are discussed. The housing of a LFIA is useful since it lowers the risk of misuse by the operator. It also helps prevent evaporation of the reagents caused by the relatively high surface area of the device. For the 3-fluid circuit, a housing is also useful for an easy application of all reagents. The housing is designed using the CAD-software SolidWorks. For fabrication of the housing, a Rapid-Prototyping machine (Stratasys Dimension SST) pertaining to the College of Engineering is used. The material used is the polymer acrylonitrile butadiene styrene (ABS). Testing of the housing is performed to measure if an improvement in reliability of the device can be attained. Finally, a method to apply all reagents with only little manipulation by the operator is developed.

3.5.1 Design of the Housing

A typical housing of a LFIA consists of two pieces, a bottom piece and a top piece. An example of a housing is shown in Figure 20.

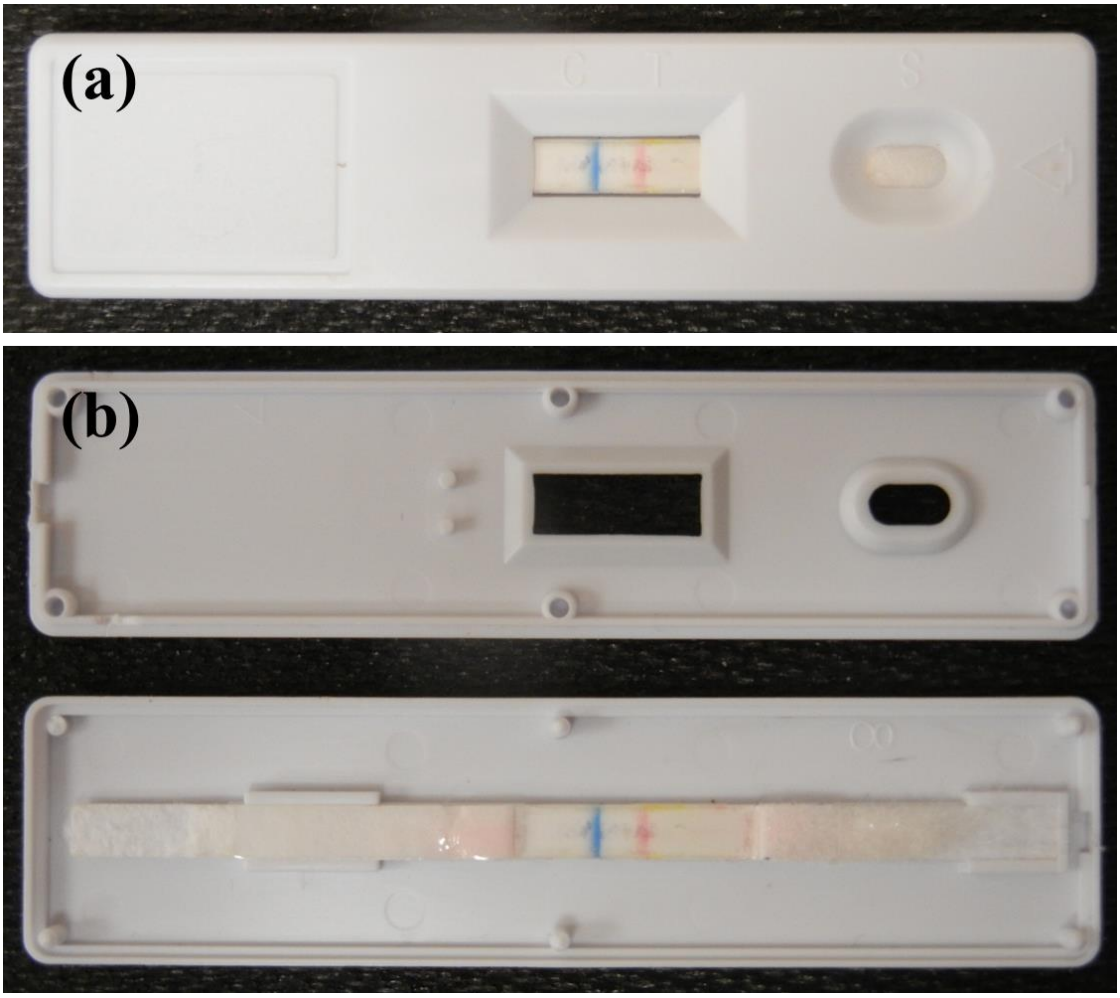

Figure 20: Housing of gluten test serving as starting product. (a) Top of test with viewing window and sample inlet. (b) Inside of housing with test strip

The test strip is placed into the dedicated area in the bottom piece. The top component is placed onto the bottom. The top part contains notches for an application of the sample and the reading of the result. Additionally, pins on the top half help to press down the test strip in order to improve the fluid flow.

The housing for the 3-fluid circuit developed in this study is build up in a similar way. In the bottom piece, an area is designed with the dimensions of the chip where the device is positioned. In the top part, a viewing window for reading the signal and notches to insert the sample, the wash and the substrate are integrated. These are

located were the inlets for the reagents are on the actual device. Pins are integrated into the top half to press down the strip at critical areas. These are the conjugate pad, the test membrane and the absorption area. These pins improve the fluid flow since they maintain a good connection between the different materials used on the strip. In order to improve the reliability of the valves in terms of a consistent opening, these are compressed. By compressing the valves, a good contact between the surfactant and the hydrophobic disc can be attained. The surfactant can then dissolve evenly into the hydrophobic disc and opens the valve. The compression of the valves is achieved by integrating semicircular shapes in the top half. After the assembly of the housing, these shapes partially surround the valves and provide good contact between the layers.

An exploded view of the designed housing for the 3-fluid device is shown in Figure 21. The shapes to compress the valves and the pins to press down the strip can be seen in Figure 22.

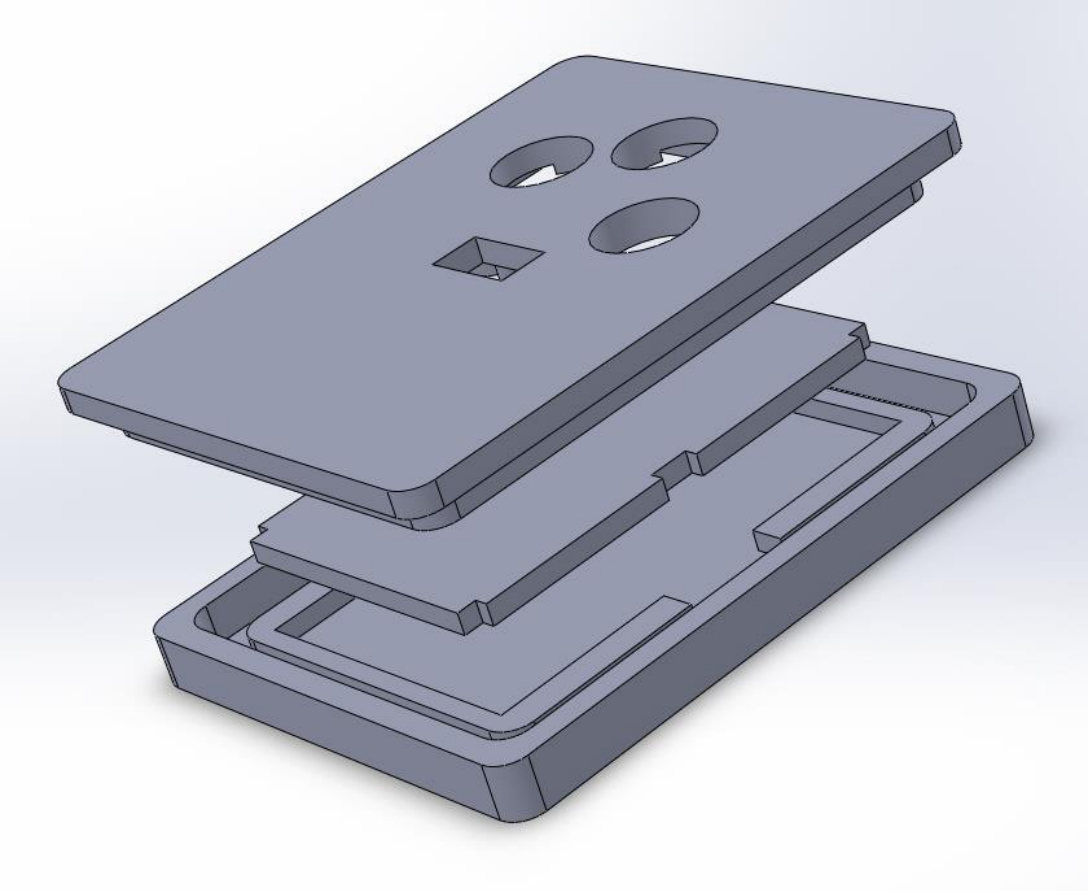

Figure 21: Exploded view of the housing for the 3-fluid chip

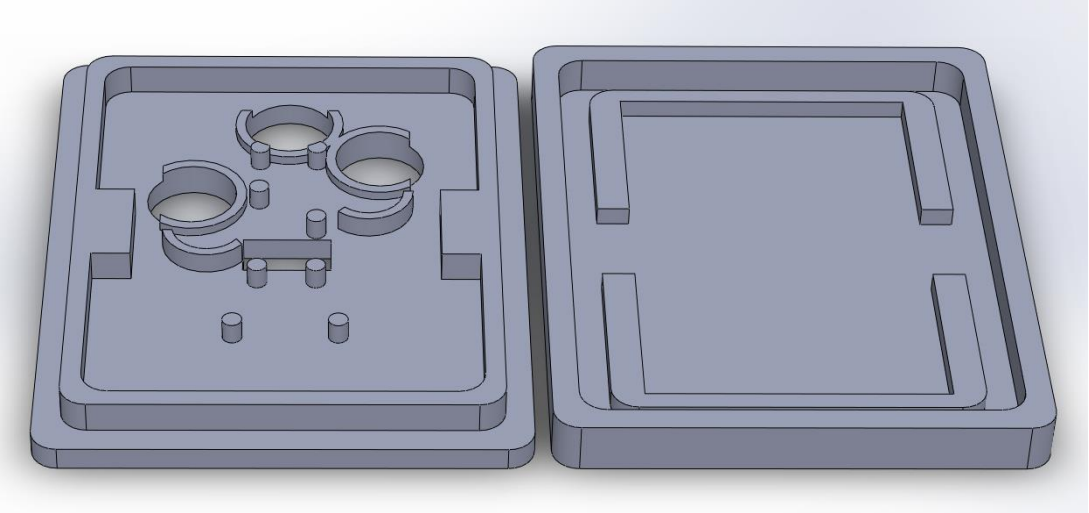

Figure 22: View on inner shapes of housing. Semicircular shapes to compress the valves and the pins to press down the chip can be seen.

3.5.2 Testing of the Housing

For testing the housing in terms of valve reliability, a test is performed. A multi-layer spot test is designed using Inkscape, the wax printer and perforated double sided tape. The spot test consists of two outer layers (top and bottom) and a valve in the center. The valve is integrated, so that the surfactant disc is located below the hydrophobic disc as in the actual device. Additionally, a housing is designed and fabricated with a feature to compress the valves. The test is performed by testing a total of 108 spots (2 tests). When bringing the bottom of the spots in contact with colored fluid, the fluid wicks through the spots dissolving the surfactant and eventually opening the valves. This can be seen when fluid is visible at the top of the spot. The opening of the valves is observed and compared to a similar test previously performed. The previous experiment was conducted using the old fabrication method and no housing to compress the valves. Comparison of the two experiments shows if an improvement of the valve reliability through compression can be attained. A CAD-drawing of the housing can be seen in Figure 23.

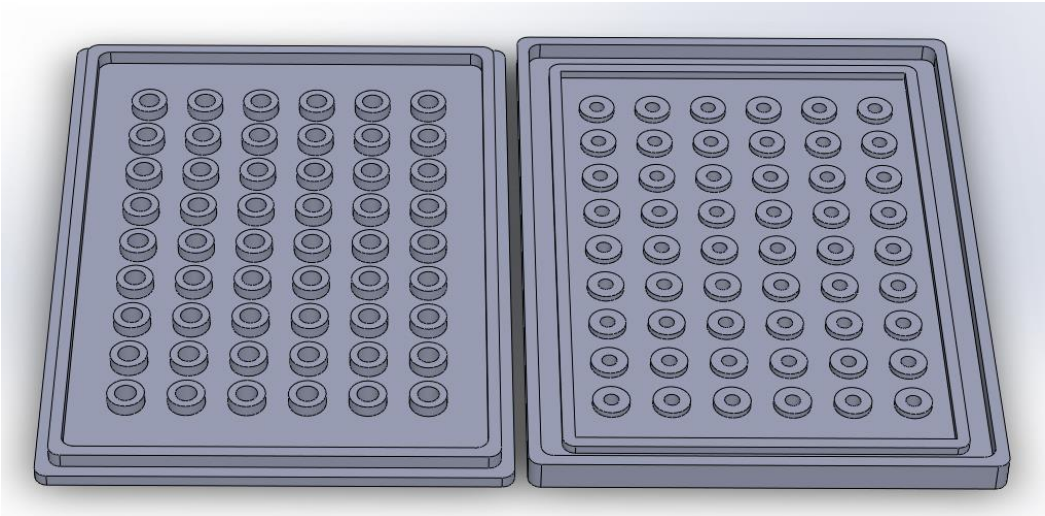

Figure 23: CAD-drawing of housing to test the compression of the valves (left: top half, right: bottom half)

3.5.3 Development of Reagent Storage for the Housing

In order to utilize the multi-fluid test in a fast manner and to reduce the misapplication of reagents, a storage is developed for the housing. The reagent storage needs to provide with an easy preparation and assembly. Because the reagents should be stored in a fridge, a separation between storage and the device is desired. The user is then only loading the reagents onto the dedicated inlets and adding the sample. To store the reagents, small plastic vials, able to hold the required amount of fluid, are used. These can be sealed and stored until needed. To utilize the test, the vials are opened and applied into the dedicated area in the housing onto the inlets of the test. If the fluid gets in contact with the inlet, the wicking process in paper begins, but eventually stops at the valve. If the sample is then applied by the user, also by using a vial, the reagents are triggered. The reagent flow out of the vial into the device due to cohesiveness of the fluid. The vials are held in the housing using a press fit. This ensures that the vials sit tight in the housing and a good contact to the inlet is provided.

