Fast Detection of Low Volumes of Mouse Antibodies Using a Nanofluidic Bioarray Chip

by
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Abstract

We have developed a novel nanobioarray (NBA) chip for the detection of antibodies (Abs) secreted from mouse immune cells. The NBA chip was constructed by sealing PDMS channel plates with a glass slide, and the intersection approach employed to develop various bioarrays whereby multiple samples each react with multiple probes using a simultaneous liquid delivery method for sample introduction. In our system, we utilized several Ab samples to react with multiple peptide probes. We were able to use the NBA chip to detect samples with a solution volume of 500 nL and of a concentration of 0.05 nM which was lower than what was detected using the conventional ELISA method. This device, when used with human samples, has applications in the detection of antibodies secreted from single immune cells and in the diagnosis of diseases.

Keywords: nanobioarray; polydimethylsiloxane (PDMS); peptide probes; antibodies; antigens; enzyme-linked immunosorbent assay (ELISA); intersection approach; disease diagnosis
Dedication

I would like to dedicate this thesis to my family and friends whose support has enabled me to complete this thesis. First and foremost, I dedicate this thesis to my parents who are my greatest supporters. When I moved to the other side of the continent to pursue a graduate degree, they were always there to share in all of my achievements and my disappointments. They never let me give up hope and always show their love and trust for me.

To my friends, they were there to help me during all of my struggles and they encouraged me and motivated me to work harder. They kept pushing me to continually pursue my goals.

Thank you all very much.
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This work wouldn’t have been possible without the help of Dr. Dipankar Sen, who allowed me to utilize the Typhoon scanner for the fluorescent detection. I would also like to thank Dr. Lin Wang for training me on the chip fabrication and chip surface derivatization procedures. I would also like to thank the past and present members of Dr. Li’s research group, without their help much of this wouldn’t have been possible.

Finally I would like to thank my friends and family who have supported me throughout the entirety of this stage in my life.
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List of Acronyms or Glossary

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
</tr>
<tr>
<td>AF-647</td>
<td>AlexaFluor 647</td>
</tr>
<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>APTES</td>
<td>(3-Aminopropyl)triethoxysilane</td>
</tr>
<tr>
<td>Bio</td>
<td>Biotin</td>
</tr>
<tr>
<td>Blotto</td>
<td>Blocking Solution made with Milk Proteins</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>Cy5</td>
<td>Cyanine 5</td>
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<tr>
<td>DI</td>
<td>Deionized</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
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<tr>
<td>EDC</td>
<td>1-ethyl-1-(3-dimethylaminopropyl)carbodiimide</td>
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<tr>
<td>ELISA</td>
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</tr>
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<tr>
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<tr>
<td>HA</td>
<td>Hemagglutinin</td>
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<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen</td>
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<td>Horseradish peroxidase</td>
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<td>Immunoglobulin G</td>
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<td>Immunogold Silver Staining</td>
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<td>IPA</td>
<td>Isopropanol</td>
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<tr>
<td>MAbs</td>
<td>Monoclonal Antibodies</td>
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<tr>
<td>N₂</td>
<td>Nitrogen Gas</td>
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<tr>
<td>NaBH₄</td>
<td>Sodium borohydride</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>NBA</td>
<td>Nanobioarray</td>
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<tr>
<td>NHS</td>
<td>N-hydroxysuccinimide</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffer Saline</td>
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<tr>
<td>PDMS</td>
<td>Polydimethyl siloxane</td>
</tr>
<tr>
<td>pVIII</td>
<td>Phage Coat Protein</td>
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<tr>
<td>RPM</td>
<td>Revolutions per Minute</td>
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<td>Streptavidin</td>
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<tr>
<td>TRITC</td>
<td>Tetramethylrhodamine Isothiocyanate</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
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1. Introduction

1.1. Introduction to Immunoassays, Antibodies, and Antigens

Immunoassays have long been utilized as a method of protein detection for biological samples (Hartmann et al. 2009). This is a method that is derived from the natural adaptive immune reaction which takes place between an antibody and an antigen. Antibodies are proteins secreted in the body (e.g. human or mouse) in order to identify and neutralize foreign objects such as viruses and bacteria. Antigens are markers on these objects that antibodies bind to in order to recognize them. The introduction of immunoassays represented a significant leap in the ability to detect low levels of as antibodies, which are clinically relevant for the detection of diseases such as hepatitis and autoimmune disorders. (MacBeath et al. 1999, Pollard et al. 2007, Robinson et al 2002) As such, immunoassays have played an important role in protein detection and clinical diagnostics.
Immunoassays can be classified as homogeneous and heterogeneous assays. In homogeneous assays, the antigen and the antibody will be mixed in the liquid phase; this method requires the chemical separation of these components from the antibody-antigen complex. In a heterogeneous assay the samples (e.g. an antibody) in the liquid phase interact with the probes (e.g. an antigen) immobilized on a solid phase. While both of these assays are in practical use, heterogeneous assays are much more common due to the ease to wash away unbound molecules from the solid substrate. The solid substrate can be a flat biochip surface or a spherical bead surface. Heterogeneous immunoassays usually take the form of the enzyme-linked immunosorbent assay.
(ELISA). In this format, an antigen is immobilized on a solid substrate for binding with the antibody as an analyte to be detected. The detection antibody has an enzyme label used for chemiluminescent detection. (See Figure 1.1). The need of sample volumes in excess of 10 µL in ELISA poses a restriction to the detection of low amounts of antibodies (usually in low volumes and low concentrations in clinical samples). This makes the development of the technique that allows for nanoliter-volume liquid handling and provides high detection sensitivity of low-volume and low-concentration samples particularly important (Chu et al. 1997).

**Table 1.1: Applications of different bioarray methods.**

<table>
<thead>
<tr>
<th>Target</th>
<th>Indications</th>
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<tbody>
<tr>
<td>Antibodies</td>
<td>Allergies, Autoimmune, Infectious disease, Human leukocyte antigen (HLA) Typing</td>
</tr>
<tr>
<td>Proteins</td>
<td>Cancer, Cardiac Problems, Neurologic (Alzheimer’s) Multiple Immunoassays</td>
</tr>
</tbody>
</table>
1.2. Technologies

The miniaturization of the immunoassay technology came to light during the 1990s when development of DNA arrays and protein bioarrays began (Ekins et al. 1998). There are many applications for protein detection using the array method (See Table 1.1). Two major methods have been developed to construct bioarrays for protein detection, namely microspot and micromosaic array.

1.2.1. Microspot Array

The most commonly developed immunoassay method is the spot array. This is a method where many probe spots are printed onto a chip substrate. The minimum volume of materials required for ELISA kits in 96-well plates are greater than 100 µL; whereas microspot arrays that handle liquids of nanoliter volumes in micron-sized channels help remove this limitation. Typically the probe spot area density is several thousand, and they are printed as a rectangular array within a single 1cm x 1cm square chip. This bioarray is then tested against a single sample, which is flowed on top of the array. A large amount of data can be obtained from this test using a single sample.
Figure 1.2: Microspot array fabrication a) Photolithography: A substrate modified with photolabile protecting groups (X) can be selected for activation by using a photomask (M1). The chip is then covered with a photoprotected protein (A-X), which will be immobilized, generating defined regions on the surface. A second mask (M2) is then applied to deprotect another set of defined regions for protein coupling. Repeated deprotection and coupling will allow for a high density protein microarray. b) Mechanical microspotting: This method requires the loading of the protein into a spotting pin via capillary action. This method can be automated using robotic control systems and multiplexed printheads. c) Ink-jet printing utilizes piezoelectric fittings (rectangles) and an electrical current to dispense a precise quantity of a loaded sample in the nozzle. By utilizing multiple jets, microarrays can be printed rapidly. (From Schena et al. 1998, reprinted with permission from Elsevier).
There are 3 common methods utilized in microspotting the substrate: photolithography, mechanical microspotting and ink-jet printing (see figure 1.2). These printing methods provide the ability to print multiple individualized spots on a single chip substrate.

