DEVELOPMENT OF A CAPILLARY BASED *HELICOBACTER HEPATICUS* BIOSENSOR

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Master of Science

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by
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HELICOBACTER HEPATICUS BIOSENSOR

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DEVELOPMENT OF A CAPILLARY BASED

HELICOBACTER HEPATICUS BIOSENSOR

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ABSTRACT

Helicobacter hepaticus is a bacterium that causes chronic active hepatitis in mice. Infections with *H. hepaticus* can invalidate research studies. Early detection is necessary to curtail the spread of infection. Current sensing mechanisms are time consuming and not suitable for preventative diagnostics. The objective of this study was to develop a quick and accurate biosensor platform for the detection of *H. Hepaticus* in fecal samples.

This research investigated an optical immunosensor using capillary waveguides. A competitive immunoassay was adapted for use in this biosensor. *H. hepaticus* was immobilized to the inner wall of the capillary. A fixed amount of *H. hepaticus* antibody was conjugated to Alexa Fluor 546 to serve as the fluorescent tracer and added to samples containing *H. hepaticus*. Sample *H. hepaticus* (analyte) bound to the antibodies in solution, thereby preventing the antibodies from attaching to the immobilized *H. hepaticus*. An increase in sample *H. hepaticus* decreased the number of antibodies bound to the capillary wall and increased the number of antibodies in solution. Several methods were utilized to analyze the fluorescence resulting from the immunoassay. Sample mixture analysis analyzed the labeled antibodies remaining in solution and had a 10 ng minimum limit of detection. Immobilized capillary analysis examined the amount of

labeled antibodies bound to the capillary walls and had a 1.0 ng minimum limit of detection with an assay time of approximately one hour. The biosensor was shown to reliably detect low levels of *H. hepaticus* in a fast and reliable manner.

CHAPTER ONE
Literature Review

1.1 Introduction to Biosensors

1.1.1 Overview

Sensors are used in wide a variety of daily tasks and are a necessary part of every day life. A sensor functions by responding to a physical stimulus and transmitting the resulting impulse[1]. Sensors are comprised of recognition and transduction components[2]. The recognition component of a sensor is responsible for detecting the presence of the measured substance, called an analyte. Upon recognition, the transduction element converts the observed stimulus into a usable signal[3]. Signal processing techniques, commonly done through a computer, transform the generated signal into meaningful information. The processed information is then provided to the user and utilized to complete a sensor specific task. Examples of sensor specific tasks include opening a door, closing a valve or sending warning messages. Sensors are divided into categories based on the recognition and transduction elements they employ.

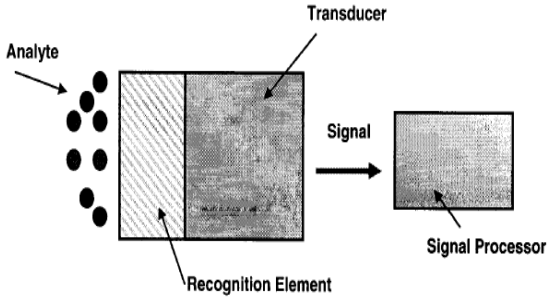


Figure 1: Representation of the recognition and transduction element of a sensor[3].

There are three main categories of sensors: physical, chemical and biological. Physical sensors measure physical characteristics such as mass, temperature, pressure and distance. A chemical sensor measures chemical substances by a physical or chemical response. Biological sensors (called biosensors) measure chemical substances using a

biological sensing element. Chemical and biosensors can measure similar substances, but the biological component distinguishes the two categories[3].

Biological recognition components can be a variety of molecules or proteins, including antibodies, enzymes, DNA and cells[2]. Using biological components increases the sensor's specificity for the analytes of interest. The biological recognition element is the foundation for the biosensor and must be carefully selected. Different recognition elements, even for the same analyte, can drastically alter the characteristics of the biosensor. There are many biological components that could potentially be used in biosensors. This creates the possibility for the detection or measurement of an equally large number of analytes with biosensors.