CHAPTER 4 - FINDINGS

In this study, a 3-fluid microfluidic device capable of running an immunoassay, based on the ELISA technique, was developed. Four valves were integrated into the device in order to reduce the assay time and improve the sensitivity. New methods for fabricating the devices were also developed and applied in order to reduce production time. Rabbit IgG, used as a sample, was tested using a colorimetric readout in order to prove the ability to run immunoassays on the device. A dose-response curve was generated and the limit of detection of the device was determined. Furthermore, the effects of the reagents used in the valves on the immunoassay were examined. For improvements in valve reliability, a housing equipped with a feature to compress the valves was developed. Based on the housing, a technique to store the reagents was integrated. This chapter presents the results of this study.

4.1. Finding of Geometric Parameters and Dimensions of 3-fluid Circuit

(1) In order to determine the length of the first trigger channel, the Washburn equation was used. For this, the effective pore size of the filter paper No. 41 needed to be determined. Small strips with a width of 1 cm were prepared using the laser cutter. By dipping the strips into colored water and measuring the time it took for the water to move 5 cm into the strip, the effective pore size was calculated using the Washburn equation. Five iterations were performed and the average time the water need to wick through 5 cm into the strip was 289 seconds. Solving the Washburn equation for the effective pore diameter D and plugging in t=289 seconds and L=0.05 m, the resulting

value for D was calculated to be 0.4763 µm. The length of the trigger channel was calculated using D=0.4763 µm and t=65 seconds (80 seconds for the sample to wick into the channel minus 15 seconds it takes at least to open the valves). The resulting value for the length of the first trigger channel was 23.7 mm.

(2) For the second fluid, the wash step was desired to have 60 µl, so that the amount of fluid between outlet 1 and the detection area could be replaced 10 times (this area can hold 6 µl of fluid). Hence, this specific amount of fluid was required to trigger the substrate. Three different channel lengths (14 mm, 15.5 mm and 17 mm) were tested in order to determine the appropriate length for the second trigger channel. Colored fluids representing the sample (130 µl) and the wash (60 µl) were applied. The trigger channel with a length of 15.5 mm showed the best results in terms of releasing the substrate after the wash was wicked into the channel. While conducting this particular test, the length for the first trigger channel was also evaluated. For instance, the applied amount of 130 µl of sample was always sufficient in order to trigger the wash. By repeating the test using colored fluids and a second trigger channel having a length of 15.5 mm, the timing of the whole circuit was measured. Timings for 5 tests were measured. These and the average times for different steps in the test are shown in Table 5. Examples of the different steps in the assay using colored fluid are shown in Figure 24. The amount of substrate used in the device is fixed to be 80 µl. This value is based on preliminary results in the laboratory. It showed the best results in terms of biological experiments using rabbit IgG.

Table 5: Timing of the 3-fluid microfluidic circuit

Test No.	Sample wicked [sec]	Wash release [sec]	Wash wicked [sec]	Substrate release [sec]	Substrate wicked/ Test Finished [sec]
1	80	84	181	184	520
2	82	85	186	190	523
3	81	83	191	197	554
4	79	80	184	191	516
5	84	87	187	199	567
Average	**81.2**	**83.8**	**185.8**	**192.2**	**536**

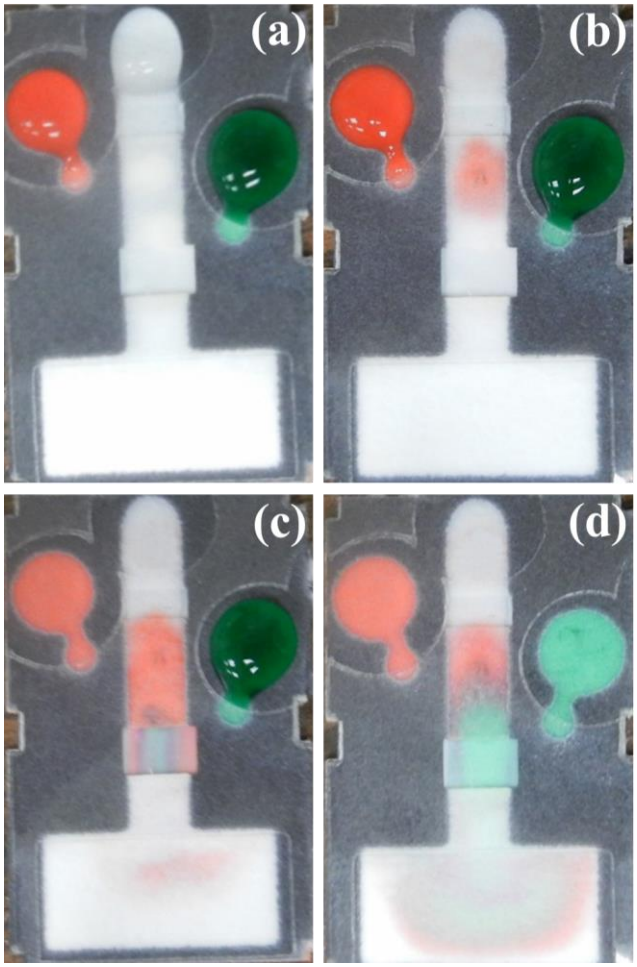

Figure 24: Pictures showing the different steps of the testing using food coloring (clear: sample, red: wash, green: substrate). (a) Start of test by applying sample. (b) Sample wicked and wash released. (c) Wash wicked and substrate released. (d) Substrate wicked/Test finished.

Having all lengths and dimensions, the size of the main channel, the trigger channels and the inlets of the chip were fixed. The main channel has a total length of 25 mm and a width of 5 mm. Trigger channel 1 is 23.7 mm long and the second trigger channel has a length 15.5 mm. Both trigger channels are 2 mm wide. The inlets for the wash and the substrate are circles with a diameter of 6.5 mm. This dimension was chosen so that the vials for the reagent storage fit onto them and do not touch the hydrophobic wax. If the liquid in the vials would touch the wax, the fluid could flow across the layer and not into the inlet.

(3) In order to determine the size of the absorption area for the device, the amount of fluid it needed to hold was tested. The whole amount of fluid applied in the test was 270 µl (130 µl sample, 60µl wash and 80 µl substrate). The amount of fluid remaining in the channels was 30 µl. The absorption area, therefore, has to hold 240 µl of fluid. Capacity tests using glass fiber and blotting paper were performed in order to see how much fluid can be absorped. It was found that 1 cm^2 of binder-free glass fiber can hold about 28 µl of fluid. Blotting paper with the same surface area can hold 50 µl, but it is slower in terms of fluid absorption rate. A stack of four pieces of glass fiber with a total surface area of 6.5 cm^2 (1.625 cm^2 per piece) was implemented into the chip. The glass fiber in the device can now hold an amount of 182 µl of fluid (75.8 % of the whole amount). The remaining 58 µl of fluid is being hold by 1.16 cm^2 of blotting paper. Since the integration of glass fiber (with a width of 18.06 mm) adds 9 mm of length to the chip, the overall dimensions of the chip became 23 mm in width and 36 mm in length. By covering the back of the chip with blotting paper, a total surface area of 8.28 cm^2 was attained. The total absorption area can therefore hold 2.5 times the

total amount of fluid used and thus, the fluid flow can be kept at a good rate. In Figure 25, all channel lengths as well as the size of absorption area and the overal dimensions of the chip are shown.

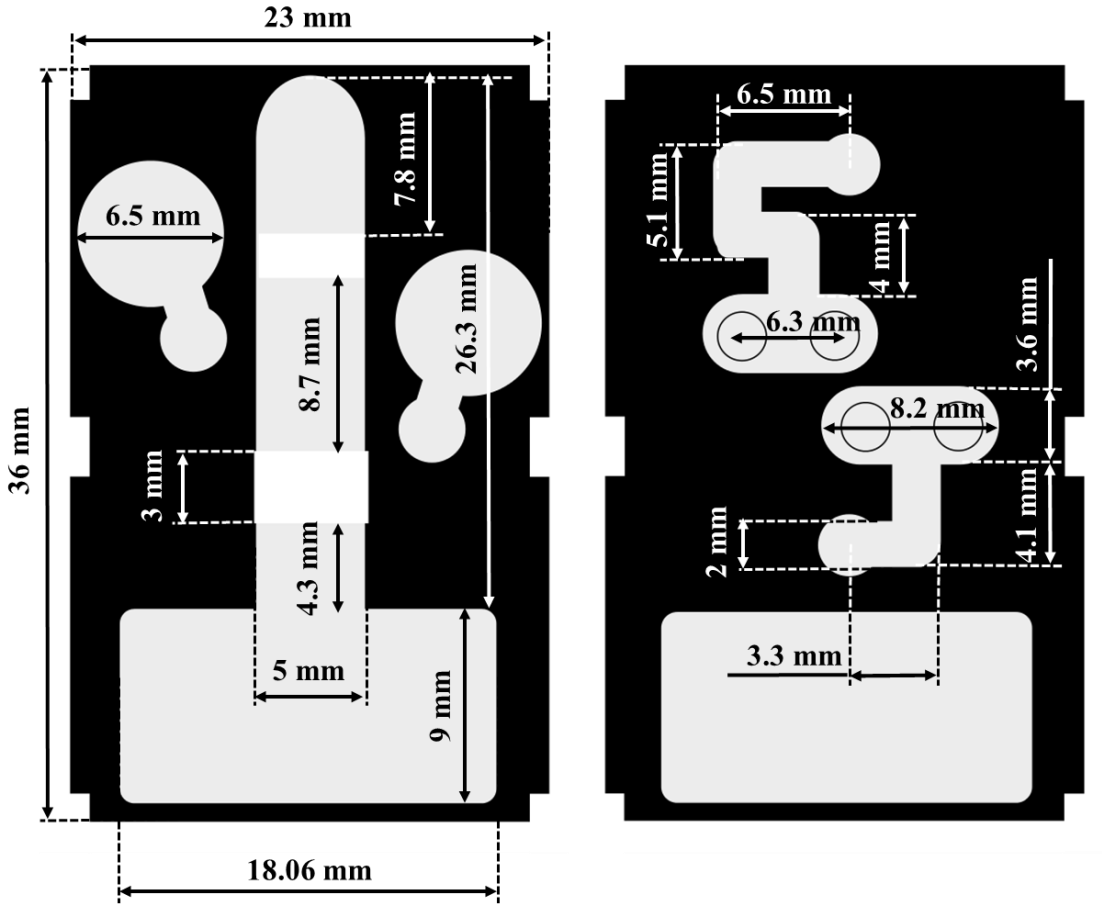

Figure 25: Dimensions of chip and lengths of channels

4.2 Improvement of Fabrication Findings

For the new fabrication method, 9 different concentrations of allyltrichlorosilane were tested with six iterations per concentration (see section 3.3). It was found that after one minute concentrations 1 to 5 already failed. This could be seen because the drop of colored water was wicking into the spot (see Figure 27) and into the blotting paper. After ten minutes, concentrations 6 to 8 also started to fail (see Figure 28). Only the highest concentration being tested was still holding the fluid on the spots even

after 20 minutes. Then the test procedure was stopped. By removing the test from the blotting paper, it was seen that most of the spots for the concentrations 1 to 7 failed by letting the liquid wick into the paper (see Figure 29). One out of six spots of concentration 8 failed, but only a small amount of fluid was visible on the blotting paper. Hence, this was not sufficient for the application in the device. Only the highest concentration tested was able to hold the reagents until the test procedure was stopped. Thus, concentration 9 (4.76 % of allyltrichlorosilane dissolved in FC-72) was chosen for the hydrophobic discs in the new fabrication method.

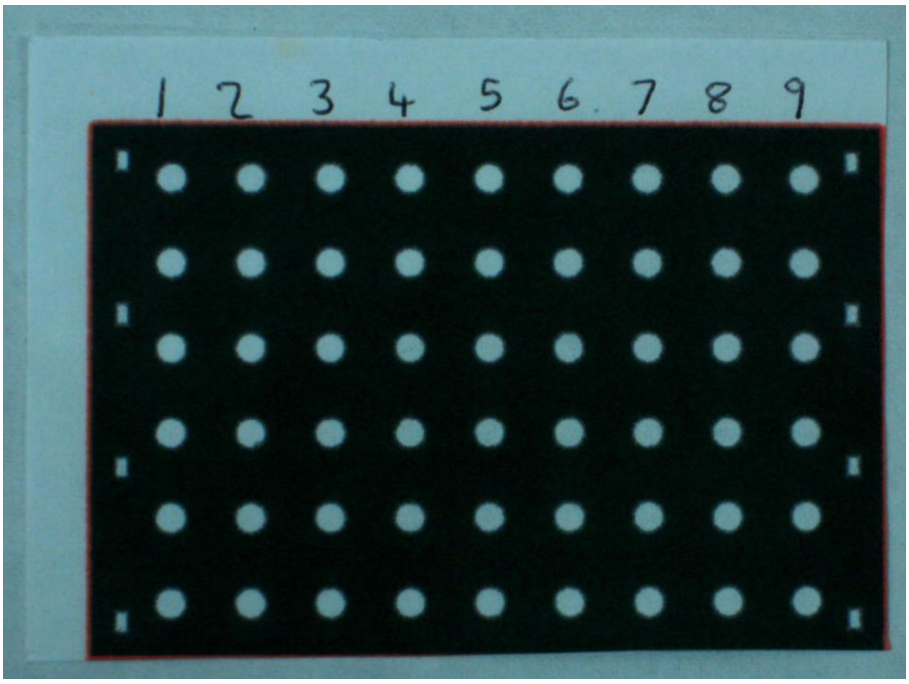

Figure 26: Spot test before starting the test procedure

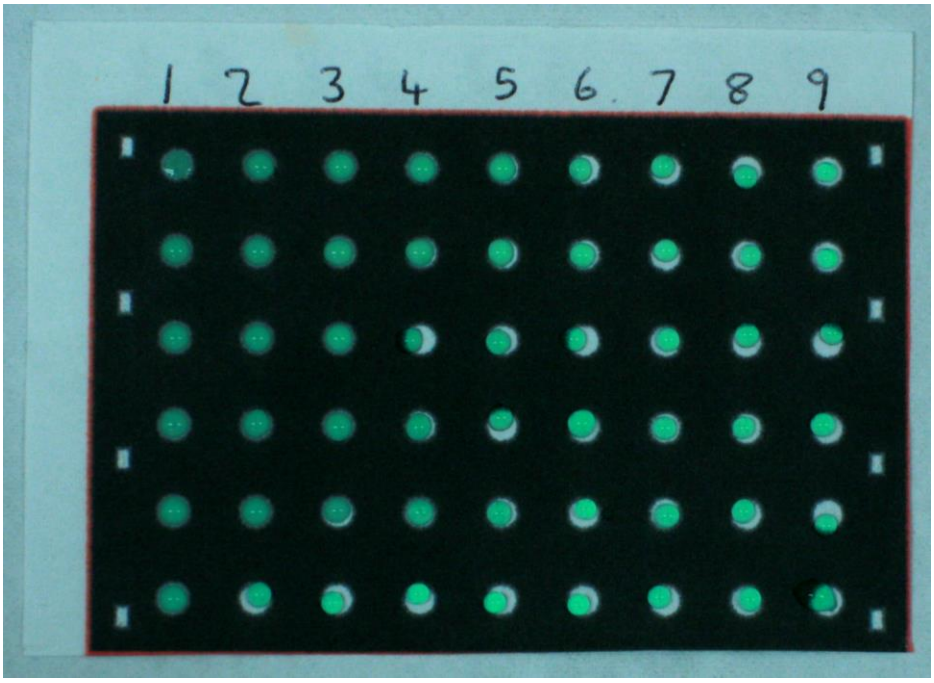

Figure 27: Spot test after 1 minute (concentration 1 to 5 already failed)