Photolithography is used to pattern many spots accurately on the surface. This method is highly reproducible, with little variations in the microspot locations (Kim et al., 2010). This method does require a photomask for fabrication, which can be costly as compared to other methods. This fabrication process is also time-consuming as each photomask must be designed and built before the chip fabrication can occur.

Mechanical microspotting utilizes a liquid droplet delivery mechanism with direct contact of spotting tips against the substrate to print the liquid from spots to spots. This method is simple and can be implemented for rapid prototyping. In addition, the microspotting procedure can be easily automated. Unlike the photolithography method, mechanical microspotting is not as precise, and so the high spot density achieved using photolithography cannot be obtained. Nevertheless, this method remains a simple method of use in research labs.

Ink-jet printing is a droplet delivery system using nozzles to transfer substances onto the substrate. This method allows for a spot density comparable to mechanical microspotting, while reducing the chip fabrication time as the ink-jet liquid delivery method is contactless. However, the delivery of the liquids is not always reliable and reproducible, due to clogging of the nozzle, which limits the use of this method.
1.2.2. **Mosaic Arrays**

The mosaic array is an immunoassay method that allows for multiple probes to react with multiple sample analytes. Rather than having a single sample to react with multiple probes, this method has several samples tested against multiple probes simultaneously.

In the mosaic array, the liquid is flowed through the PDMS channels placed on top of the substrate (see figure 1.3). This method is simple to use and it utilizes micron-sized channels to define precisely and accurately the regions of the probes utilizing nanoliters of reagents. This method requires the removal of the first chip and the assembly of a second chip before detection can occur. In other words, the channels in the chips must be intersecting in order to conduct the multiple probe, multiple sample tests. The linear channel bioarray proposed by Bernard et al. (2001) was able to detect the antibodies to levels down to 0.5nM.
Figure 1.3: Steps to perform a micromosaic immunoassay: a) Different antigens are patterned on the substrate in single lines using a microfluidic chip network. b) Any areas not patterned in step a) are blocked with BSA to prevent any non-specific binding. c) The first microfluidic network is replaced with another chip and antibodies are flowed through to bind to the patterned antigens. d) The mosaic generated will allow for the detection and quantification of antibodies on the surface e) In this method, a 200µg/mL rabbit IgG labelled with TRITC is immobilized on the surface using the microfluidic network. The substrate was then blocked with BSA, and then the second chip was positioned across the printed antigen lines and a FITC-labelled anti-rabbit-IgG solution was applied to allow for immunobinding. (From Bernard et al 2001, reprinted with permission from American Chemical Society)
In both methods of microspot and micromosaic arrays, a high spot density can be obtained. In the mosaic bioarray, the multiple probes will not only detect proteins in one sample, but also detect multiple samples; whereas the microspot array will not be able to detect multiple samples (Murphy et al., 2008).

Furthermore, the need for simultaneous detection of a large number of analytes led to the development of multianalyte binding assays. Nanofluidics conducted in micron-sized channels provided a system in which a minimal volume of liquids could be utilized while maintaining or even improving the sensitivity of the protein detection in the liquids. The development of such a technology that can perform multianalyte detection in nanoliter-volume of samples is called the nanobioarray (NBA) chip which will be described in detail in subsequent sections.

The NBA chip was evolved from the linear chip (Wang et al., 2007) and spiral/radial chip (Wang et al., 2010) previously developed in Dr. Li’s group for DNA detection. The linear chip is described in Fig 1.4. The radial/spiral chip is described in Fig 1.5 and it can handle more samples up to 96.
Figure 1.4: Linear channel Bioarray chip system. (From Wang et al. 2007, reprinted with permission from American Chemical Society)

Figure 1.5: Radial/Spiral Bioarray chip system. (From Wang et al. 2010, reprinted with permission from Elsevier)
1.3. Immobilization Chemistry

In heterogeneous immunoassays, there are various methods of immobilizing the probes onto the surface. Covalent attachment, adsorption and bioaffinity attachment are the most common methods used (see Fig 1.3).

Figure 1.6: Diagram of various Immobilization techniques. (A) Physical immobilization occurs by the directly exposing the sample onto the surface through physisorption. (B) Covalent attachment requires functionalization of the surface before exposing the sample for immobilization. (C) Bioaffinity utilizes an immobilized biomolecule to create a bond of the sample through another biomolecule onto the substrate. (From Rusmini et al. 2007, reprinted with permission from American Chemical Society)

Adsorption is a simple method in which physisorption is commonly used to immobilize proteins onto a surface. This method can be achieved by adding proteins with high affinity to a particular substrate that is able to adsorb materials well. The drawback of this method is the occurrence of nonspecific binding. In addition, the weak binding force of physisorption means that protein desorption can easily occur through changes in reaction conditions, such as temperature, pH, and solvent composition.
(Kusnezow et al., 2003). These drawbacks make this method rather limited in applications. While adsorption is a viable method in microspot arrays, it is not as ideal as in micromosaic arrays.

In bioaffinity attachment, the most common method is through the use of biotinylated groups coupled to probes, which will attach to the immobilized avidin analogues bound on a solid substrate. The high binding affinity of biotin to avidin and the ease of biotinylation of various probes make this a simple method for anchoring probes on solid substrates. In addition, the biotin-avidin link is very robust and is able to withstand many extreme conditions that may be encountered in a reaction (Moy et al. 1994). The drawback is that the probes must be biotinylated prior to being used which requires an additional purification step. In order to avoid steric hindrance due to biotin on the active group of the probe, a carbon chain linker is usually used in between the biotin moiety and the probe.

Covalent attachment requires surface functionalization in order to attach the probes. Depending on the amino acids present in the protein or peptide probes (see Table 1.2), there are several methods of surface functionalization. Rusmini et al (2007) lists 2 of these methods as (1) N-hydroxysuccinimide (NHS) with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide) (EDC) chemistry and (2) aldehyde chemistry to couple with amino groups in proteins. In the former method, EDC is used with NHS to create an amine-reactive NHS ester, which is easily attached to proteins through forming a stable amide bond. In the aldehyde method, glutaraldehyde is used for the modification of the surface. Then, the aldehyde will interact with an amino group on the proteins. We adopted to use the aldehyde method for peptide antigen immobilization.
Table 1.2: Comparison of Immobilization techniques used to anchor proteins containing various types of amino acids

<table>
<thead>
<tr>
<th>Side Groups</th>
<th>Amino Acids</th>
<th>Surfaces</th>
</tr>
</thead>
<tbody>
<tr>
<td>-NH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Lys, hydroxyl-Lys</td>
<td>Carboxylic acid</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Active ester (NHS)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Epoxy</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aldehyde</td>
</tr>
<tr>
<td>-SH</td>
<td>Cys</td>
<td>Maleimide</td>
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<tr>
<td></td>
<td></td>
<td>Pyridyl disulfide</td>
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<tr>
<td></td>
<td></td>
<td>Vinyl sulfone</td>
</tr>
<tr>
<td>-COOH</td>
<td>Asp, Glu</td>
<td>Amine</td>
</tr>
<tr>
<td>-OH</td>
<td>Ser, Thr</td>
<td>Epoxy</td>
</tr>
</tbody>
</table>

1.4. Detection Methods

The common detection methods for immunoassays are based on tags that produce signals due to chemiluminescence, fluorescence, colorimetry, electrochemistry, or radioactivity (Pollard et al 2007).

Chemiluminescent detection can be obtained by a horseradish peroxidase (HRP) tag, which is an enzyme that catalyzes the chemiluminescent reaction of substrates such as luminol. The HRP can be tagged onto the detection antibody which will be applied to the system (Noguera et al., 2011). This method allows for a two orders of magnitude increase in detection ability as compared to the use of a fluorescent tag. The drawback of this method is the short lifetime of the chemiluminescent reaction. The detection must be completed rapidly upon the introduction of the substrate. In other words, this method of detection will be inaccurate if the substrate is not introduced to all detection regions or wells simultaneously. This method is excellent for high throughput detection, but light shielding from other wells (usually white) is required in order to prevent any residual light from bleeding into other wells, causing false positives (Lee et al., 2010). This
requirement for optical isolation from other chemiluminescent reaction sites precludes its use for a solid-surface bioarray.