The biological recognition components serve as a receptor for the analyte and are in contact with the transducer[4] (Figure 2). Facilitated by the transducer, the biological recognition elements convert a biological state into a useful signal[5]. The transducer produces a quantifiable optical or electrochemical signal after analyte recognition[2]. Signal processing refines the signal and provides information to the user about the biological process occurring in the sample.

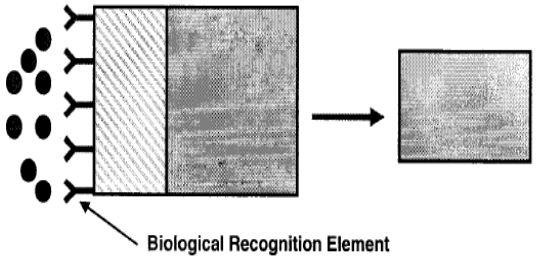


Figure 2: Schematic of how the recognition element is incorporated into the classical sensor design[3].

The biological components contribute to both the strengths and weaknesses of the biosensor. Biosensors are capable of producing quick, sensitive and specific measurements[3]. Specificity refers to the sensors ability to distinguish between the

analyte of interest and similar molecules[2]. The biological components of a highly specific biosensor should only produce a response from the analyte of interest. Sensitivity is the sensor's response capabilities to incremental changes in analyte of detection[2]. This is similar to a sensor's limit of detection, which is the minimum concentration resulting in a detectable sensor response. Detection time is largely dependent on the binding kinetics of the analytes to the recognition element[6]. Biosensors response times should approximately range from several seconds to 10 minutes[3]. Response times may be longer, but this may reduce the usefulness of the biosensor.

A major drawback to biosensors is that they are limited to use in specific environments. Biological components are sensitive to their surrounding environment. Harsh conditions, such as extreme pH levels and temperatures, can alter or destroy the biological components. This may affect the output signal from the biosensor and produce inaccurate results. In addition, biosensors have a limited dynamic range compared to other types of sensors. Biosensors are developed with these factors in mind and should only be used under proper conditions[2].

Biosensors use a variety of different transduction methods. Examples of these methods include electrochemical, optical and thermal[2]. Electrical transducers measure voltage changes generated by the biological components. Thermal transducers use thermoresistors to measure heat generated by the chemical or biochemical processes. Optical transducers use optical techniques to measure the resulting changes from the biological or chemical processes. Each of these methods has its own place in biosensor development. This research project focused on optical based biosensors, which are discussed in further detail below.

1.1.2 Optical Biosensors

Optical biosensors transduce optical signals resulting from biological interactions. The optical waveguide is a crucial component of the biosensor[4]. An optical waveguide is a material that transports light down its path from one location to another[4] . Various substrates are utilized as optical waveguides, and each type of optical waveguide is unique and largely determines the optical properties of the biosensor. The biological components are attached to the optical waveguide. The resulting binding event changes the light properties of the recognition components, which are captured by the optical waveguide. The optical biosensor then analyzes the changes resulting from the examined biological process. Optical biosensors are categorized by the optical waveguides and light principles used in the sensor.

Optical fibers were the first optical waveguides used for biosensors and have been widely studied[4]. Many different optical fiber sensors have been studied for a variety of different applications including clinical[7,8], environmental[9,10] and warfare agent analysis[11,12,13]. Other examples of waveguides include biosensors that utilize surface plasmon resonance (SPR), which is a valuable tool widely used in medical diagnostics[2]. Capillary-based biosensors are advantageous for small volume application and have been studied increasingly in the last two decades[14]. Capillary biosensors were the focus of this research and a more comprehensive description of their functions will be presented subsequently.