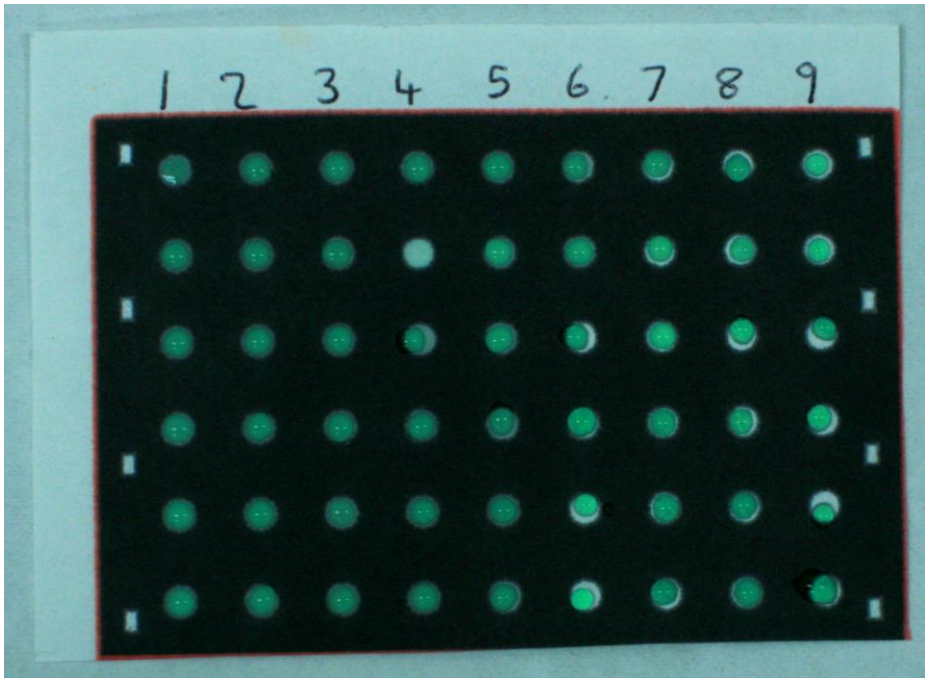

Figure 28: Spot test after 10 minutes (concentration 6 to 8 start to fail)

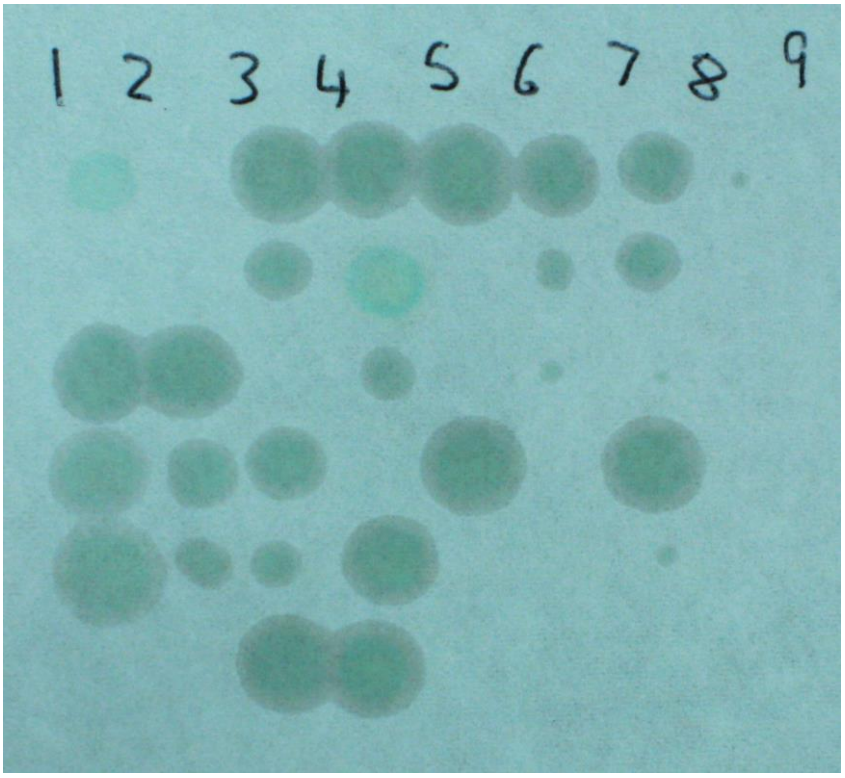

Figure 29: View on blotting paper (concentrations 1-8 failed)

4.3 Results of Biological Experiments

In this section, the findings of the biological testing are shown. At first, the results of the experiments for determining the best material for the conjugate pad and reagent concentrations are discussed. Using the determined parameters, the testing was performed using rabbit IgG. The calibration curve was created and the limit of detection was determined. Finally, the findings of the experiments to determine the influences of allyltrichlorosilane and Tween 20 on the sensitivity of the immunoassay are shown.

4.3.1 Improvement of Conjugate Release Findings

Two different kinds of sugars, trehalose and sucrose and a mix of the trehalose and sucrose in different concentrations were tested. The results of the different concentrations tested can be seen in Figure 30.

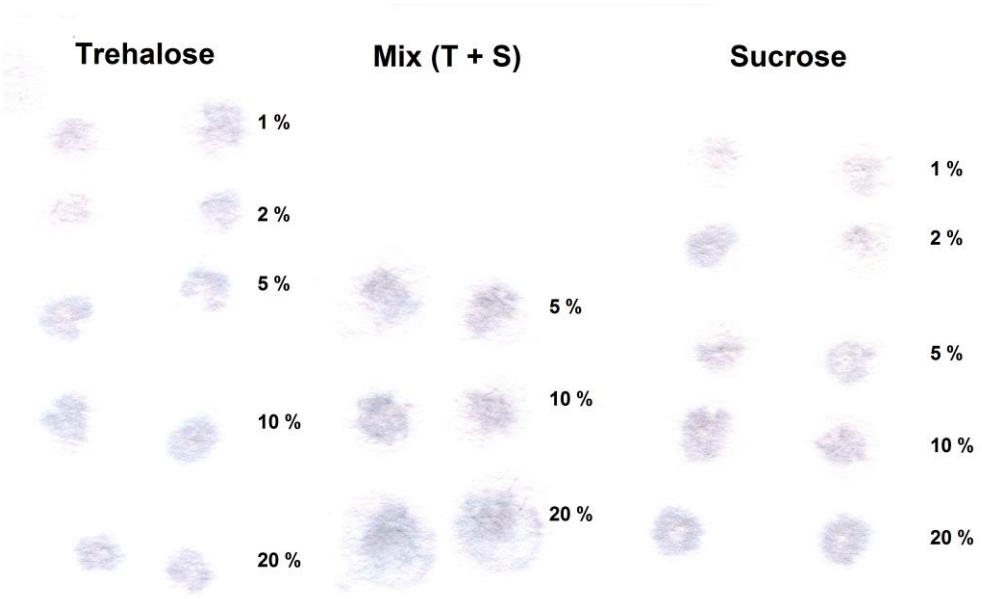

Figure 30: Results of sugar testing

The color intensity (mean gray value) was measured using ImageJ. The mean values of the two measurements per concentration and the standard deviation were then calculated. In Figure 31, the measurements of the experiments were plotted. It can be seen that the results for each sugar type can be approximated by a logarithmic trendline. Also, the mix of trehalose and sucrose is consistently better in releasing the conjugate of labeled detection antibodies than one sugar type alone.

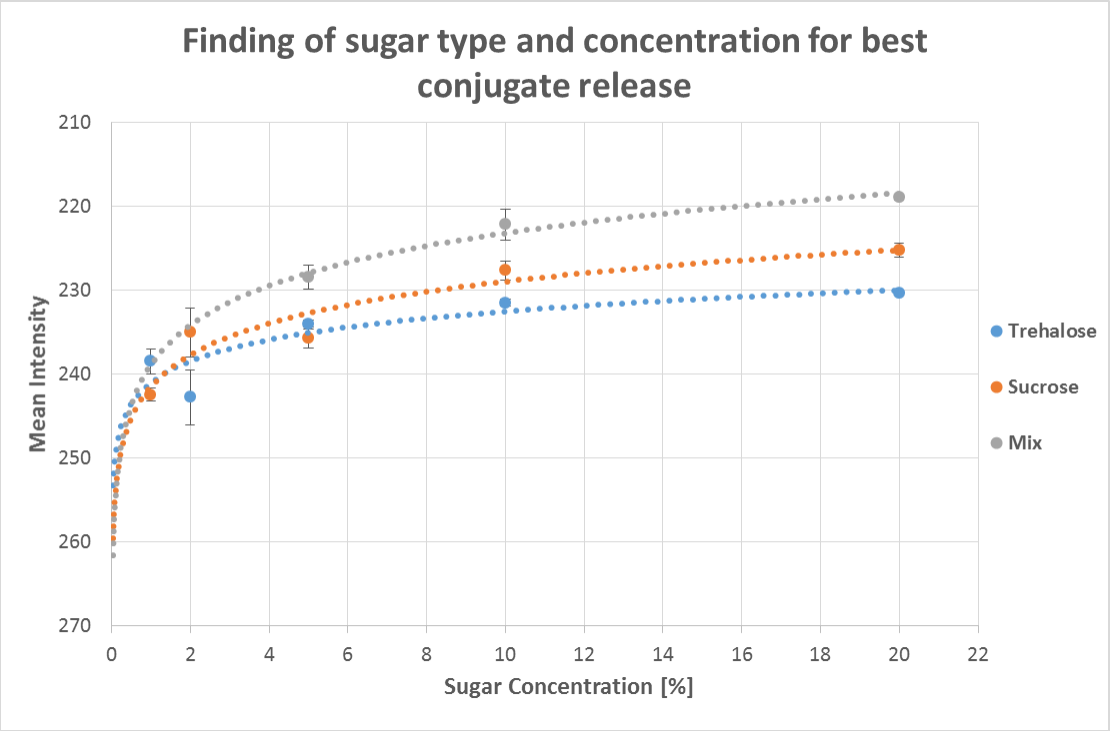

Figure 31: Plot of mean color intensity for different types of sugar and different concentrations

The logarithmic function used to approximate the results for the mix of trehalose and sucrose can be found as:

$$f(x) = -6.829 \ln(x) + 238.91 \quad [6]$$

The mean intensity attained with the 20 % concentration was 218.45. For a 50 % and a theoretical 100 % concentration of the sugar mix, these values were calculated to be 212.2 and 207.46, respectively. These values are slightly better in terms of the release rate compared to the 20 % result. However, they would cause difficulties in fabrication of the solution and the conjugate pads. Concentrations of sugar dissolved in SuperBlocker higher than 20 % are becoming very viscous. Furthermore, the

solubility of trehalose in liquid is limited. Additionally, the fabricated conjugate pads are hardly drying. Therefore, a 20 % solution of trehalose and sucrose was used.

Based on the most promising sugar type and concentration, the concentration of labeled detection antibodies was optimized. Four different concentrations of detection antibodies with 3 iterations per concentration were tested. The analyte concentration was constant at 500 ng/ml. One result per concentration is shown in Figure 32.

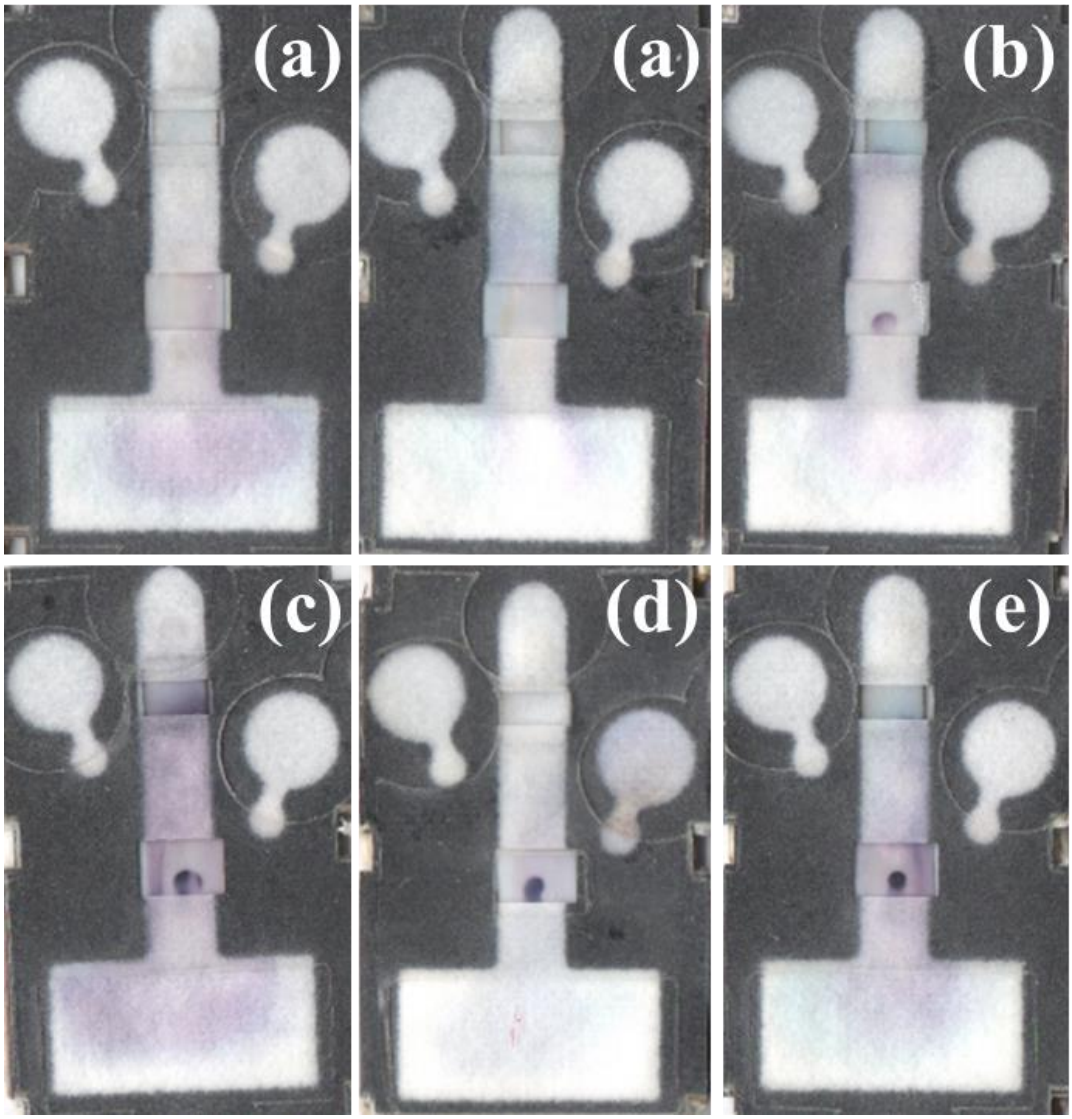

Figure 32: Experimental results of detection antibody testing. Concentrations are: (a) negative controls, (b) 20 µg/ml), (c) 30 µg/ml), (d) 40 µg/ml and (e) 50 µg/ml

The mean gray values of the signal and the background were measured using ImageJ. The Signal-to-Background ratio was calculated by dividing the signal strength by the background noise for every iteration per concentration. The mean values were then determined and the standard deviation was calculated. A plot of the Signal-to-Background ratio for every concentration is shown in Figure 33. A high ratio means good visibility of the signal compared to the background noise.