Fluorescence offers a solution to the issues of the chemiluminescence by removing the need to introduce the HRP substrates as well as the need for reagent localization. The most direct method is attaching the fluorescent tag to the detection antibody (Ghafari et al. 2009). This method is the simplest and requires the least amount of time to detect. However, this method does not generate light as bright as chemiluminescence which has chemical amplification. Nevertheless, fluorescent detection can be enhanced in many ways. One way is to multiply the number of fluorescent tags by adding a chain reaction or by using an antibody with multiple fluorescent tags. Another way is by utilizing fluorescent beads for detection, increasing the detection sensitivity by 2 orders of magnitude. Fluorescence is a versatile method that can be utilized in a high throughput design. Therefore, we adopted to use fluorescence as the detection method for antibodies.

Other approaches include colorimetric detection which can be achieved by a variety of methods. The simplest method is the introduction of a colorimetric substrate such as 3,3’5,5’tetramethylbenzidine that can be catalyzed by HRP to produce a colored compound (Frey et al. 2000). Another method for colorimetric detection is immunogold silver staining (IGSS), which uses silver nanoparticles to aggregate on the gold-tagged detection antibody. (Holgate et al. 1983; Hayat 1995; Lackie 1996) This method has a detection ability that is visible to the naked eye even at concentrations of 0.005nM. While normal colorimetric methods are not as sensitive as chemiluminescence or fluorescence, silver staining enhances the detection ability to a comparable level. In addition, the ease of detection allows them to be viable for low cost detection equipment.
such as scanners, when large and bulky instruments are unavailable. The issue with this method is that it is difficult to quantify, as this reaction utilizes a catalytic reaction in order to determine the presence of a compound in a solution.

Electrochemical detection utilizes the reduction and oxidation abilities of the materials for immunoassay detection. The method requires electrodes (usually the working electrode) to be fabricated directly onto the nanofluidic device. This method is more sensitive than colorimetric detection, and it does not require transparent material to fabricate the device. However, it is dependent on flow conditions within the channels and the electrodes must be treated to prevent inaccurate results due to biofouling.

Radioactivity is another method that can be used for immunoassay detection. In this method the requirement for a transparent material for the nanofluidic system can be bypassed as this method does not depend on optical detection methods. In addition, it requires the radiolabelling of the protein, which allows for tracing of the protein in the nanofluidic system. However, this method requires specialized equipment that reduces its compactness. In addition, radioactive hazard is a concern that requires specialized safety training.
1.5. Thesis Motivation and objectives

1.5.1. Thesis motivation

There is a need to obtain a cost effective yet multiple sample multiple probe system in order to detect the presence of antibodies within the biological samples. Nanofluidics is important in achieving our goal as we are interested in the detection of low volume samples such as the secretome obtained from a single cell. The requirement for a multiple sample multiple probe system is to have the sample to interact with as many probes as possible while being able to test multiple samples simultaneously. This method is not achievable as in the ELISA method using a 96 well plate where only one sample reacts to one probe. The requirement for large sample volume (>100 µL in 96-well plates or >10 µL in 384-well plates) makes it near impossible to detect anything secreted at the single cell level. The time required to utilize a 96-well plate is at a minimum of 6 hours to complete, and so a rapid test in an hour would be ideal. With this in mind as our motivation, we hope to develop a device that will be able to rapidly detect multiple antibody samples at low volumes using multiple peptide probes.

1.5.2. Thesis objectives and outlines.

Our objective is the development of a device for the rapid detection of mouse antibodies. Our system, which involves the NBA chip and utilizes a secondary antibody for fluorescent detection, is similar to the micromosaic approach. The micromosaic utilizes air pressure and as such delivery of liquids in multiple linear channels is not always simultaneous. However, with the NBA chip we are able to deliver the liquids simultaneously and without the need for air pressure. By utilizing a spiral NBA chip we
hoped to increase the amount of tests we would be able to see using a single chip. The linear channel chip was first used for simplicity and as a facile method of development for proof of concept, and then it was optimized for utilization in the spiral channel chip. The linear channel chip features a 16 by 16 array to generate 256 reactions, as compared to 96 reactions in the conventional 96-well plate. Then, by utilizing the spiral chip we are able to have a 24 by 48 array. Furthermore, we believe the sensitivity of detection is enhanced by using the spiral channels because they are longer and so the residence time of the antibody-antigen reaction is increased. The chip has been made from PDMS; as it will need to be reversibly sealed onto the glass substrate. In addition, the PDMS is clear so that we may readily monitor the liquid delivery along the chip channels. Since PDMS is unreactive to aqueous materials, this is ideal as most of the reagents we require are in an aqueous medium. A glass substrate will be used as an anchor for the peptides. We will utilize a flat-bed scanner to quantify our results.

Our objectives for the development this NBA chip include the following:

- Fast (under 2 hours for testing)
- Able to test multiple samples on multiple probes (>96 tests)
- Low sample volume (<1µL of material)
- Comparable detection sensitivity or better than ELISA

With these objectives in mind we will be able to rapidly test multiple samples of low volume with good detection sensitivity.
2. Experimental Section

2.1. Materials

For our tests we utilized 2” x 3” glass slides obtained from Fisher Scientific. The silicon wafers were purchased from University Wafer (Boston, MA). NBA chips were fabricated by PDMS utilizing the Sylgard 184 silicone elastomer kit; including both crosslinking silicone polymer and curing agent from Dow Corning (Midland, MI). Cleaning solvents ethanol (EtOH) and isopropanol (IPA) were obtained from Commercial Alcohols (Chatham, ON). The peptides (HA, bioHA and biopVIII) were obtained from GL Biochem (Shanghai). The detection antibodies were obtained from Invitrogen (Burlington, ON). Tween-20, Triton X-100, and bovine serum albumin (BSA) were obtained from Sigma (Mississauga, ON). Disodium hydrogen phosphate (Na$_2$HPO$_4$), monopotassium dihydrogen phosphate (KH$_2$PO$_4$), sodium chloride (NaCl), potassium chloride (KCl) and sodium borohydride (NaBH$_4$) utilized in the phosphate buffer saline (PBS) and the reducing solution were obtained from Caledon Labs (Georgetown, ON). The blocking reagent (Blotto) was obtained from BioRad. The (3-aminopropyl)triethoxysilane was obtained from MP biomedicals. Concentrated sulfuric acid, 30% hydrogen peroxide, and 25% glutaraldehyde was obtained from Caledon Labs. Deionized (DI) water (18MO cm$^{-1}$) was obtained from a Waters Millipore filter system. Repel Silane ES was obtained from GE Healthcare Life Sciences (Quebec, CA). The fluorescent beads, which were streptavidin-coated polystyrene microspheres (mean diameter of 0.97µm) embedded with a dye called Flash Red were obtained from Bangs Laboratories Inc. (Fishers, IN).

The anti-pVIII sera were obtained from the mouse (Mus musculus strain: BALB/c), from Charles River Supplier (cat. no: Strain code 028).
2.2. Mask and chip fabrication

In order to fabricate our polymeric nanobioarray (NBA) chips, we utilized the casting method using a mold master, see Figure 2.1. The mold master was prepared using the photolithography method using a photomask, see Appendix A. The photomask utilized in the development of the linear channel chips was prepared using a design incorporating 16 parallel channels each with a width of 200\(\mu\)m (see Figure 2.2). The designs for the chip were drawn on the computer utilizing Visual Basic and other drafting software programs. The chip designs were then used to create photomasks printed at resolutions of 3385dpi (Coles Litho-Prep Ltd. Vancouver, BC).

Figure 2.1: The mold master and a PDMS chip (a) The mold master was fabricated on a Si wafer with the center PDMS region cut and removed, (b) The PDMS chip (5cm x 5cm) was sealed with a glass slide, with channels filled with a blue dyed solution.
Figure 2.2: The photomask design: (a) The design of 16 horizontal channels (b) the design of 16 vertical channels. The channels are 200 µm in width and the straight portion of the channel is 25mm in length.