Commonly used optical techniques include absorption and fluorescence spectroscopy, chemiluminescence, and refractive index changes[3]. Fluorescence is widely used in biosensors, including in this project, because fluorescence spectroscopy allows

for higher sensitivities compared to other techniques[3]. Fluorescent molecules are a special class of typically aromatic chemical compounds known as fluorophores[15]. These compounds absorb light energy, resulting in an electron being placed in a higher energy level. The electron will leave the excited state and return to the more stable ground state, emitting a photon. As shown in Figure 3, the Jablonski diagram classically depicts this process.

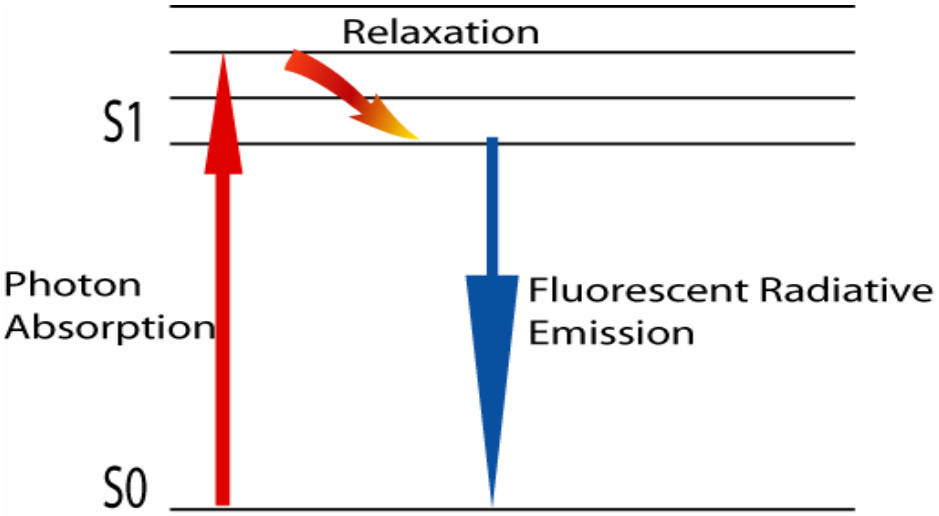


Figure 3: Jablonski Diagram representing the fluorescence process [15].

Fluorophores absorb energy over a range of wavelengths called the absorption or excitation spectrum of the fluorophore. Absorption occurs at shorter wavelengths than the emission. Optimal excitation occurs at the wavelength corresponding to the peak of the absorption spectrum. Light is emitted by the fluorophore in a characteristic spectrum as the molecule returns to ground state. The wavelength corresponding to the peak of the emission spectrum is analyzed for measurement of fluorescent intensity. Figure 4 shows the absorption and emission spectrum for Alexa Fluor 546, the fluorophore used in this research.