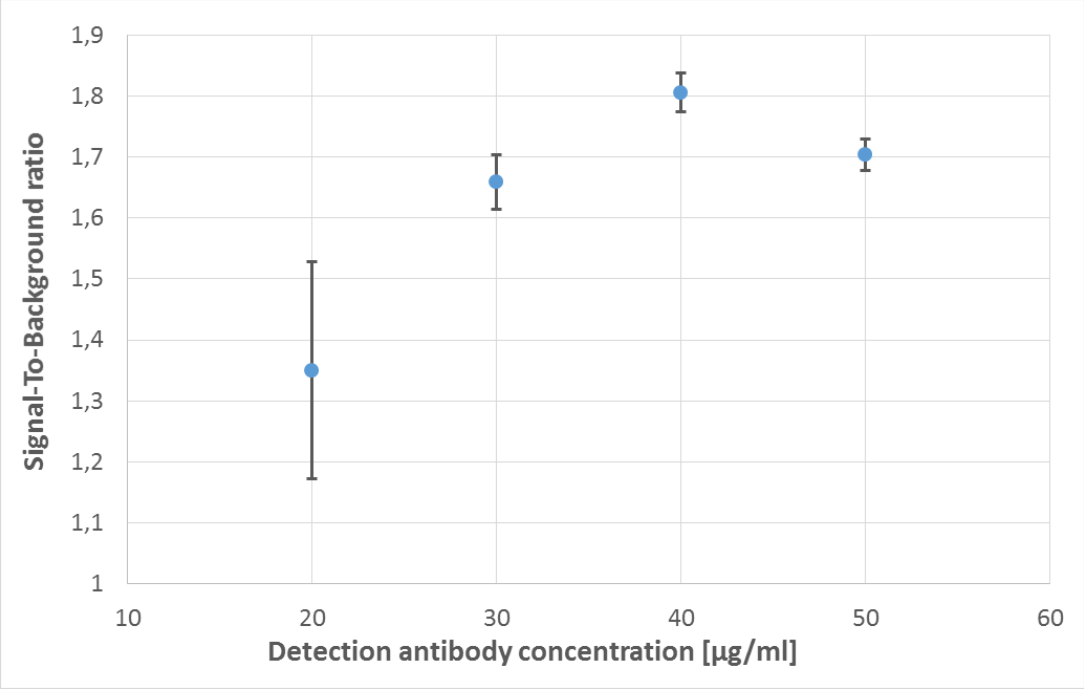

Figure 33: Graph showing the mean values of the Signal-to-Background ratio for all concentrations of detection antibody tested

Based on this results it can be seen that the optimal value of detection antibody, giving the best Signal-to-Background ratio, is at 40 µg/ml. If higher concentrations are used, the background noise intensity increases faster than the signal strength leading to a lower Signal-to-Background ratio. Concentrations below 40 µg/ml also yield a lower Signal-to-Background ratio. For instance, the signal strength decreases faster than the associated background noise.

4.3.2 Generation of Calibration Curve and Determination of Detection Limit

The calibration curve was created using ten different analyte concentrations and a negative control. Three iterations were performed per concentrations. Examples of the results for every concentration tested and the negative control can be seen in Figure 34.

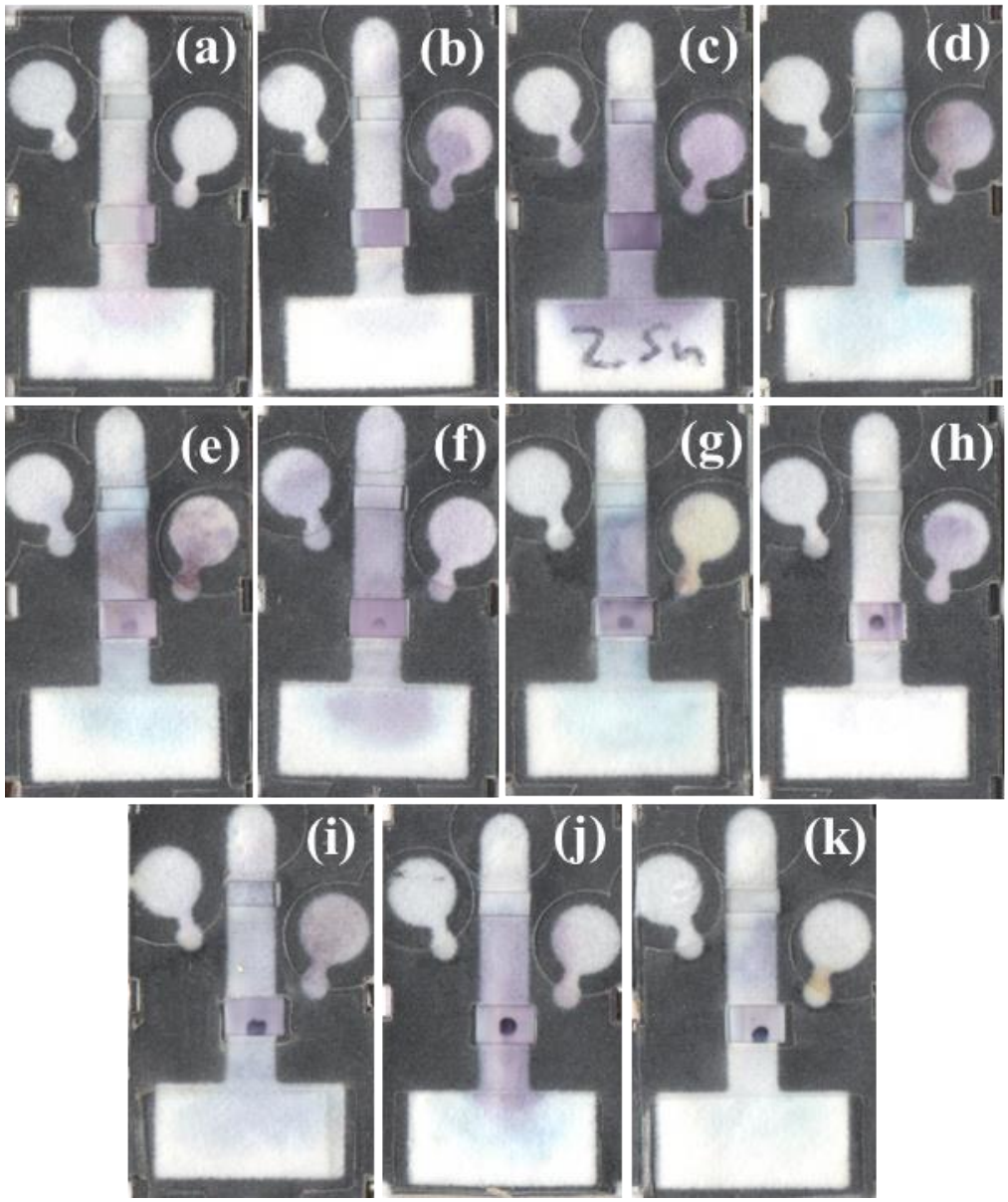

Figure 34: Examples of the results of different concentrations of rabbit IgG in the developed 3-fluid circuit. (a) Negative control. (b) 1 ng/ml. (c) 2.5 ng/ml. (d) 6 ng/ml. (e) 8 ng/ml. (f) 10 ng/ml. (g) 50 ng/ml. (h) 100 ng/ml. (i) 500 ng/ml. (j) 1 µg/ml. (k) 5 µg/ml.

The mean intensities of signal and background were measured using ImageJ. The Signal-To-Background ratio was then calculated for every single test. The negative control has a value of one (no signal compared to the background). Samples containing analyte result in higher ratios. The mean value for every concentration was determined and the standard deviation of the values to the mean. The data points were approximated using the sigmoidal Weibull model and the calibration curve was attained. In equation 7 the resulting Weibull function used to fit the data points is shown.

$$Y = 2.03 - 1.08\, e^{-3.7 * x^{0.63}} \quad [7]$$

A plot of the calibration curve for rabbit IgG in the 3-fluid circuit is shown in Figure 35.

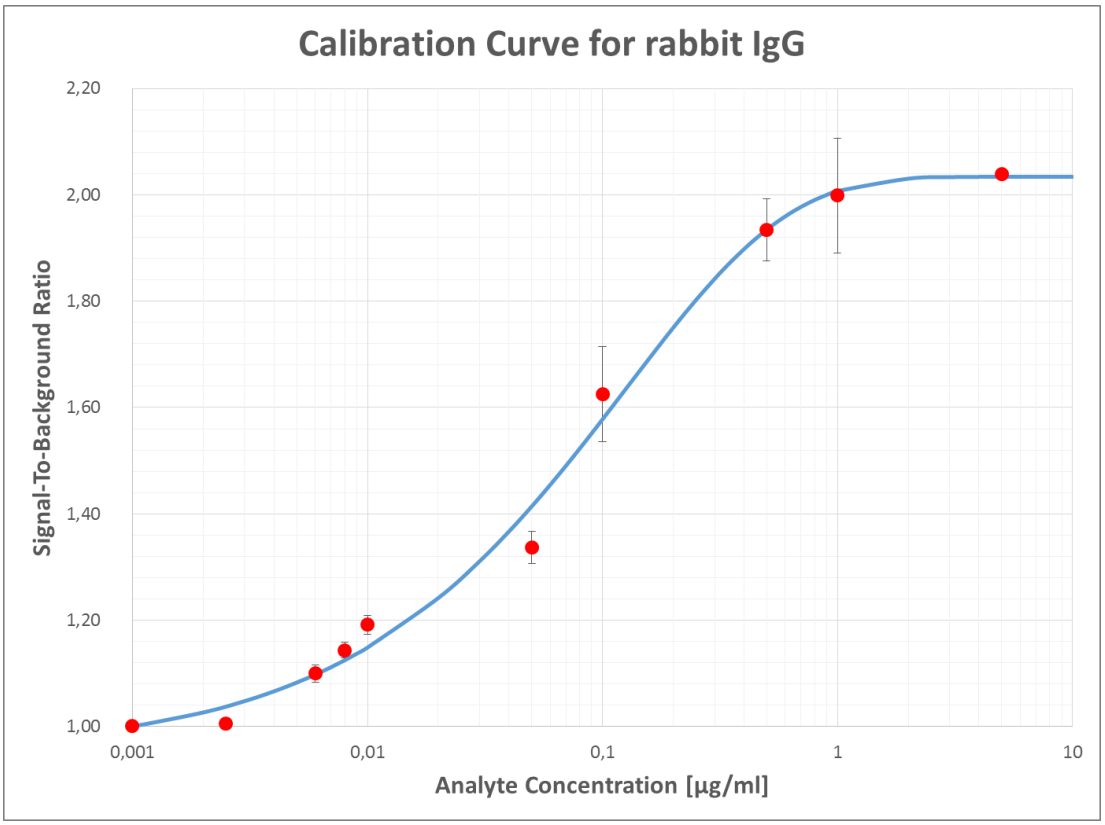

Figure 35: Calibration Curve for rabbit IgG

The limit of detection for this device using rabbit IgG was detected visually and by using the blank determination method. By evaluating the results in Figure 34, it can be seen that down to a concentration of 6 ng/ml a signal could be detected visually. At a concentration of 2.5 ng/ml, no signal was visible. The LOD for this device, using rabbit IgG, is therefore in a range between 6 ng/ml and 2.5 ng/ml.

A calculation of the LOD was performed using equations 5 and 7. The intensities of the negative control were measured and the mean value was calculated. By dividing every value by the mean, a ratio was attained. For these ratios, the mean and the standard deviation were determined. This standard deviation is used for caluating the LOD since it indicates the variations of background in the test. In Table 6, the

intensity values of the negative control and the calculated means and the standard deviation can be seen.

Table 6: Calculated values for the negative controls used to attain the LOD

Iteration	Intensity of Negative Control	Ratio Intensity/Mean Intensity
1	188.565	1.015386
2	186.365	1.003539
3	182.193	0.98469
Mean	185.708	1
Standard Deviation		0.02592327

Plugging the mean and the standard deviation into equation 5, the corresponding value of the Y-axis was attained. The calculated value was 1.0778 and it predicated the Signal-To-Background ratio at the theoretical LOD. The corresponding X-value for the calculated ratio was attained by solving equation 7 for x. The calculated value was 4.7 ng/ml. This theoretical LOD for rabbit IgG in the 3-fluid device matches the results of the experiments and the visible signals. For a concentration of 6 ng/ml, a signal was seen by the naked eye, whereas for a 2.5 ng/ml, no difference between signal and background could be measured. The overall LOD for this device using rabbit IgG was therefore set between 4.7-6 ng/ml.

4.3.3 Effects of Valve Reagents in the Immunoassay Findings

Devices containing no valves and therefore, no allyltrichlorosilane and Tween 20 were fabricated. Antigen concentrations of 1 µg/ml and 100 ng/ml were tested. Examples of the attained results can be seen in Figure 36.