In order to improve fabrication quality by removing the possibility of dust contamination, the mold master fabrication for the micron-sized structures was completed inside a clean-room enclosure (577 series, Clean Air Products, Minneapolis, MN) equipped with an air filtration system.
The mold master was prepared using 10cm-diameter circular silicon wafers. The pre-oxidized Si wafer was cleaned using a piranha solution (70% H₂SO₄:30%H₂O₂). Then it was rinsed with DI water followed by EtOH. The wafer was then rinsed again with DI water and blown dry using N₂ gas. This was then baked in a convection oven at 120°C to remove the residual solvent. The wafer was then placed onto a spin coater. A SU-8 2035 photoresist was obtained from Microchem (Newton, MA) along with a SU-8 photoresist developer solution. The SU-8 with a depth of 35µm is applied onto the surface and spun to evenly distribute the photoresist. In order to obtain the proper thickness of SU-8 according to company specifications, the wafer was spun at 500rpm for 10 seconds, ramping up to 4000rpm for 30 seconds. The wafer was then transferred
from the spin coater onto a hot plate at 65°C for 3 minutes and then on another hot plate at 95°C for 6 minutes. This soft bake was done to remove the solvent. The coated wafer was placed underneath the UV exposure system (Model LS-150-3, Bachur & Associates, San Jose, CA). The mask was then placed on top of the wafer and held in place with a blank glass plate. The wafer was then exposed to UV light ($\lambda=365$nm) for 12 seconds. After exposure, the crosslinking was completed with a post-exposure bake: The wafer was put onto the 65°C hot plate for 1 minute and then onto the 95°C for another 6 minutes. Once this was complete, the wafer was placed into a glass container and covered fully with the SU-8 developer solution for 10 minutes. It was gently agitated for 10 minutes during this process. The wafer was then removed from the solution. If there was still uncrosslinked photoresist, the developing process was repeated. After the mold master has been developed, it was rinsed with IPA followed by a DI water wash.

The master was then blown dry with a steady stream of $N_2$ gas. Silicone glue (Dow Corning 732 multi-purpose sealant, Dow Corning, Midland, MI) was applied around the mold master to form a barrier. This barrier was used to prevent the PDMS casting solution from flowing off the mold master. The barrier was left to dry for at least 24 hours before any PDMS chip fabrication began.
Figure 2.4: The spin coater used for preparing the SU-8 mold master.

Figure 2.5: A SU-8 mold master being developed.
2.2.1. **PDMS chip Fabrication**

The volume of casting mixture required to fabricate a 1.5mm-thick chip was calculated, and the PDMS prepolymer and curing agent were mixed together in a 10:1 ratio. In order to remove any air bubbles, the mixture was stored at -20°C overnight. A thin layer of the repel silane ES solution (a solution of dimethyldichlorosilane) was then applied on to the mold master for 5 minutes, and this was to allow for the facile release of the mold after casting. The mold master was then rinsed with EtOH followed by DI water, then dried by N₂ gas. The casting mixture was then poured onto the mold master. Any large bubbles were removed using autoclaved pipette tips. The mold master containing the mixture was then placed on a plane surface and cured at room temperature for 24 hours. The channel depth of the PDMS was the same as that of the SU-8 thickness (i.e. 35 µm). Once the PDMS was cured, it was cut from the molding master and peeled off gently. A sharpened flat-tip needle was used to punch out holes to make the solution reservoirs.

*Figure 2.6: A SU-8 mold master fabricated on a Si wafer (10cm in diameter) with the silicone barrier.*
2.3. Immobilization of peptide probes on glass surface

The glass was rinsed by water and ethanol. The glass was first hydrolyzed and then aminated. It was followed by aldehyde functionalization. The details are given in appendix B.

For our tests we immobilized the HA and the bioHA peptides on to the glass surface by coupling primary amines groups on the N-terminal of the peptides to the aldehyde groups on the glass surface. The amino acid sequences of the peptide probes are shown in Fig 2.7. The HA peptide probe (12 amino-acid residue) was derived from influenza hemagglutinin (HA). This probe will bind with the sample that is the anti-HA mouse antibody produced by the mouse 17/9 strain. This would allow us to determine whether the detection of antibodies would be compatible with the nanobioarray platform or not.

HA peptide (from flu virus hemagglutinin A glycoprotein):
H-Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala-Gly-Ala-Gly-Cys-NH$_2$

bio-HA peptide
H-Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala-Gly-Orn(Biotin)-Gly-Cys-NH$_2$

bio-pVIII peptide (from a phage coat protein):
H-Ala-Glu-Gly-Asp-Asp-Pro-Ala-Lys-Ala-Ala-Phe-Asp-Ser-Leu-Gln-Ala-Ala-Orn(Biotin)-Gly-Cys-NH$_2$

Figure 2.7: Amino acid sequences of peptide probes. The left is the N-terminal and the right is the C-terminal with the amide. (The red letters represent the amino acids of the binding epitope)

2.4. The immunoassay for antibody detection

Our experimental procedures for antibody detection were adapted from the (enzyme-linked immunosorbent assay (ELISA) method conducted at Dr. Jamie Scott’s laboratory (Simon Fraser University, BC).
For the linear channel chips, 0.5µL of the peptide was applied into the PDMS channels and allowed to react for 15 minutes. Once this was completed, the probe solution was removed and PDMS chip was removed. The glass was then submersed into a reducing solution of sodium borohydride for a total of 15 minutes. The reducing solution was removed and a 5% BSA solution in 1X PBS was applied for 15 minutes to block the surface. A wash using 0.1% Tween-20 (in 1X PBS) followed by 1X PBS was applied. The chip was then dried with a stream of nitrogen gas. A second PDMS chip was applied onto the surface with its channels perpendicular to the previous chip. Then, 0.5µL of the antibody solution was flowed into the channels over the probes. The solution was removed after 15 minutes and 1.5µL of a wash solution of 1X PBS containing 0.1% Tween-20 was applied into the channels followed by 1.5µL of 1X PBS solution.

In the radial/spiral chip system, 0.5µL of the peptide was applied into the radial PDMS channels and spun at 700 rpm for 10 minutes. The spinning platform used was shown in Figure 2.8. The chip was held in place utilizing a magnet and the spinner speed was adjusted using a variable knob and confirmed using a stroboscope. Once this was completed, the probe solution was removed and radial chip was removed. The glass was then submersed into a reducing solution of sodium borohydride for a total of 15 minutes. The reducing solution was removed and a 5% blocking solution in 1X PBS was applied for 15 minutes to block the surface. A wash using 0.1% Tween-20 (in 1X PBS) followed by 1X PBS was applied. The chip was then dried with a stream of nitrogen gas. A spiral PDMS chip was applied onto the surface with its channels perpendicular to the previous chip. Then, 0.5µL of the antibody solution was flowed over the probes, by spinning the disc at 1250rpm followed immediately with 1.5µL of a wash solution of 1X PBS solution containing 0.1% Tween-20. The secondary antibody solution was removed after 15 minutes and 1.5µL of a wash solution of 1X PBS solution containing 0.1% Tween-20 was applied into the channels followed by 1.5µL of 1X PBS solution.
Figure 2.8: The spinner platform utilized for liquid delivery.