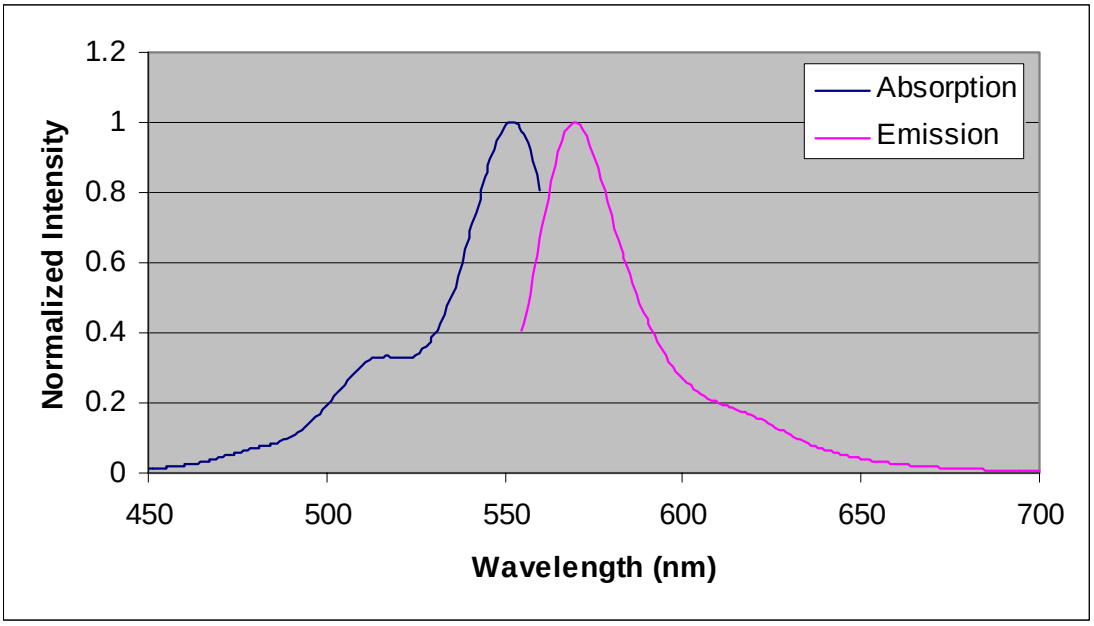


Figure 4: Absorption and emission spectra for Alexa Fluor 546. The emission and absorption peaks are approximately 546 and 570 nm, respectively.

There are many fluorophores covering the entire visible light spectrum, thus making them a valuable resource for optical biosensor development. Fluorescent molecules can be conjugated to biological components or to the analytes that respond to the biological components. The fluorescent changes mediated by the biological components attached to the transducer represent the event analyzed by the biosensor. Fluorescent intensity measurements serve as a representation of the biological process being monitored.

Optical biosensors are a valuable tool in many fields. Progress in the optical biosensor field is reflected by the increased number of commercially available products. Examples of successful commercial products include the BIACORE SPR based machines[2] and the optical fiber based Analyte 2000[13]. There are many advantages to optical biosensors that make them attractive products. Biosensors produce fast, almost real time results. Some biosensors are compact and portable, allowing for testing outside

of the laboratory[13]. Most biosensors are able to test analytes with a degree of accuracy comparable to other current analytical methods.

There are also some potential complications in developing optical biosensors. Parasitic optical signals, such as background fluorescence and stray ambient, have a major impact on the accuracy of optical biosensors. The parasitic signals interfere with the measured optical signal, resulting in increased variability and inaccurate results. Biosensors are designed to reduce these factors and are specified for use in certain conditions[3].

1.1.3 Total Internal Reflection and Evanescent Wave Theory

Total internal reflection (TIR) and evanescent wave excitation are optical techniques often used in optical biosensors. Reflection or refraction occurs when a light wave strikes an interface between two mediums with different indices of refraction. As demonstrated in Equation (1)[5], light traveling from a higher (n_1) to lower (n_2) index of refraction material will be reflected if the incident angle (Θ) is less than the critical angle (Θ_c). Light is refracted if Θ is greater than Θ_c.

$$\theta_c = \sin^{-1}\left(\frac{n_2}{n_1}\right) \qquad \text{Eqn. (1)}$$

TIR is a special case in which all incident light is reflected back into the higher index of refraction material. TIR is very useful in making waveguides. Light can propagate down the waveguide into a detector with minimal scattering and loss of intensity. Optical fibers are a classic example of this technology. They have a glass core surrounded by a

cladding made of a lower index of refraction material that allows the fiber to transmit light efficiently[3].

An evanescent wave is a by-product of TIR. When light is reflected back into the lower index of refraction material, there is a finite electrical field that crosses the boundary[3]. The electrical field is confined within a penetration depth extending from the surface defined by equation (2)[5]:

$$d_p = \frac{\lambda}{2\pi\left(n_1^2 \sin^2 \theta_1 - n_2^2\right)^{\frac{1}{2}}} \qquad \text{Eqn. (2)}$$

The evanescent wave is used to excite fluorophores within the penetration depth[16]. Fluorophore emission is captured by the waveguide and transmitted to a detector. This is the basis behind optical fiber biosensors. Evanescent wave excitation has also been applied to capillary biosensors[14].