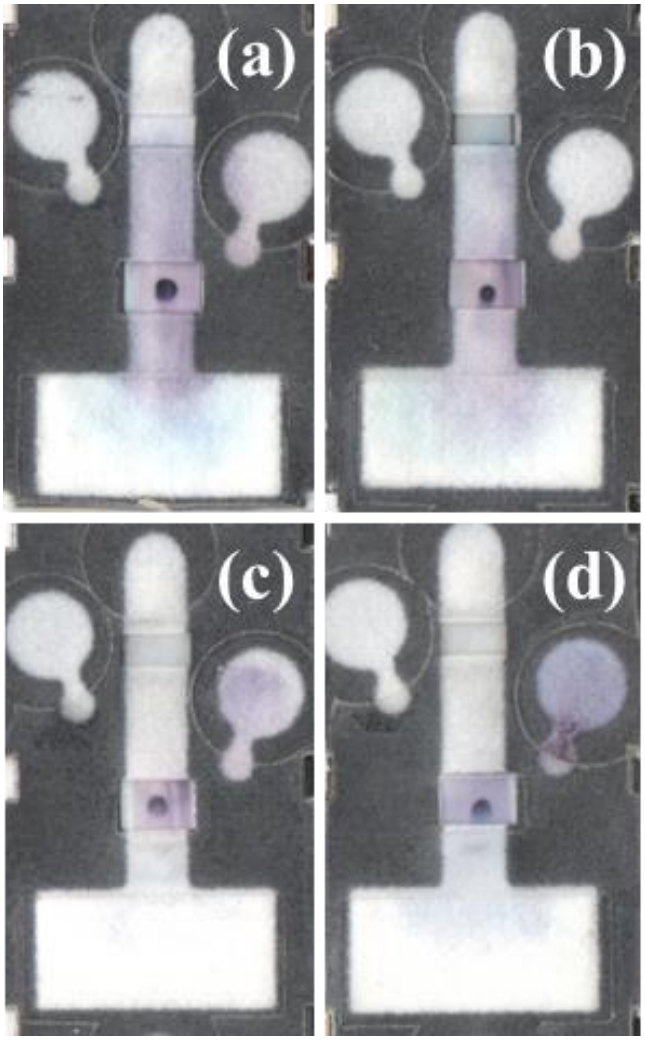

Figure 36: Example results for testing of the 3-fluid device without PBMV. For comparison, the results from section 4.3.2 are shown again (with PBMV)

The mean grey values of signal and background were measured for every test and the Signal-To-Background ratio was calculated. The mean Signal-to-Background ratios for both concentrations with and without valves are shown in Figure 37.

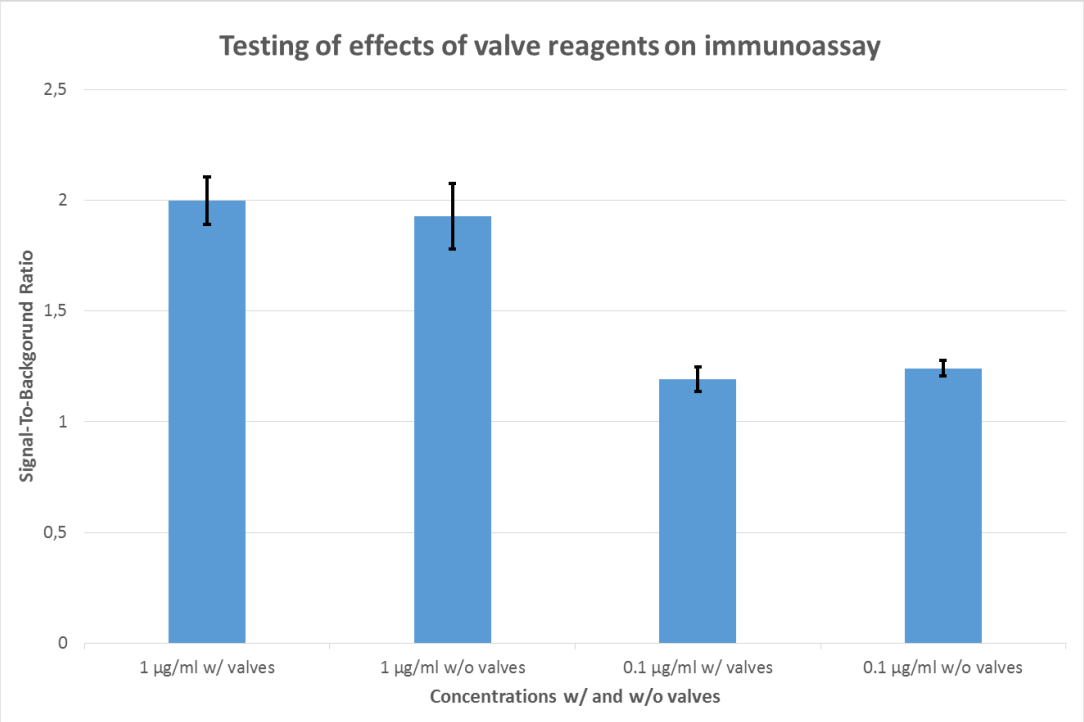

Figure 37: Mean Signal-To-Background Ratios for experiments conducted with and without the PBMV technology

For both antigen concentrations tested, the Signal-To-Background ratio was very similar. Only slight differences in the ratio and standard deviation could be measured. It can be seen that for the low analyte concentration, the results in the device using no PBMV technology are slightly better. For the high analyte concentration, the device with valves gives better results in terms of Signal-to-Background ratio and standard deviation.

After conducting this experiment, it can be seen that the valve reagents allyltrichlorosilane and Tween 20 do not affect the results in the biological tests for concentrations used. Both results are almost similar. Neither an improvement nor a decrease in the assay sensitivity was seen.

4.4 Housing Findings

The housing for the 3-fluid device was designed in SolidWorks and fabricated using the rapid prototyping machine at the URI College of Engineering. A picture of the finished product, including a chip, is shown in Figure 38.

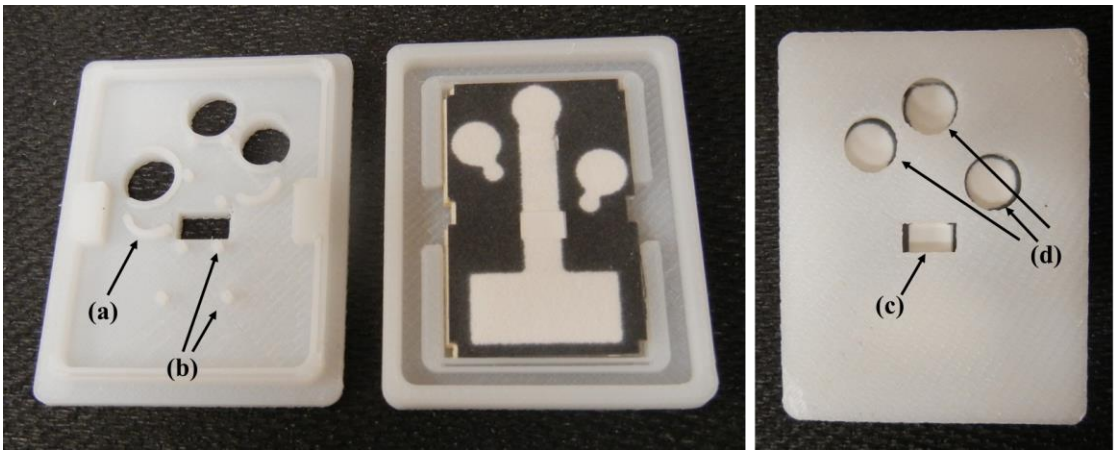

Figure 38: Photo of housing including the 3-fluid device. The left picture shows a disassembled housing, the left picture shows an assembled housing. (a) Semi-circular shapes for compressing the valves. (b) Pins to press down the device at the connections between materials. (c) Viewing window. (d) Fluid inlets

In order to see if the feature of compressing the valves improves the reliability and lowers the time to open the valves, the method described in section 3.5.2 was applied. The housing used for this procedure is shown in Figure 39.

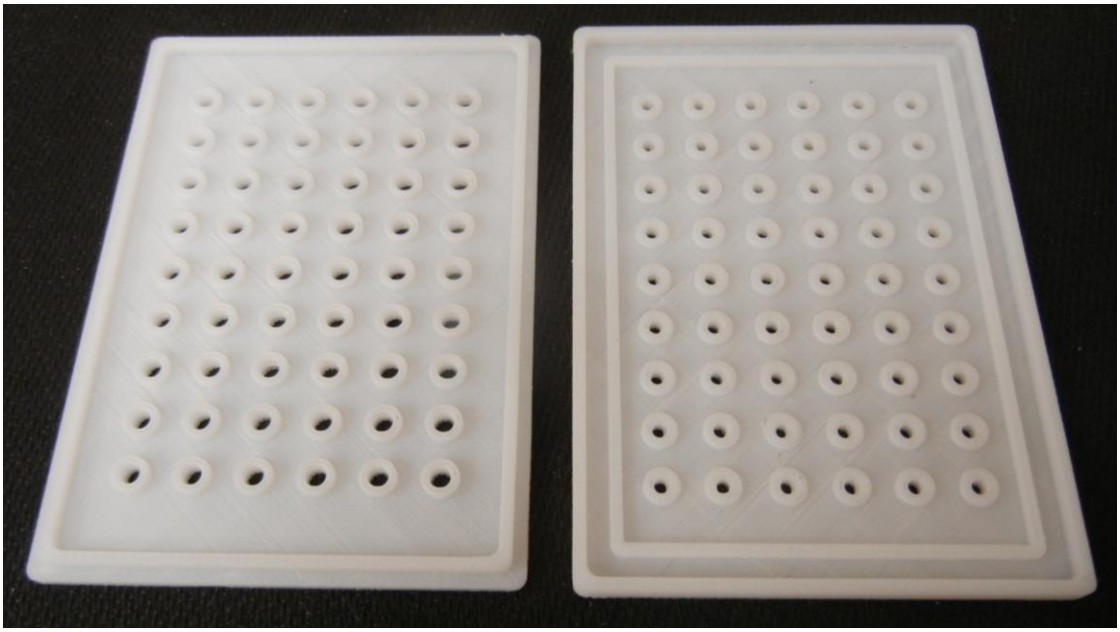

Figure 39: Housing fabricated to test the feature of compressing the valves (left: top part, right: bottom part)

After submerging the housing from the bottom into colored fluid, a measurement of the time it took the valves to open was taken. The test was performed two times in order to attain reliable data. Pictures taken at different times during testing are shown in Figure 40. A white spot means the valve is not opened. A green spot indicates an open valve which allows fluid to pass through. During testing the housing was compressed using binderclips in order to attain even pressure on all valves.

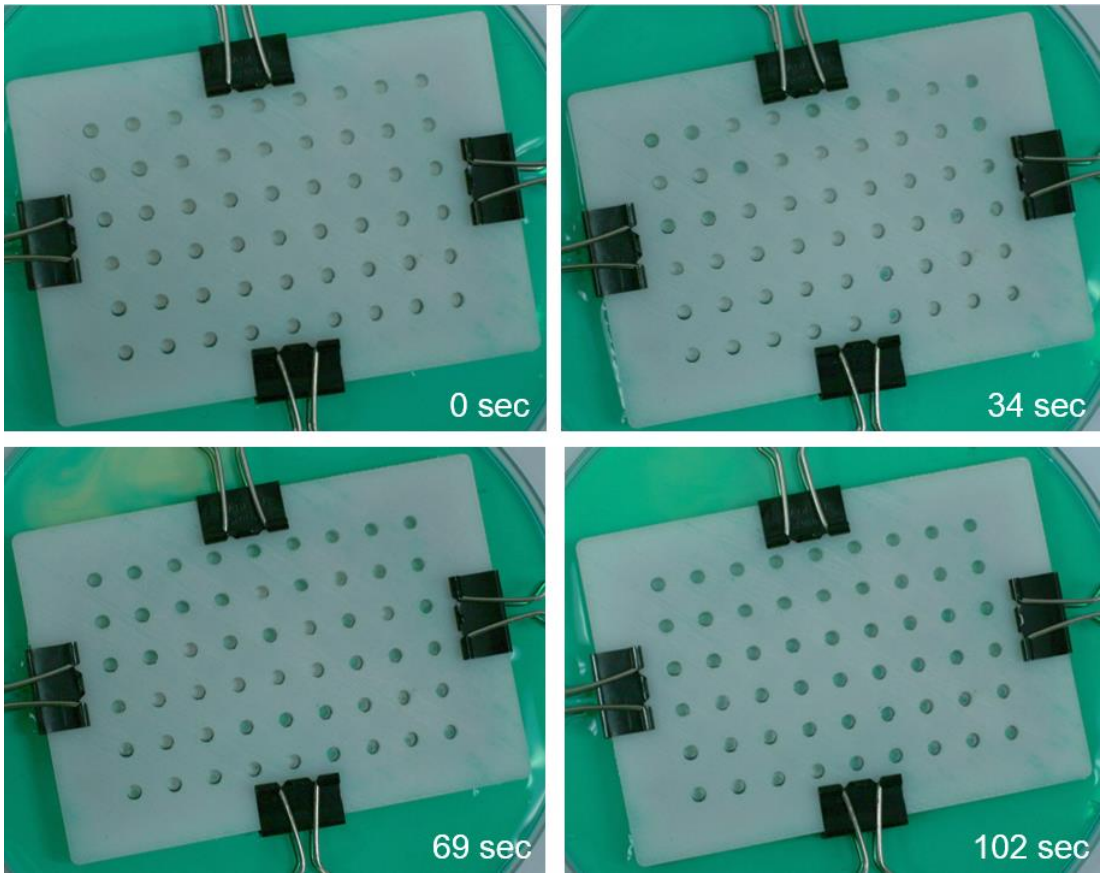

Figure 40: Photos of Valve Testing taken after 0, 34, 69 and 102 seconds (enlarged versions and pictures of other sampling points are shown in the Appendix)

The percentage of openend valves was calculated and plotted over time for both tests and can be seen in Figure 41.