The instrument utilized for the detection of our samples was a flat-bed fluorescent scanner (Typhoon FLA 9000 biomolecular imager, GE Healthcare Life Sciences, Quebec, CA). Our fluorescent dyes were the AlexaFluor-647 (AF-647) and Cyanin-5 (Cy-5). This required the excitation wavelength from the system to be at 650nm while the emission wavelength was set at 670nm. The sample concentrations were measured using the graphical data obtained from the fluorescence detector. By averaging the values we were able to obtain an approximate number of counts from the samples. These values were then compared against the background of the entire channel as well as the chip at various areas, upon which a normalized value could be obtained for comparison. In each case, multiple replicates would be taken in order to determine an averaged value for one concentration.
The Typhoon 9000 flat bed scanner. The chips were placed atop the scanner and a 10µm pixel size was selected to obtain the proper resolution to detect our materials on the NBA chips.
3. Linear Channel Chip Results and Discussion

The linear channel chip was first used to create the bioarray for antibody detection. By using the linear microchannels, we utilize pressure-driven liquid flow in order to deliver the solutions to the channels. The method is shown in fig. 3.1

A: Probe Immobilization

1) Chip 1 sealed against an aldehyde glass chip
2) Flow in streptavidin followed by peptide probe solutions
4) Remove Chip 1
5) Reduction of Schiff base linkage followed by blocking step

B: Target Binding

1) Chip 2 sealed against glass chip
2) Flow in samples followed by detection antibodies
4) Remove Chip 2
5) Scan with confocal fluorescence scanner

Figure 3.1: Schematic of the linear channel micromosaic system.

3.1. Peptide immobilization

In order to test the presence of the peptide probe on the surface, we use the peptide bioHA so that its presence can be detected using streptavidin (SA) which is conjugated with the fluorescent dye Cy5. On the other hand, the HA probe would not produce any
signals. The tests were completed utilizing the linear channel chip in figure 3.2. The peptides were immobilized along the horizontal channels, and the SA-Cy5 flowed through the vertical channels. Since the patches were observed at the intersection between the horizontal BioHA probe lines and the vertical channels, this allowed us to determine the Schiff base chemistry was successful for this immobilization reaction. Our results showed that we were able to immobilize bioHA onto the surface. We also note that the signals of the patches are stronger when a 50 or 100 µg/ml, rather than 25 µg/ml, of BioHA is used.

![Figure 3.2: Immobilization of peptide probes. Various concentrations of HA and bioHA were tested to determine if the probe could be properly immobilized onto the surface and then detected using a fluorescent tag. Utilizing various concentrations of bioHA and SA-Cy5, we were able to see patches at the intersections. The HA peptide does not show any patches because there is no biotin for binding with SA-Cy5. The spots are 200µm by 200µm.](image_url)
3.2. Test of antibody samples

Our results in the peptide immobilization allowed us to proceed to the next step of antibody detection. We use HA as the probe because the antibody sample (MAb 17/9) can now be detected using an AlexaFluor 647-labelled detection antibody. We use 5% BSA for blocking of non-specific binding. As shown in Figure 3.3, rectangular patches occurred at the intersections of vertical channels and horizontal peptide probe lines. This indicated the binding occurred between the antibody sample flowed in the vertical channel and the peptide molecules printed as a horizontal strip on the chip. The signal was greater when a higher amount of HA was used. Mab 17/9 did not bind to bioHA, and this can be explained by the steric effect of the biotin molecule close to the binding region of the peptide as depicted in Figure 2.7.
Figure 3.3: Binding of Mab anti-HA from mouse 17/9 (10 nM) with peptide probes. BioHA in 50 µg/mL and HA in various concentrations were used; the 50 µg/mL HA was missed because of a solution preparation error. The three left channels were flowed with the antibody sample (MAb 17/9) and the control channel on the right was flowed with PBS. In all cases, AlexaFluor 647-labelled detection antibody (10 nM) was flowed for detection of the presence of MAb 17/9. The spots are 200µm by 200µm. The right top inset shows the intersection of 10-nM anti-HA (in 3 replicates) with the HA and bioHA peptide probes (in 3 replicates) The right bottom inset shows the expected results.
An issue observed in this experiment was that there was some streakings along the length of the vertical channels. We believe this issue was caused by non-specific binding of AlexaFluor 647-labelled detection antibody on the region not previously immobilized with the peptide probes. It was because streaking also occurred in the control channel flowed with PBS but not MAb 17/9. So it would be necessary to improve the blocking step in between the probe immobilization and target binding steps in order to prevent the streaking. Therefore, milk powder or casein (Blotto) was used in the blocking step and the results were improved as shown in Figure 3.4. Here, the rectangular patches were cleanly separated from each other without the streaking line joining between them.

Initially, BSA was used and this provided some blocking but because the Blotto solution contained various milk proteins, a more effective blocking solution was obtained. The
wider variety of proteins in the milk solution seems to be more effective at preventing the streaking.

In the experiment shown in Figure 3.4, we also found that binding by MAb 17/9 is specific to the biotinylated HA peptide, with no binding detected to the biotinylated pVIII peptide, which served as a negative control.

To reduce streaking further, a non-ionic surfactant was added to the antibody sample solutions. The effect of the surfactant is to assist in the solubilisation of the antibody so that the specific binding is enhanced, and to create a protective layer on the surface so that the non-specific binding is reduced (Batteiger et al., 1982). We tested 2 surfactants, Tween-20 and Triton X-100. As shown in fig.3.5, Tween-20 had resulted in less streaking than Triton X-100 when added to the antibody samples. Moreover, Tween-20 had resulted in higher signal-to-background, see Table 3.1. The use 0.25% Tween-20, which has produced a higher signal-to-background than 0.5%, was adopted for use in subsequent experiments.
Figure 3.5: The effect of various surfactants on the streaking effect. Tween-20 and Triton X-100 were tested at various concentrations to test the effect of removal of non-specific binding. The spots are 100µm by 200µm.

Although the background streaking was removed by the use of Blotto, there was a reduction of the signal. This signal loss was rather significant and this reduced our ability to detect lower concentrations of the antibody samples. In order to enhance the signal, we felt that the probe immobilization needed to be enhanced.
Table 3.1: Results of binding of 10 nM of MAb to HA probe using either Triton X-100 (TX-100) or Tween-20 (T-20) as the surfactant mixed with the antibody sample. Error is standard deviation from 9 replicates

<table>
<thead>
<tr>
<th></th>
<th>10nM Anti-HA 0.25% T-20</th>
<th>10nM Anti-HA 0.5% T-20</th>
<th>10nM Anti-HA 0.25% TX-100</th>
<th>10nM Anti-HA 0.5% TX-100</th>
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</thead>
<tbody>
<tr>
<td>25μg/mL HA</td>
<td>4.7 ± 0.6</td>
<td>3.7 ± 0.5</td>
<td>4.1 ± 0.4</td>
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<tr>
<td>50μg/mL HA</td>
<td>7 ± 1</td>
<td>7 ± 1</td>
<td>5.4 ± 0.6</td>
<td>4.9 ± 0.4</td>
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<tr>
<td>75μg/mL HA</td>
<td>6.8 ± 0.9</td>
<td>7 ± 1</td>
<td>4.8 ± 0.4</td>
<td>4.7 ± 0.6</td>
</tr>
</tbody>
</table>

All fluorescent count values were multiplied by 10²

3.3. Signal enhancement

3.3.1. Effect of glutaraldehyde concentration on probe immobilization

The aldehyde surface plays an important part in the successful immobilization of the peptide probes (See fig 3.6). Two factors that may influence the binding efficacy are salt concentration in the glutaraldehyde solution and the reaction time. The concentration of the PBS solution that contained the glutaraldehyde was changed from an initial concentration of 9X PBS to determine if using a 1X PBS solution would produce similar signal intensities in subsequent antibody binding. Based on some observations, the tests indicated that 1X PBS led to lower signal intensity, thus we kept the 9X PBS concentration. The reason for this is that the higher salt concentration stabilizing the glutaraldehyde. This prevents the glutaraldehyde from reacting with itself allowing for the APTES to bind with the glutaraldehyde.