1.1.4 Immunosensors

An immunological approach is commonly applied in the development of biosensors. Immunosensors are immunologically based biosensors that use the antibody-antigen relationship as the foundation of the biological recognition component of the sensor[2]. The transducer measures the antibody binding event[16]. This approach takes advantage of an antibody's affinity for a specific antigen[3]. Biosensor researchers benefit from immunological techniques developed in other fields. Utilization and adaptation of these well-established techniques provide a valuable resource for biosensor researchers.

Antibodies help defend the body against infection and are a crucial part of the immune system[17]. A representation of a secreted IgG, the antibody type typically used in biosensors, is shown in Figure 5. The immune system develops many antibodies for protection against possible foreign substances. An antibody forms after injecting a host animal with the antigen. The immune system will recognize the antigen as foreign and stage an immune response using B-lymphocytes. Antigen specific B-cells recognize the antigen and transform into plasma cells that produce antibodies specific for the introduced antigen. Produced antibodies can then be removed from the animal and purified. The antibodies are then ready to serve as the biological recognition component for the biosensor.

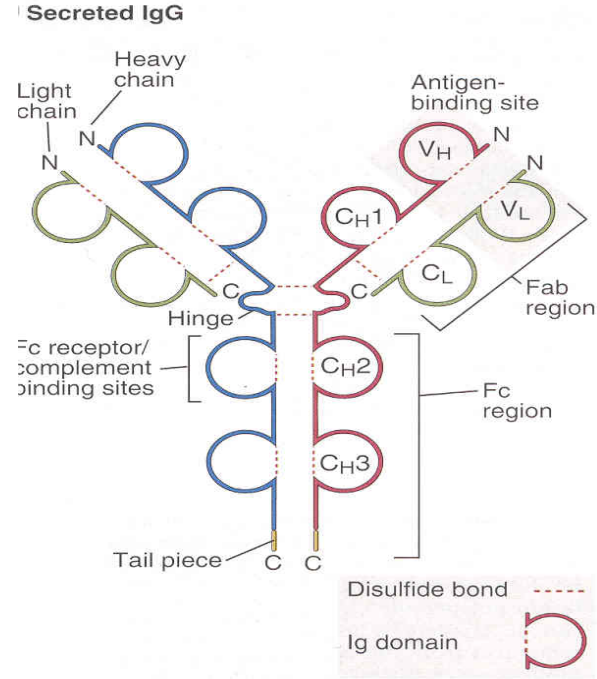


Figure 5: Representation of an antibody. Note the antibody binding sights in each variable domain[17].

Important characteristics of the antibody-antigen relationship include the bond strength and specificity of the antibody for the antigen. Antibody-antigen interactions are largely the product of weak electrostatic and hydrogen bonding between the antibody and antigen[16]. Antibodies with weak affinity for their antigen will hold the antigen with less strength than one with a higher affinity. Specificity is the antibody's ability to distinguish between similar antigens[17]. A highly specific antibody will only bind to the desired antigen where as less specific antibodies may bind to multiple antigens.

Specificity is measured by determining the cross reactivity of the antibody with similar antigens[17]. Affinity and specificity are important factors to keep in mind when selecting an antibody for use in a biosensor. Most biosensor applications require an antibody with both high affinity and specificity[1].

Fluorescently based immunosensors (fluoroimmunosensors)[18] are examined in this research. At least one part of the measured biological system must be conjugated to a fluorophore in order to produce a fluorescent signal. In most cases, either the antibody or the antigen can be labeled with a fluorophore. If the labeled component is immobilized to the transducer, then the change in fluorescence from the binding event can be measured. Alternatively, if the labeled element is in solution, then the collected signal monitors the binding to the immobilized component. In both cases, the transducer is measuring changes occurring in the optical characteristics of the antibody antigen complexes[16].