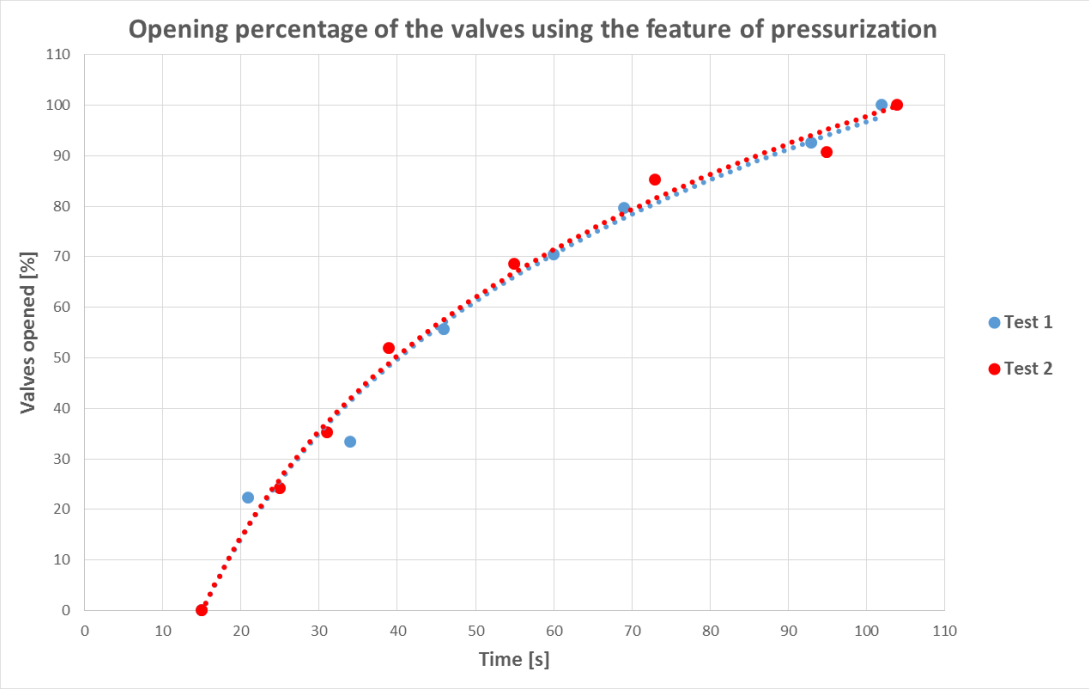

Figure 41: Plot of valves opened (in percent) over time

Figure 41 shows that both iterations of the test procedure gave similar results regarding valve opening. The deviations between both tests are very small. With a time of 15 seconds it took the first valve to open, the data points can be approximated by a logarithmic function. Both functions almost match.

By comparing the results of the preliminary tests conducted in the laboratoy, which used anold fabrication method and no housing, to the new results of this study, an improvement was attained. Although, the both preliminary and final tests were performed in the same way, by submerging the bottom of the test into colored fluid, they showed dramatic differences. The data collected during the preliminary experiments showed, that after 65 seconds, 50 % of the valves were open. However, during the final test which included the use of a housing and a new fabrication method, 75 % of the valves were open at this point. Moreover, after 102 seconds, only 75 % of the valves were open in the preliminary tests compared to 100 % in the final

method using the housing. It took 170 seconds in the old method to attain a opening rate of 95.8 %. 4.2 % of the valves failed complety by not opening even after 250 seconds.

Based on results, it can be seen that the usage of the housing combined with the new fabrication method can improve the consistency of the multi-fluidic system by reducing the failure rate of the valves and the timeframe in which the valves open. With an improved contact between the layers of the chip, more reliable results in regards to time and fluid release rate are possible.

The reagent storage was implemented into the housing using small vials available in Dr. Faghri's laboratory. The vials are pressed into the dedicated port in the housing. The ports are designed to be of the same diameter than the vials in order to attain a press fit when inserted. A lip at the vials helps keep them tight at the respective spot in the housing. After the test, the vials can be removed, cleaned and reused, in order to reduce waste. Photos showing the steps of preparing the housing including the reagent storage are shown in Figure 42.

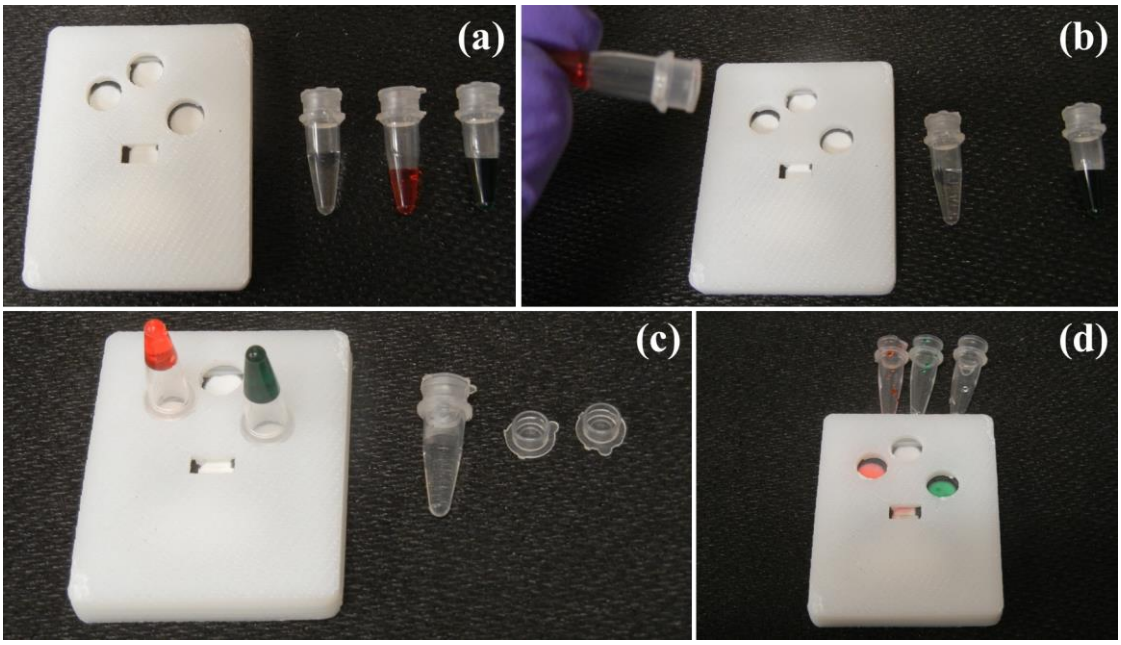

Figure 42: Photos showing the usage of the reagent storage. (a) Housing with inlets and the vials for antigen (clear fluid, 130 µl), wash (red fluid, 60 µl) and substrate (blue fluid, 80 µl). (b) Inserting the vials into the respective inlets. (c) Vials inserted into the housing. (d) Removed vials after the test

CHAPTER 5 - CONCLUSION

This research demonstrated that an autonomous 3-fluid microfluidic device is achieved by using the PBMV technology developed in Prof. Faghri's laboratory. By including two valves per fluid, the time to finish the assay was decreased compared to previous designs. Another advantage using two valves instead of one valve was that the introduction of the substrate into the main channel below the conjugate pad. The background noise was lowered. Furthermore, the introduced wash step as second fluid decreased the background noise even more.

Since the new design included four valves per chip, new fabrication methods were developed in order to reduce the assembly time and decrease errors during assembly. A new method for assembly and fabrication was developed in a different study performed by Roman Gerbers [15]. This method was applied during this project. For the new technique, the reagent volumes for the hydrophobic discs were determined. Using the new method, the assembly time and the risk of misalignment were reduced.

In order to prove the ability to use the 3-fluid device for immunoassays, the model analyte rabbit IgG was applied. Using different concentrations between 5 µg/ml and 1 ng/ml, a calibration curve for rabbit IgG in the respective device was attained. By choosing a colorimetric readout, the signal was able to be read without the need for specialized equipment. The visual limit of detection for the 3-fluid device was determined to be 6 ng/ml for rabbit IgG with a sample volume of 130 µl. The

theoretical LOD was then calculated using the blank determination method. The obtained value for the Signal-To-Background ratio of the theoretical LOD was 1.0778. The respective concentration of the theoretical LOD was determined as 4.7 ng/ml. Since theoretical and visibly detected LOD were almost matching, the overall LOD was set in the area between 4.7-6 ng/ml. This value was found to be significantly better than the detection limits on paper-based devices for rabbit IgG found in the literature. The LOD of the device was found to be even better as in a conventional ELISA using microtiter-plates (8.6 ng/ml). However, no specialized equipment was needed and the time to result was very low compared to the conventional technique. A signal could be seen after less than 10 minutes. This makes the device suitable for the use in resource-poor environments. Furthermore, the test runs autonomously after adding the sample and is not needed to be conducted by laboratory personnel. Moreover, less reagent volumes are required compared to a conventional ELISA. Finally, the materials used in the 3-fluid device are mostly biodegradable and, therefore, the device has a smaller footprint compared to the material used to fabricate microtiter-plates.

An experiment was performed in order to determine the effects of allyltrichlorosilane and Tween 20 in the system. Several devices were tested without these reagents by manually adding each reagent. Only marginal differences between the results with and without valves were seen. Therefore, effects of the valve reagents on the sensitivity of the immunoassay in the 3-fluid device were foreclosed.

For the new fabrication method using disc-holding layers, instead of single discs, a housing was developed in order to improve the reliability of the valves. It was

proven that the compression of the valves lowers the opening time of the valves and increases their reliability. Using the old method and no housing, a total opening rate of 95.8 % after 170 seconds was observed. 4.2 % failed completely. In the new method no single valve failed and after 102 seconds all valves were opened. After this time, only 75 % of the valves using the previous method were opened.

Based on the compression feature, a housing for the 3-fluid device was produced. Furthermore, a technique for storage the reagents was integrated. Wash and sample were stored in small vials, which only had to be pressed into the dedicated area in the housing. After adding the sample, the test finished autonomously and the signal could be read.

5.1 Future Work

To further improve the 3-fluid microfluidic device developed in this study, improvement of the materials used could be done. A material called Fusion 5, developed by Whatman, could be tested as material for the channels and conjugate pad. It offers a high test sensitivity and a low background noise due to low protein binding in the fibers. The release of the detection antibodies from the conjugate pad could be improved and the binding of antigens in the channels could be prevented. Due to its ability to be used as conjugate pad, the channels and the detection area component contact issues could be eliminated.

To further improve the sensitivity of the device, an amplified system could be applied. An amplified system provides with several fold increased sensitivities by labeling large molecules with enzymes and conjugating it to the detection antibody. One detection antibody would then carry multiple enzymes. Thus, less detection

antibodies at the detection site are needed to attain a signal. The sensitivity could also be improved by changing the material for the detection area. The nitrocellulose used in this study had limitations in binding the capture antibodies. A material with a larger internal surface area would provide with more binding sites for the capture antibodies. Hence, the sensitivity of the device could be increased. The above mentioned material, Fusion 5, could be a material providing with this property. Because of its low protein binding characteristics, latex beads with a diameter of about 2 µm are needed. They bind to the material and can be used to immobilize the capture antibodies at the desired location.

Another way of increasing the sensitivity of the 3-fluid design is to change from a colorimetric readout to an electrochemical detection. First attempts to include an electrode into the device were conducted in collaboration with Prof. Munge from Salve Regina University in Newport, Rhode Island. In their current design for electrochemical detection of the biomarkers Interleukin 6 and Interleukin 8 (head and neck cancer), expensive equipment such as pumps are needed. By including the electrode into the 3-fluid design, this equipment would not be necessary anymore, since the multi-fluid circuit is able to sequentially load the reagents without the need of pumps. Only a potentiostat to measure the signal would be required.

A very important property for microfluidic devices is the possibility of multiplexed analyses. By running several tests simultaneously in a single run, multiple analytes can be measured at the same time. The developed 3-fluid design could be adapted for multiplexed analyses. Several chips equipped with different antibodies to test for several analytes could be produced. A housing with the capability to hold these

devices in a circular-shaped array could be developed. In the center of this array, the sample could be added. Channels in the housing filled with a porous material could lead an equal fraction of the sample to every inlet of a device. The devices would then all be run at the same time, after the sample was added. By equipping the housing with the developed reagent storage for every device, all tests would be completed autonomously after adding the sample.

APPENDICES

In this appendix enlarged figures of the photos taken during the valve testing are shown. For every picture the number of valves opened is given. In Figures A9 – A12 drafts of the different housings developed in this study are shown. Dimensions are in millimeters.

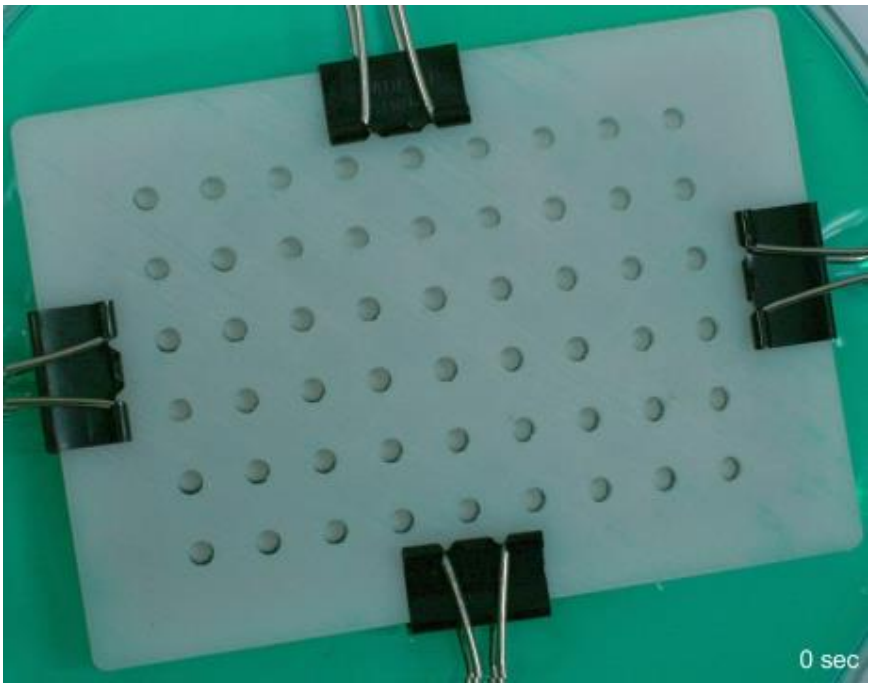

Figure A1: Photo of valve testing taken after 0 seconds (0/54 valves opened)