In the initial studies of the glutaraldehyde, the glass was allowed to react overnight. Due to signs of possible polymerization on the surface, the reaction time was reduced to 1 hour to determine the possible effect on the glutaraldehyde. The result showed no polymerization and the fluorescence signals were enhanced in subsequent experiments. This leads to a possibility that the extended reaction time has reduced the free aldehyde groups because more time could have allowed both ends of the glutaraldehyde to react with the APTES modified surface.
3.3.2. Effect of antibody incubation time

The incubation time of the antibodies after introduction in the NBA chip is an important parameter of the process. We have studied the results obtained at two incubation times, i.e. 15 min. and 20 min, in order to maximize the fluorescence signal. Since a longer incubation time will also lead to more non-specific binding, we will need to use surfactants mixed in the antibody sample. The results displayed in Figure 3.7 indicated that the surfactant Tween-20 performed better than Triton-X under a longer incubation time. The increase in the Tween-20 results, but not in the Triton-X results, suggest that the signal can be enhanced by introducing a longer incubation time when Tween-20 is mixed with the antibody sample. Figure 3.7 also showed that the results in using 50µg/mL of the peptide probe on the surface were similar to 75µg/mL, suggesting signal saturation.

Figure 3.6: Experimental Schematic Diagram.
Figure 3.7: Effect of sample incubation time on signal. When Tween-20 was used, a longer incubation time resulted in a significantly higher signal. The error bars are standard deviations obtained from 9 replicates.

3.3.3. Effect of using streptavidin to immobilize peptide that was biotinylated

Since we had the biotinylated version of our HA probe, we used the high binding affinity between streptavidin and biotin to increase the peptide probe immobilization on the surface (Peluso et al., 2003, Ramachandran et al., 2004). As shown in Figure 3.7, by using the bioHA immobilized on streptavidin (i.e. SA/BioHA), an increase in the fluorescence intensity was observed, as compared to HA immobilized on an aldehyde surface. It is suggested that it could be the removal of some steric hindrance, caused by the Schiff base reaction binding of the N-terminal of the peptide on to the aldehyde surface blocking the probe epitope, thus preventing the antibodies from binding. By
utilizing streptavidin to bind with biotin on bioHA, the epitope is less hindered allowing the probe to bind more effectively with the antibody.

![Image of antibody binding](image)

**Figure 3.8:** Enhanced signal detected from specific binding by MAb anti-HA from mouse 17/9 using a streptavidin-coated NBA chip. Binding by anti-HA is specific to the biotinylated HA peptide, with no binding detected to the biotinylated pVIII peptide. The right inset shows the region of anti-HA binding to bioHA. The spots are 100µm by 100µm.

### 3.4. Limitation of Detection ability of the Sample in linear channels:

The linear channel chip is unable to detect any of the fluorescent tags when the antibody concentration is lower than 0.5nM. This result is not as good as an equivalent ELISA assay. In order to increase the signal, a radial/spiral chip is utilized, using centrifugal liquid pumping. The centrifugal force provides dynamic liquid flow rather than a stop flow, allowing for the antibody sample to pass the channel continuously. In addition, this NBA chip has a longer channel allowing for the sample to remain in the channels for a longer period of time, offering a longer residence time. In order to determine how much of a signal enhancement could be obtained, the system was tested with the antibody sample ranging from 10nM to 0.05nM. Initial tests displayed a decrease in the detection limit from 0.5nM to 0.1nM of the antibodies. This was a significant improvement, bringing us to a level similar to that of the ELISA test result (0.1nM). Fluorescent imaging shows very specific patches and binding only occurs where a correct probe-sample pair intersects. Initial tests of the system in comparison to
the linear channels also displayed much lower background streaking, even with BSA as
the blocking agent. The dynamic interaction of the sample with the probe shows a
significant increase in the detection ability as compared to a stop-flow interaction.
4. Spiral Channel Chip Results and Discussion

4.1. Fabrication of Radial and Spiral Chips

In order to enhance the signal, a method that enhanced the dynamic interaction and increased the residence time was required. In order to do this, a spiral channel chip was introduced. The spiral chip was designed with numerous inner reservoirs to accommodate the samples to be injected. No outlet reservoirs were used as they caused potential backflow problems in the chip. The chip design incorporated the dimensions of 100µm width x 35µm height with a length of 4cm and 10cm for the radial and the spiral channels, respectively. The chip designs are as shown in figure 4.1.

![Designs of the radial/spiral chips](image)

Figure 4.1: Designs of the radial/spiral chips: (a) radial chip with 48 channels and (b) spiral chip with 24 channels. In both cases the outer circle has a diameter of 10cm.
Figure 4.2: Mold master (spiral chip) fabricated with SU-8 on a silicon wafer. The right top inset shows several positive relief structures, and right bottom inset shows each positive relief was of 100\(\mu\)m width and 35\(\mu\)m height.

Our chip would allow for the liquid to be delivered to each of the samples simultaneously by using centrifugal liquid pumping. The dimensions of the channels were made to allow for the best possible liquid delivery. The height of each of the channels would ensure that the liquid flow remained closed to the surface throughout the channel. The channel width of 100\(\mu\)m was made in order that a lower resolution of fluorescent scan (i.e. 25 \(\mu\)m) or an in-house made optical device would be able to measure the signals. If the channels were any smaller, it would be difficult to detect the signals. The radial design allowed for adequate physical separation between each of the channels and reservoirs such that there would be no backscatter resulting from unwanted fluorescence in adjacent regions. The spiral channels were designed with an equal force spiral in mind, and this would allow for the proper application of liquid delivery force, such that it would not simply accelerate uncontrollably through the channel.

The reservoirs were designed such that it would hold more liquid than would be needed to pass throughout the channel once. This was done to ensure that the possibility of a depletion of sample would not occur when applied in the channel. It was necessary to remove the outlet reservoirs because previous attempts to utilize the spiral channels with outlet reservoirs resulted in back pressure causing the liquid not to be
properly delivered along the spiral. In the fabrication of the chips, we utilized photolithography to fabricate the mold master on a silicon wafer substrate, see Figure 4.2. Subsequent casting of PDMS conducted onto this mold will result in the radial and spiral chips, see Figure 4.3.
Figure 4.3: Molded radial and spiral PDMS chips (a) Radial PDMS Chip of 7.5cm diameter. (b) Spiral PDMS Chip of 7.5cm diameter.
4.2. Signal Enhancement Using the Spiral Chip

While we were only able to detect sample concentrations of 5nM 17/9 antibody in the linear channels, the spiral channels demonstrated a significant enhancement, allowing us to detect 0.5 nM of the antibodies. We are able to compare our result with that of a comparable ELISA (i.e. 0.1 nM), see Fig. 4.5. The initial tests to compare to the results from the linear channels were made without streptavidin or a blocking agent. From these results, we were able to obtain a detection of 0.5nM of anti-HA antibody. While this was still 50 times greater than our intended detection limit of 0.01 nM, we had improved our detection limit by 10 times just switching from the use of the linear chip to the spiral chip. Another observation was that the initial test on the spiral chip was done without a blocking reagent. This is important advantage of using the spiral chip as the streaking in the channel was significantly reduced.
Figure 4.4: Results obtained on a spiral chip. (a) Initial results obtained from the whole NBA chip, with the left inset showing the binding of antibody samples in several spiral channels intersecting with 3 radial probe lines. The spots are 100µm by 100µm. (b) This is a sample schematic of one of our runs. The left one shows the intersection of anti-HA of 5 concentrations (in 3 replicates) with the HA peptide probes (in 5 replicates.) The right schematic shows the expected results.
**Figure 4.5:** **ELISA results obtained using an antibody standard (Anti-HA) at various concentrations. The detection limit is estimated to be around 0.1nM of the 17/9 monoclonal Anti-HA mouse antibodies.**

Results were provided by Dr. Naveed Gulzar.

Our tests indicated that if the glass chips were immobilized for 1 hour rather than 9 hours, a much more effective binding was demonstrated to the peptide probes, see Figure 4.6. In this way, we were able to obtain higher signals obtained from the markers (both radial and spiral) and from the binding by antibodies. Previous experiments had produced very faint radial marker lines which were difficult to locate, possibly because the aldehyde surface has been blocked preventing the peptide probes from binding onto the surface. With the radial markers more visible and simpler to detect, it would be possible to automate this method. More importantly, the signals obtained from antibody binding were much more intense.
Figure 4.6: Whole NBA chip scan for experiments conducted by glutaraldehyde treatment of 1 h versus 9 h before peptide immobilization.