Inherent antibody properties largely determine immunosensor sensitivity[16]. Several controllable factors can be optimized to maximize the sensitivity of the system. For example, the amount of antibody and antigen used in the system can be adjusted, thereby altering both the signal and the amount of time needed for binding event completion. The immunological technique used will also affect the sensitivity of the sensor. Some techniques are capable of higher sensitivity than others[16]. The immobilization method of the biological receptor components is also an important factor in determining sensitivity. Characteristics of these techniques can be found in the literature and research should be performed when selecting the proper technique for each biosensor application.

1.1.5 Immobilization Techniques

Immobilization of the biological recognition component to the transducer is an important aspect in biosensor development. It is important that the immunological characteristics of the biological component be unaltered and securely attached to the sensor waveguide or platform. There are three categories of immobilization techniques: physicochemical, electrostatic and covalent attachment[5].

Adsorption is the primary method of physicochemical immobilization. This method relies on weak electrostatic and Van der Waals forces between the biological component and transducer[5]. This is the simplest method of attachment. The optical transducer (waveguide) is placed into a solution containing the biological component and adsorption will occur automatically. Immobilization using adsorption is not a permanent linkage and the biological components can be removed with extensive washing[3]. Additionally, this technique does not insure proper orientation of the recognition components and binding may be inhibited as a result[16].

Electrostatic immobilization is very similar to absorption[5]. This method relies on the formation of an ionic bond between positive and negative charged regions on the sensor waveguide and biological components. Electrostatic immobilization has the same advantages and drawbacks as absorption. The strength of electrostatic bonds is greater than adsorption, but still is not permanent.

Covalent attachment is the most stable form of immobilization and is used in this research[16]. Immobilization using this method results in a covalent bond between the optical transducer and the biological components[5]. The bond is long-lasting, but may degrade over longer periods[3]. This technique is more complex than the others, but the

resulting immobilization is better suited for biosensor applications. In covalent attachment, the surface of the optical transducer is modified to allow for the biological component attachment. While there are many methods for covalent attachment, this discussion is limited to silanization, which is the method utilized in this research project. This immobilization process was first described by Bhatia et al[19,20].

The first step in silanization is to add a silane film to the waveguide surface. Glass has easily modifiable hydroxyl (Si-OH) groups. Silanization substitutes the hydrogen bound to the oxygen of the Si-OH with a silyl group to form a silane film. The silane can then be further modified without altering the glass substrate[5]. A heterobiofunctional crosslinker can be used to connect the silane film to the protein. Heterobifunctional crosslinkers have active sites on both ends of the molecule that bind to different functional groups. GMBS was used in this research (see materials for full chemical name). GMBS has maleimide and N-hydroxysuccinimydyl (NHS) ester groups that react with thiol and amine groups, respectively. The cross-linker binds to the thiol group of the silane through the maleimide end of the cross-linker. The opposite end of the GMBS molecule containing the NHS ester functional group binds the biological recognition component through the amine groups present in the protein. This method is widely used in the biosensor field[14,21,22].

1.2 Capillary Biosensors

Capillary biosensors utilize capillary tubes as the optical waveguide. The biological components are immobilized to the inner surface of the capillary. Filling the capillary with an aqueous medium helps preserve the biological components allowing for

long-term storage. The capillaries can be made in advance and used when needed, a desirable attribute for a commercial product. Both glass and plastic capillary tubes are used as biosensors.

Polystyrene capillaries allow for the adsorption of the biological components with out capillary surface modification[23]. This is advantageous when making patterned capillaries that have the biological components immobilized only to certain sections of the capillary. The use of polystyrene capillaries in optical biosensors is limited[23,24,25] and a commercial supplier could not be found for this project.

Glass capillary tubes are used in this research because of their availability and because the biological components can be covalently immobilized using the silanization process described above. Covalent immobilization is desirable because of the strong, long lasting attachment of the biological components to the capillary surface. The majority of capillary biosensors reported in literature utilize glass capillary waveguides. The outer surface of the capillary is sometimes coated to increase light propagation. In several reports Teflon coated capillaries are used to improve evanescent wave excitation and light propagation capabilities[6,14]. The Teflon coating also increases fluorophore emission capture and propagation down the length of the capillary by increased internal reflection.