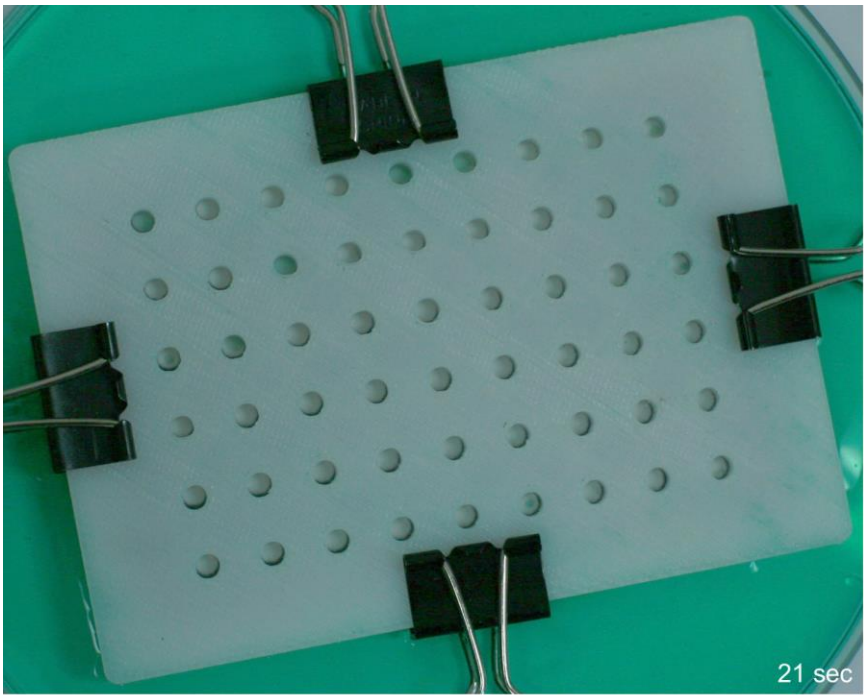

Figure A2: Photo of valve testing taken after 21 seconds (12/54 valves opened)

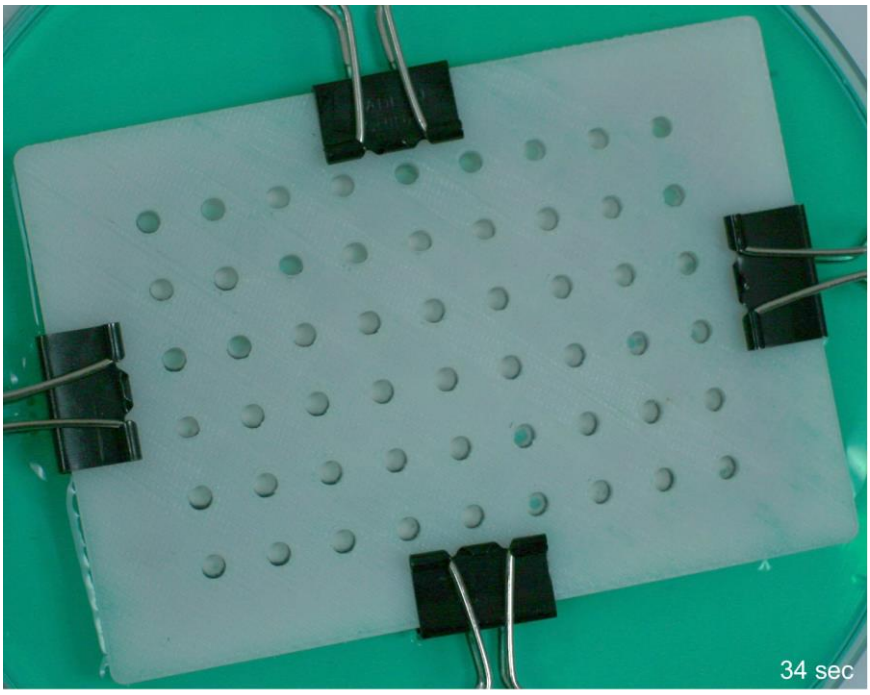

Figure A3: Photo of valve testing taken after 34 seconds (18/54 valves opened)

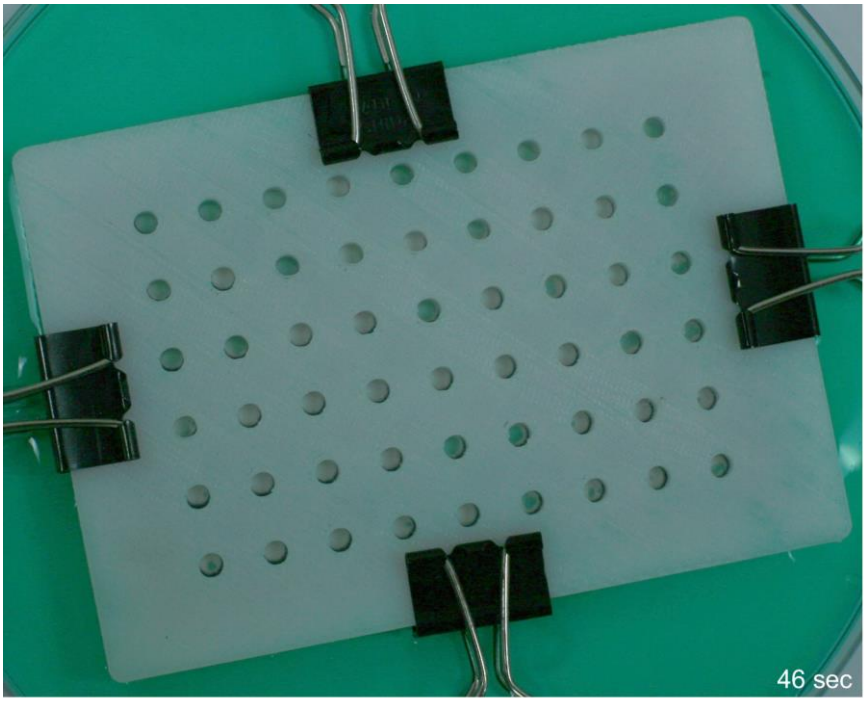

Figure A4: Photo of valve testing taken after 46 seconds (30/54 valves opened)

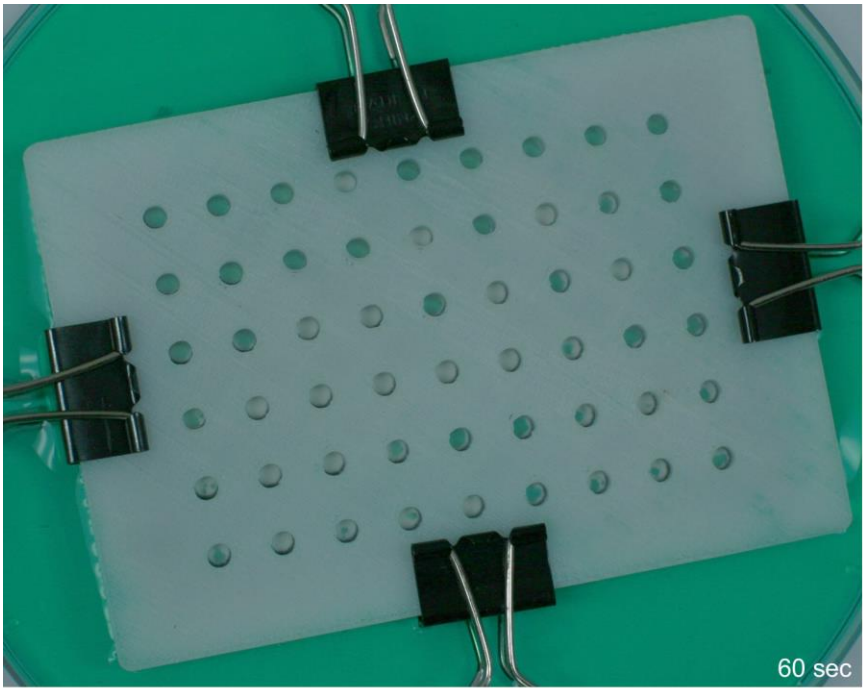

Figure A5: Photo of valve testing taken after 60 seconds (38/54 valves opened)

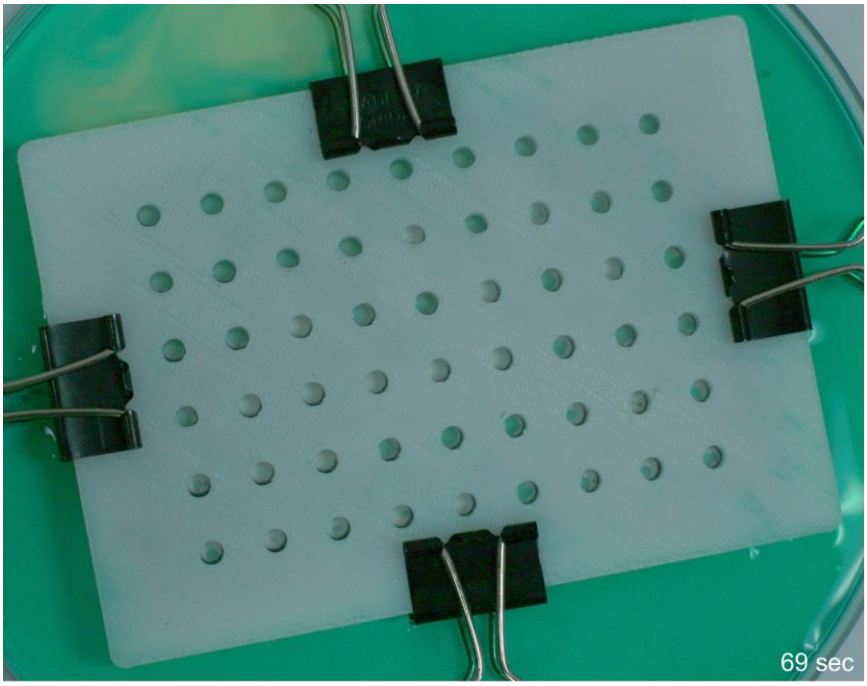

Figure A6: Photo of valve testing taken after 69 seconds (43/54 valves opened)

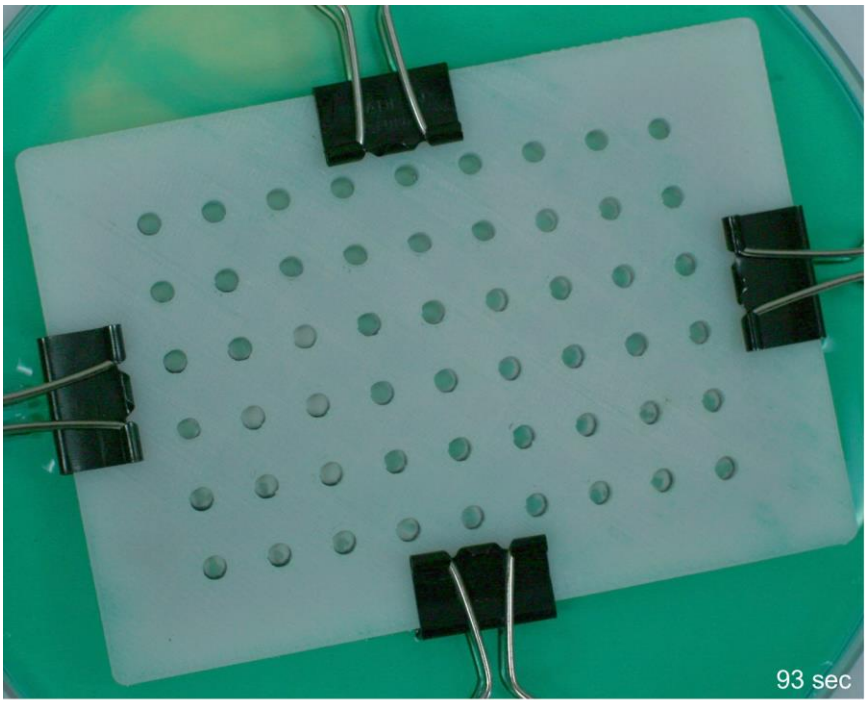

Figure A7: Photo of valve testing taken after 93 seconds (50/54 valves opened)

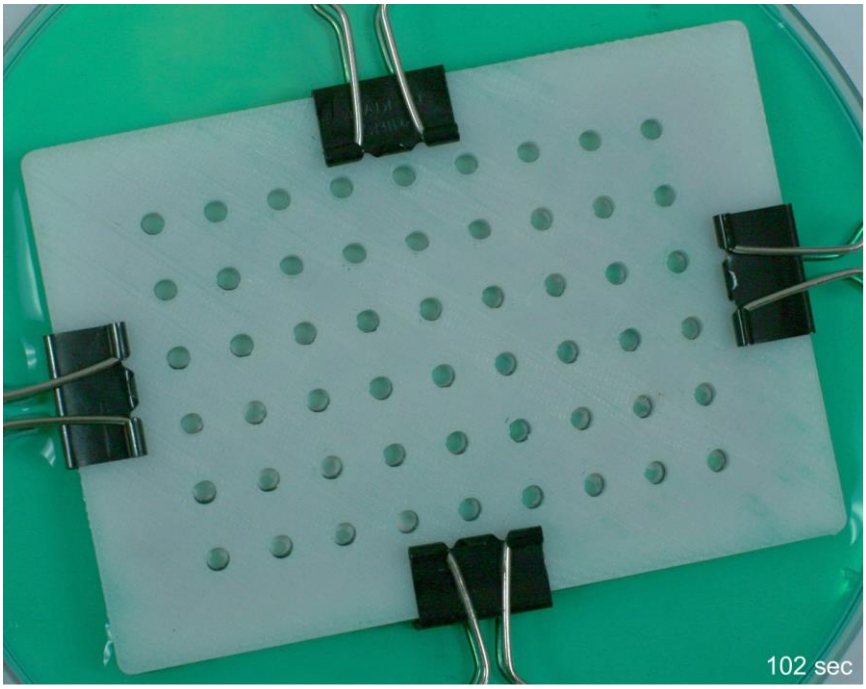

Figure A8: Photo of valve testing taken after 102 seconds (54/54 valves opened)