When the streptavidin and the blocking agents were applied in the spiral chip, we were able to lower the detection limit 5-fold to 0.1nM of the MAb 17/9 sample, see Figure 4.7. While this is comparable to an ELISA assay, it has not yet achieved our goal of detection limit of 0.01 nM. This detection limit should be able to allow us to analyze the sample that comes from a single cell. Based on calculations obtained from Corti et al., the average cell secretes 6 attomoles per hour, see below.
((1000 molecules secreted/s)*(3600 s/h)/(6.022x10^{23} molecules/mol))

= 6 \times 10^{-18} \text{ mol or 6 amol}

In order to detect samples with this amount of material in our volumes (0.5\mu L or 500 nL) we would require a detection limit \sim 0.01 nM of material. Thus we sought to optimize our design to detect such a concentration.

**Figure 4.7:** Detection of antibody against peptide probe lines containing immobilized complexes of streptavidin and biotinylated HA peptide. Detection of concentrations as low as 0.1 nM in 0.4 \mu L of sample have been achieved. 800rpm radial spin speed, 1500rpm spiral spin speed. Blocking was 45min using blotto. The results were obtained from 9-replicate measurements on various regions of the chip. The spots are 100\mu m by 100\mu m.

In each of our results, we tested a variety of locations in order to determine reproducibility throughout the chips. Duplicates, and often triplicates, were obtained for each sample on a single chip. This would result in six to nine areas being measured for
each concentration per chip. The measurements were obtained and normalized against the background of each of the individual channels in order to determine the signal before plotting them.

Thus we began to optimize the spiral chip parameters. Many of the parameters were tested on the linear channels first and then brought over to the spiral chip. Several of the parameters that were brought over from the linear channels were from substrate preparation, peptide immobilization, as well as sample preparation. The parameters that would need to be optimized utilizing the spiral chips were the spin speed at which the chips would be spun to deliver the liquid and the ability to switch the use of the radial and spiral channel chips in terms of which would be utilized first for peptide immobilization.

From our tests involving dynamic interaction of samples, the longer the incubation time, the better the signal. The spin speed of the spiral channel is important to determine the incubation time. This can affect the antibody binding results drastically because the slower rotation speed allows for a longer incubation time. A significant increase in the signal was observed at 1250rpm, see Figure 4.8. The significant jump in signal is attributed to the oscillation in the liquid flow. Signals cannot be obtained from spin speeds lower than 1250rpm as there is not enough force to deliver the liquid.

![Figure 4.8: Signal enhancement by using lower spin speeds for the spiral chip during antibody introduction and binding. Radial spin speed 800rpm. All results were obtained on the same chip.](image)

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Figure 4.9: Fluorescent signals obtained from an NBA chip loaded with various antibody concentrations. (a) histogram showing the average fluorescent counts and the fluorescent image of the patches shown in the inset. (b) a graph obtained from the fluorescence counts along one radial line. The results were obtained from various spiral channels filled with various concentrations. The radial spin speed is 800rpm, spiral spin speed is 1300rpm. 17/9 = Anti-HA monoclonal mouse antibody obtained from mouse strain 17/9.

After several tests using various chips, we were able to obtain fairly reproducible results. In addition, we found that there was no significant decrease in the signals obtained from near the inlet (front) to near the edge (end) on the same spiral channel, see Figure 4.10. Similar signals obtained from near the inlet and from near the edge showed that there was little to no depletion of antibody samples in the dynamic binding process along the spiral channel. This allows for replicates to be completed along one channel from one end to another without loss of reproducibility.

While the flow at 1250rpm provided the highest signal intensity we utilized 1300 rpm as it demonstrated the most consistent results. The flow disruption caused some channels to stop flowing properly. At any speeds below 1250rpm, a complete disruption of flow was observed.
Figure 4.10: The signals obtained from the patches near the channel inlet near the chip centre (front) are compared against those near the edge (end) of the spiral chip. Similar signals were obtained from each antibody concentration, showing no sample depletion along the channel. The spin speed for the chip tests acquired was set to 1300rpm. The error bars were obtained from standard deviation of 18 replicates (3x3 replicates from 3 radial probe lines and 3 spiral channels from 2 chips done on the same day).

So far, we have been using the radial channel chip first for peptide immobilization followed by the spiral channel chip for antibody detection; we called this the radial-first method. In order to determine if the radial-first method has provided a better antibody detection capability, we reversed the chip application process. In this method, the spiral channel chip was first used to immobilize the peptide probes, while the radial channel chip will be utilized to introduce the samples; we called this the spiral-first method. We found that the spiral-first method displays higher signals than the radial-first method at higher antibody concentrations. On the other hand, the radial-first method displays higher signals than the spiral-first method at lower sample concentrations. This may be explained by more peptide probes being immobilization on the surface when a spiral channel chip was used, allowing the detection of higher antibody concentration without signal saturation. On the other hand, with the limited amount of peptide probes provided by the radial channel chip for immobilization, a lower antibody concentration required the longer residence time offered by the spiral channel chip to react completely with the
peptides to generate sufficient signals. Our method selection is to continue to utilize the radial-first method because this allows us to obtain better signals at lower concentrations, making it a better choice for our goal to achieve a low antibody detection limit.

Figure 4.11: Comparison of the signal obtained from the radial-first method and the spiral-first method. The spiral-first method shows higher signals at higher antibody concentrations, while the radial-first method shows higher signals at lower concentrations. Spin speed was set to 1300rpm. The error bars were obtained from the standard deviation of 9 replicates.
4.3. Detection of Mouse cell supernatants/Mouse ascites samples

In a test for the multiple detection abilities of the chip, bioHA and biopVIII were utilized as the probe lines for the detection of various concentrations of the mouse 17/9 anti-HA antibody, and this antibody in 17/9 mouse ascites and 17/9 hybridoma supernatant. The fluorescent signals obtained from various concentrations of anti-HA was shown in Figure 4.12. From this information, the amount of 17/9 antibody in the mouse samples can be estimated. The serum sample obtained from the mouse immunized with the phage coat protein consisted of anti-pVIII and should not have a response on intersecting with the bioHA probe line. In the biopVIII probe regions, we are able to identify the binding of anti-pVIII in the mouse serum, but none of the anti-HA antibody binding was detected. The fluorescent signals upon binding with biopVIII are shown in Figure 4.13. The selective binding of different antibodies in each of the individual channels shows that we are able to selectively identify multiple antibody samples on the same NBA chip.
Figure 4.12: (a) Results from using bioHA to test samples containing anti-HA MAbs from mouse 17/9 along with ascites and supernatants obtained from immunized 17/9 mice. The consisted of anti-pVIII and should not have a response. The error bars were obtained from the standard deviation of 9 replicates. The spots are 100µm by 100µm. (b) The fluorescent counts were plotted against various anti-HA concentrations.
Figure 4.13: Detection of samples containing anti-pVIII, which is the mouse serum immunized using the phage coat proteins. BiopVIII (50µg/mL) was used as the peptide probe lines for detection. The spots are 100µm by 100µm.

4.4. Signal Enhanced by Using Fluorescent Beads for Detection

The use of a fluorescently tagged detection antibody allowed us to detect the anti-HA antibody concentrations as low as 0.05nM. However in our attempt to detect lower concentrations, the alternative of utilizing a 0.97µm streptavidin (SA)-coated bead embedded with the Flash Red fluorescent dye (or so called SA-bead thereafter) is adopted and it is able to lower our detection limits beyond 0.05nM. Since we did not have biotinylated anti-HA; as a proof of concept, a biotinylated peptide (i.e. biopVIII) was immobilized on the NBA chip to be tested by either SA-Cy5 or SA-bead. As shown in Figure 4.14, the bead provided almost 5 times of fluorescent signal enhancement as
compared to Cy5. We believed this method can be applied to antibody detection to achieve a lower detection limit.

**Figure 4.14:** The use of a SA-bead compared to SA-Cy5 displayed a significant enhancement in the signal of the detection when immobilized biotin from biopVIII (50 µg/mL) was used. The error bars were obtained from the standard deviation of 9 replicates.
5. Conclusions and Future Directions

We have been able to develop a NBA chip platform that is able to detect antibodies with a detection limit below that of a comparable ELISA. It is able to be run each sample using 0.5µL on the NBA chip, as compared to the 100µL of sample that an equivalent ELISA on a 96-well plate requires (see Table 5.1). Detection of antibody samples based on dynamic flow using the spiral chip allows for consistent results obtained from the center of the chip to the edges. We have also demonstrated that for detection of antibodies at low concentrations, a radial-first method (i.e. radial probe lines intersected by samples in spiral channels) gives better results than the spiral-first method. More importantly, we have applied the developed method to detect antibodies in serum and ascites obtained from immunized mouse samples. In addition, we are able to demonstrate the multiple-probe, multiple sample approach on the NBA chip. As shown in Table 5.1, detection of 16 samples on 16 probes has been demonstrated in the linear channel chips (see Fig 3.5), as compared to detection of 16 samples on 6 samples on the 96-well plate platform. The capability of the NBA chip is more, as the detection of 24 samples on 48 probes is feasible on the spiral channel chips.

<table>
<thead>
<tr>
<th></th>
<th>Sample Volume</th>
<th>Number of Samples</th>
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<tbody>
<tr>
<td>MMA</td>
<td>≤0.5 µL</td>
<td>16 samples x 16 probes or more</td>
</tr>
<tr>
<td>96-well plate</td>
<td>100 µL</td>
<td>1 sample x 96 probes or 16 samples x 6 probes</td>
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With the detection of 0.05nM of the 500-nL anti-HA sample demonstrated, future work will include the detection of anti-HA secreted from a single antibody-producing B cell. With the need to rapidly generate monoclonal antibodies, the ability to detect antibodies from a single cell sample allowing for the ability to quickly isolate the antibody-producing cell for single-cell genetic sequencing. To assist in this goal, application of the biotinylated secondary detection antibody for the application of the streptavidin-coated fluorescent bead will be important in enhancing the detection ability. This method will also require the optimization of the probe immobilization step, as streptavidin can no longer be used to anchor the peptide probes without causing interference.

Current applications are focused on samples that have the ELISA kits developed. However, there are a wide variety of antibodies that are not so easily captured. In addition to proteins that are difficult to analyze, many samples need to be purified before they can be analyzed. However, using our system it should be able to simultaneously analyze various types of unpurified biological samples such as the secretome produced by a single cell.

In terms of technology advances, there will be needs to improve the immunoassay systems for ease of automation. This will lead to much higher throughput and more reproducible results. While most of the instruments have automated operations, dispensing of the materials do not, which can lead to variations in detected signals. Another area that needs to be improved is the sensitivity of the instruments. It has been shown that the use of fluorescent detection results in lower detection limits in micro or nanofluidic devices than in conventional methods like ELISA. To obtain a better
signal enhancement, or a stronger fluorescent signal, a variety of methods such as fluorescent beads may be utilized.

Furthermore, there will be a need for a high throughput system that will be able to analyze a large number of samples in a short amount of time. In addition, only a single aspect of these samples is currently analyzed. If instruments are able to completely characterize a sample, it will provide significantly more information. This will vastly improve our ability to understand the role of the antibodies in a variety of physiological environments. If possible automation would be useful in cutting down the time required for analysis.
6. References


Appendices
Appendix A. NBA chip fabrication

The NBA chip fabrication was made utilizing a silicon (Si) wafer as a mold master.

The silicon wafer was cleaned using water and Liqui-Nox detergent. The wafer was rinsed and placed into a piranha solution (70% H₂SO₄ 30% H₂O₂). The silicon wafer was then dried with N₂ gas. After cleaning, the wafer was placed onto a spin coater and a photoresist layer of SU-8 was applied. The wafer was spun at 500 rpm for the first 10 seconds followed by ramping the speed up until 4000 rpm for the final 30 seconds. The wafer was then removed from the spin coater and placed on a hot plate at 65°C for 3 minutes. After, the wafer was placed on another plate at 95°C for an additional 5 minutes. After the wafer was cooled to room temperature, the photomask was placed on top of the wafer and UV light was applied to crosslink the SU-8 epoxy polymer.
Appendix B. Glass Surface Modification

1. Glass Cleaning
   a. Wash glass slides utilizing a 10% Liqui-Nox solution.
   b. Rinse with distilled water.
   c. Rinse with ethanol.
   d. Rinse with 18MΩ-cm deionized water.
   e. Dry using nitrogen gas.

2. Hydrolyzing the glass surface
   a. Place 2 glass capillary tubes parallel to each other on the bottom of the crystallizing dish. (125mm x 65mm)
   b. Place a clean glass slide on top of the capillary tubes.
   c. Place 2 additional capillary tubes on top of the clean glass slide.
   d. Place a clean glass slide on top of the capillary tubes.
   e. Repeat steps c, d for as many slides as necessary. (We have utilized a maximum of 4 slides)
   f. To the dish, add 100mL of piranha solution (30mL of 30% hydrogen peroxide and 70mL of concentrated sulphuric acid) to the dish. Cover the dish with glass cover.
   g. Place the crystallizing dish on a hot plate.
   h. Keep solution at approximately 80-90°C (do not boil) for 15 minutes. Swirl the dish gently every 2-5 minutes.
   i. Remove the dish from the hot plate and allow it to cool.
   j. Remove the glass chips from piranha solution and rinse with distilled water.
   k. Rinse with 18MΩ-cm deionized water.
   l. Dry with nitrogen using a zigzag motion, from side to side working from top to bottom, ensuring no streaks remained along the surface.
3. Amination of the glass surface using 3-aminopropyltriethoxysilane (APTES)
   a. Place 2 glass capillary tubes parallel to each other on the bottom of a crystallizing dish. (125mm x 65mm)
   b. Place a hydrolyzed glass slide on top of the capillary tubes.
   c. Place 2 additional capillary tubes on top of the hydrolyzed glass slide.
   d. Place a hydrolyzed glass slide on top of the capillary tubes.
   e. Repeat steps c, d for as many slides as necessary.
   f. Add 100mL of APTES solution (2mL of 3-aminopropyltriethoxysilane, 98mL of anhydrous ethanol) to the dish. Cover with Parafilm.
   g. Purge the dish with N₂ gas for 60 seconds.
   h. Keep the glass slides in the dish for 20 minutes. Swirl the dish gently every 2-5 minutes.
   i. Remove the glass slides from the APTES solution and rinse with 95% ethanol.
   j. Dry with nitrogen using a zigzag motion, from side to side working from top to bottom, ensuring no streaks remained along the surface. *NOTE*: If streaks occur in this case, rinse again with ethanol and dry using the zigzag motion. Using a higher gas pressure makes this easier to do without leaving streaks.

4. Ethanol Removal
   a. Place the dry glass into a crystallizing dish. The edge of the glass must be placed on the bottom with the top corners against the side of the dish.
   b. The APTES modified glass is placed into an oven at 120°C for one hour.
   c. Remove the dish from the oven and allow the glass to cool.
5. Glutaraldehyde Modification
   a. Place 2 glass capillary tubes parallel to each other on the bottom of a crystallizing dish. (125mm x 65mm)
   b. Place APTES-modified glass slide on top of the capillary tubes.
   c. Place 2 additional capillary tubes on top of the APTES-modified glass slide.
   d. Place APTES-modified glass slide on top of the capillary tubes.
   e. Repeat steps c, d for as many slides as necessary.
   f. Add 100mL of 5% glutaraldehyde solution (10mL of 50% glutaraldehyde solution, 90mL of 10X phosphate buffer saline solution) to the dish. Cover it with a glass dish cover.
   g. Purge the dish with N₂ gas for 60 seconds.
   h. Place the dish immediately into the crisper drawer of a refrigerator.
   i. Allow the glass surface to react at least 1 hour in solution before removal.
   *NOTE*: The glass can be left in dish for >2 weeks. After removal of the glass from the dish, purge it with N₂ gas to ensure remaining chips remain in the inert environment.