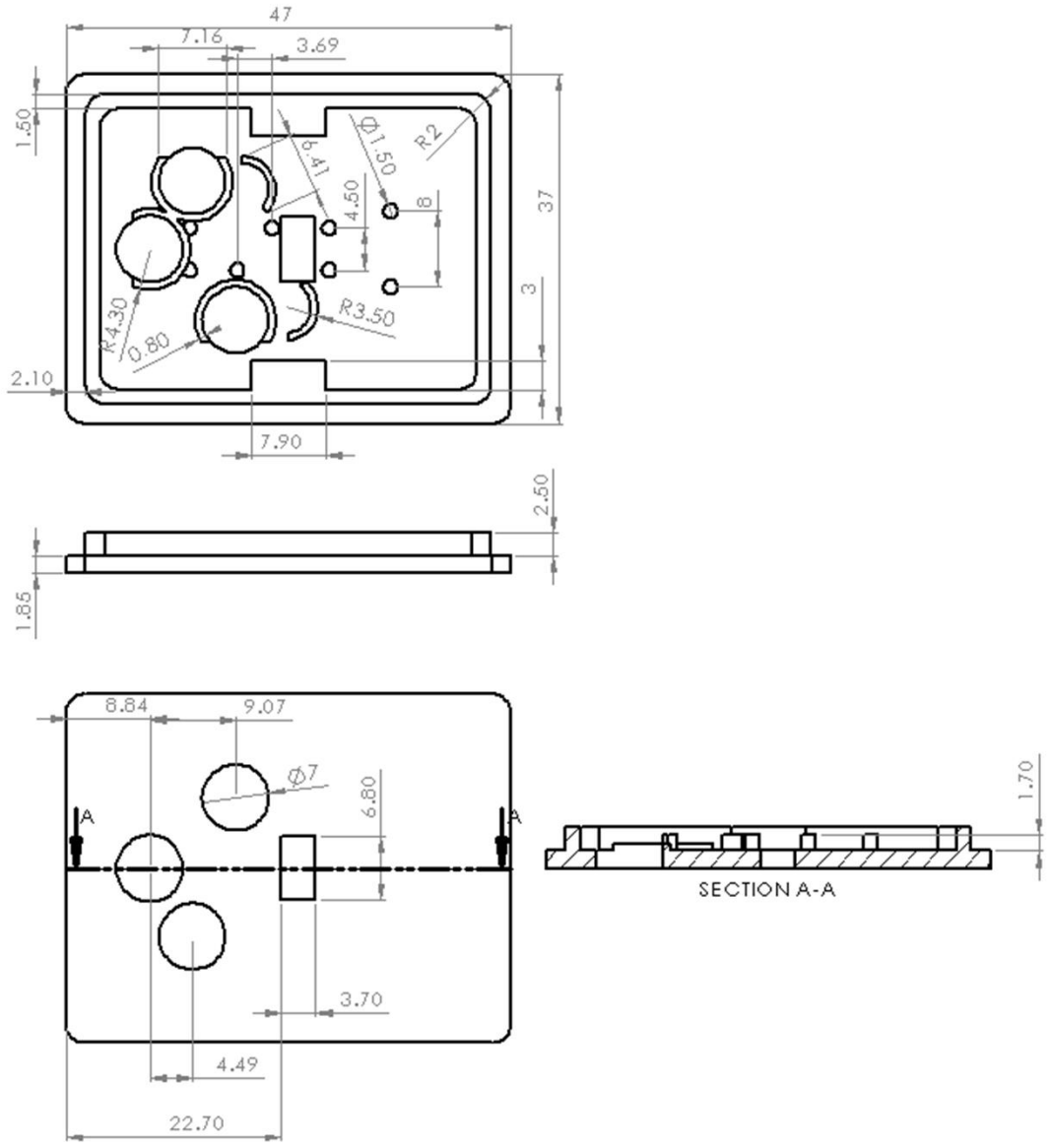

Figure A9: Drawing of top half of housing with dimensions

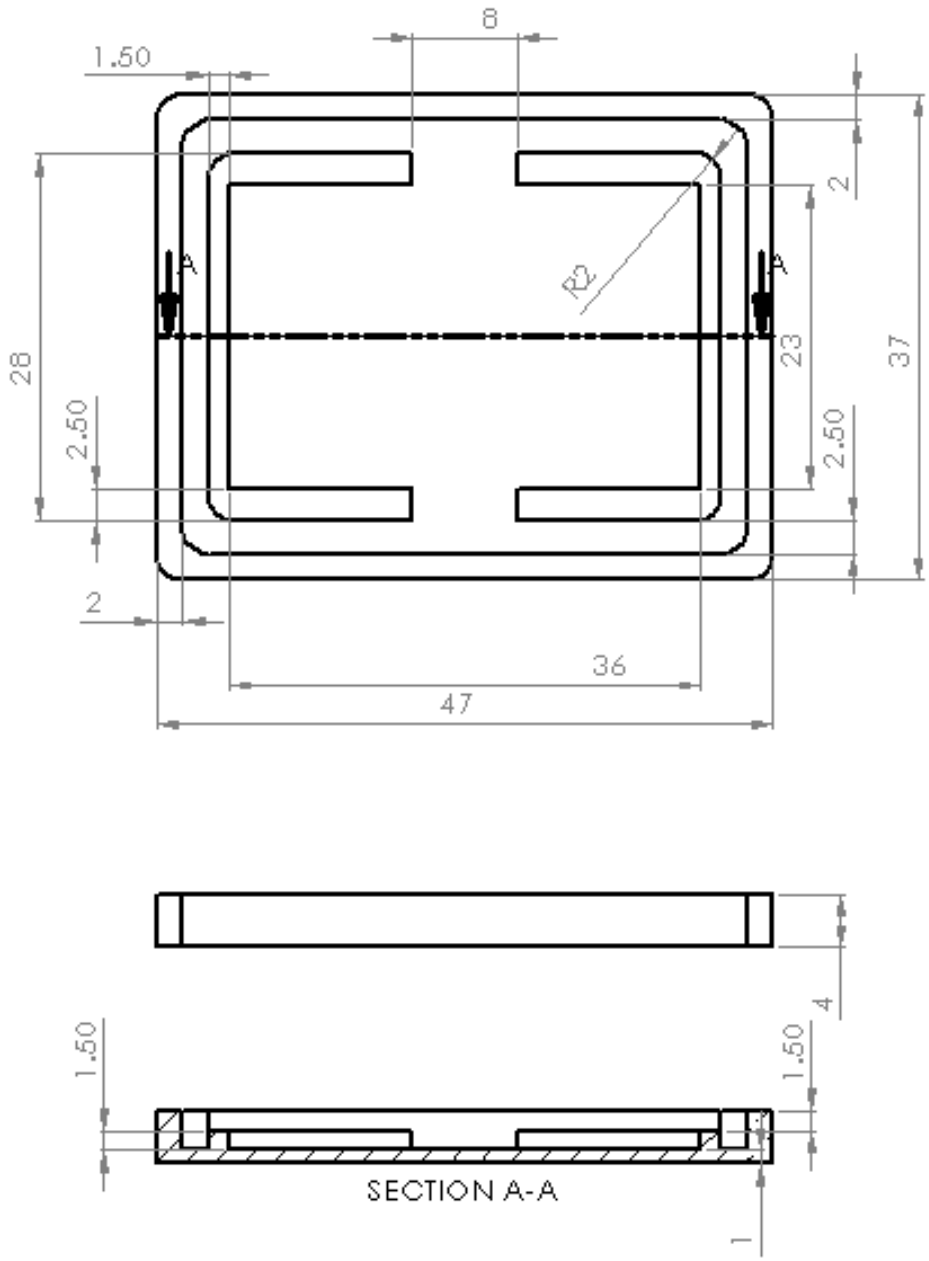

Figure A10: Drawing of bottom half of housing with dimensions

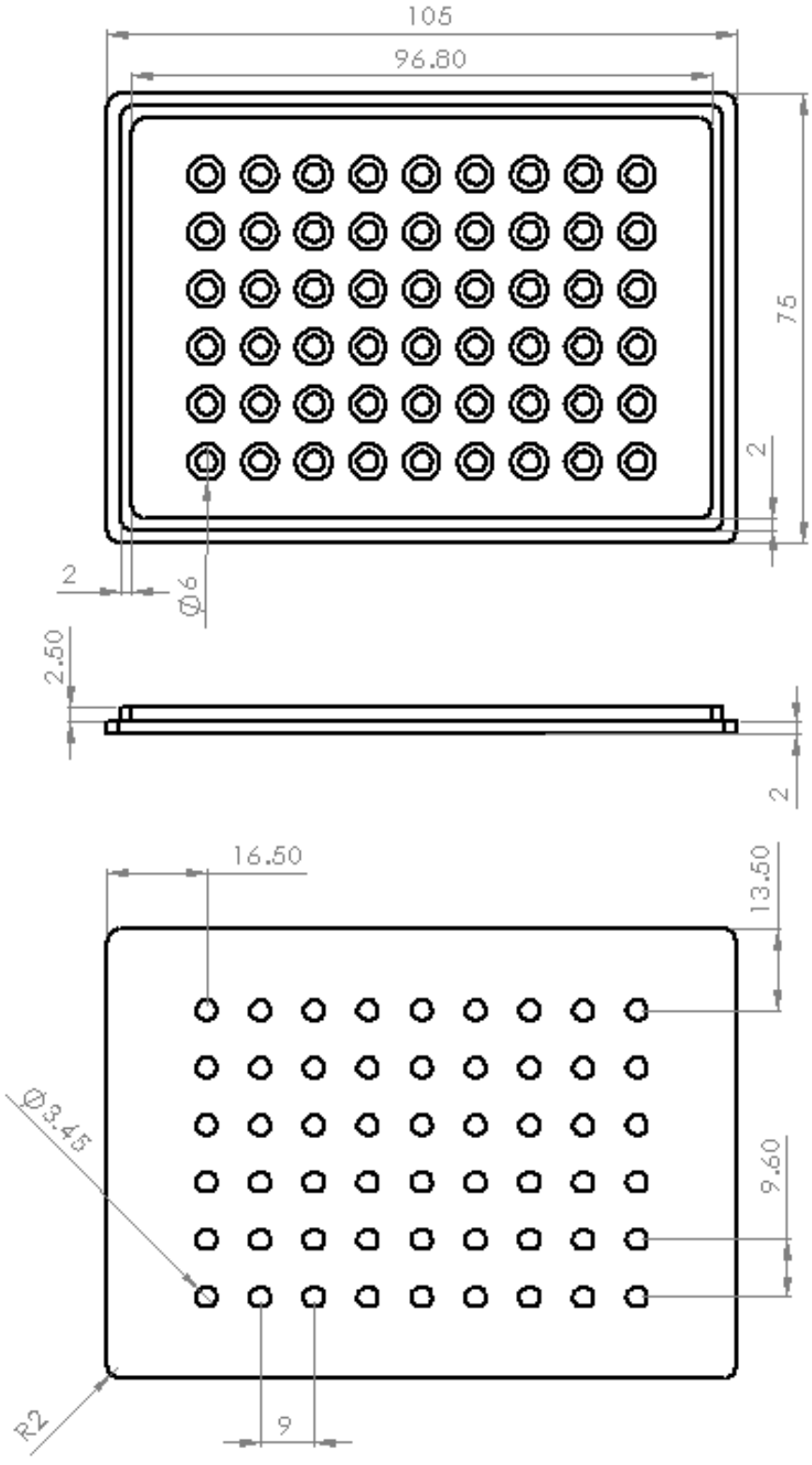

Figure A11: Drawing of top half of housing for testing the valve opening time with dimensions

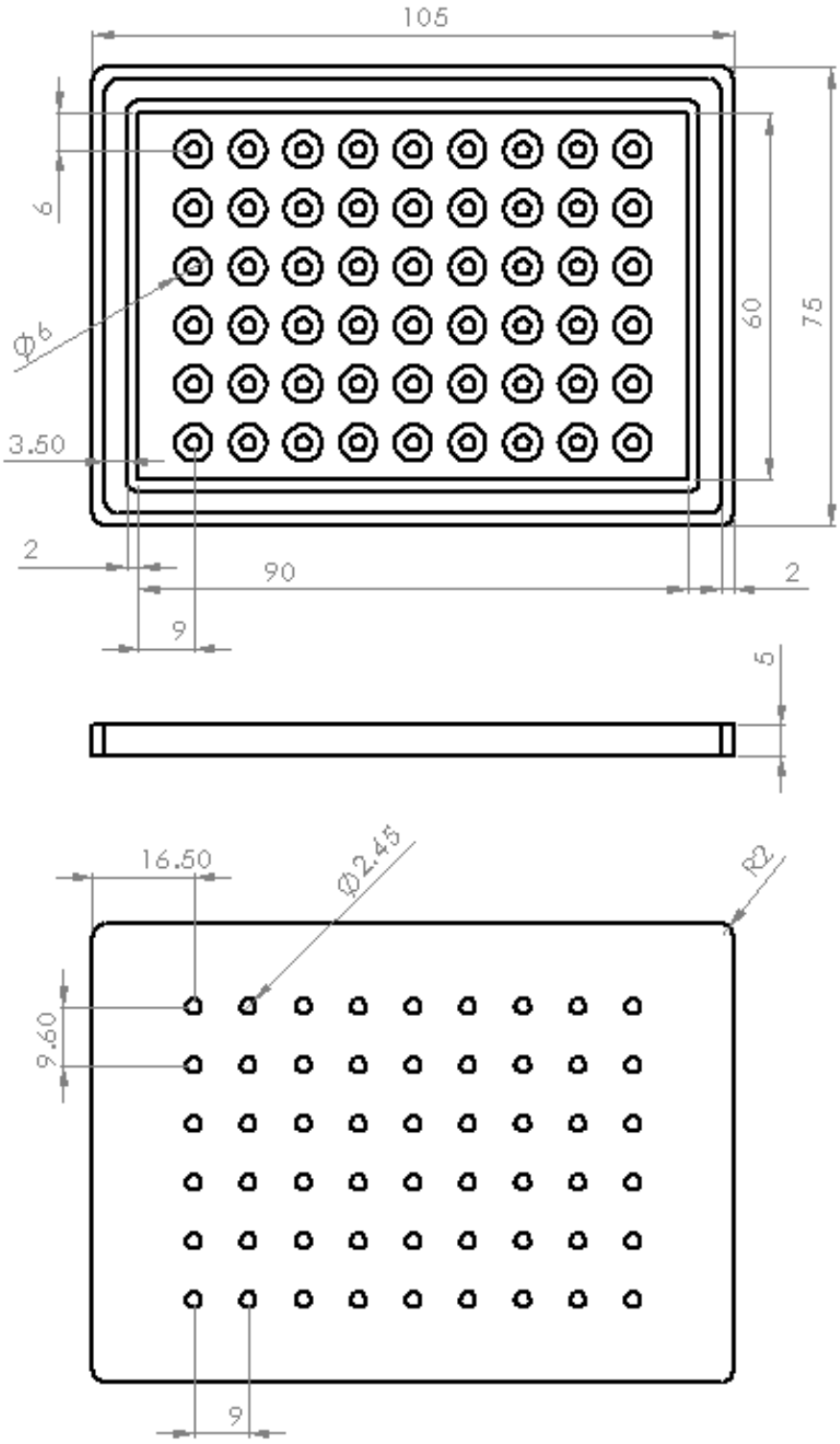

Figure A12: Drawing of bottom of housing for testing the valve opening time with dimensions

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