Capillary biosensors are integrated sensors in that they serve as both a sampling device and an optical waveguide[26]. The capillary tubes support fluid flow through the interior and light propagation in the walls[26]. This allows for both sampling and analysis in one location. Capillary tubes can easily be adapted to automated devices. The capillary tubes allow for efficient filling and fluid transport by incorporating pumping

devices into capillary biosensors[6,27,28]. Eventually, it is envisioned that entire capillary biosensor systems will be small, portable, automated devices capable of sampling and analyzing with minimal human intervention.

Using capillary tubes provides several advantages over other waveguides. Capillary design allows them to hold small, fixed volumes of approximately 1-100 µl. Reduced sample volumes use less reagents and shorten required incubation times[29]. Immunoreagants are expensive and using less lowers assay costs. Capillaries have increased surface to volume ratios compared to traditional well plate immunoassays. This reduces immunoreagant diffusion and allows for better assay kinetics [23,30]. Capillary tubes are less expensive compared to other optical waveguides such as optical fibers. Low cost and mass production allows capillary tubes to be a disposable component of the biosensor that is high throughput and cost effective[29].

Biosensor applications utilize capillaries in two primary ways. One, capillaries have been used as flow cell and two, integrating waveguide immunosensors[14]. In both cases, the biological components are immobilized to the capillary, but the analysis methods are different. Flow cell immunosensor analysis occurs outside of the capillary and waveguide capillary analysis occurs within or using the capillary. This research uses aspects of both designs.

Several flow cell immunosensors have been reported in literature. Koch et al. in 2000 reported an optical flow-cell multi-channel immunosensor to detect biological warfare agents using a sandwich assay design[28]. This system essentially did a traditional Enzyme-Linked ImmunoSorbent Assay (ELISA) within the capillary tube. This sensor measures the colorimetric change resulting from the immunoassay by absorption

detection. This system incorporated peristaltic pumps for fluid transport that allowed semi-automation. Assay time for this biosensor was 30 minutes per sample with the ability to test several samples simultaneously.

The optical flow capillary sensor most relevant to this research was a displacement flow fluoroimmunosensor to detect explosive, Tri-Nitro-Toluene (TNT), reported by Narang et al. in 1997[27,31]. In this technique, antibodies were immobilized to the inner capillary walls. The antibodies were then loaded with a fluorescently labeled TNT analogue. Samples were flowed through the capillary and the run off collected. Sample TNT displaces the fluorescently labeled molecule from the antibody. The labeled TNT were collected in the flow through and analyzed. As expected, this method showed increased fluorescent intensity with increased TNT concentration.

Capillary waveguide biosensors have also been developed[14]. This technique used the capillary as both a location for the immunoreaction and signal transduction. The biological components were immobilized to the inner walls of the capillary and serve as capturing agents. There are different excitation and emission collection techniques that have been used with the capillary waveguide biosensors.

The first capillary waveguide chemical sensor measured carbon dioxide and was reported by Weigl and Wolfbeis in 1994[26]. Substances that produce a color change when exposed to carbon dioxide were immobilized to the inner surface of the capillary. A modified optical fiber coupler holds the capillary and optical fibers. The optical fibers were used for both excitation and collection. Both the excitation and collection fiber were perpendicular to the capillary, but in line with each other separated by the capillary. This method allowed for sensitive, accurate and repeatable absorption measurements.

Ligler et al in 2002 provided a summary on four different potential excitation and collection methods for integrating waveguide Teflon coated capillaries[14]. Two of the techniques were analogous to methods used in this research project. An evanescent wave is used for excitation of bound fluorophores. Fluorescent emission can be captured by the capillary, propagated down its axis and collected at the end of the capillary. This technique is analogous to techniques used with optical fibers. Alternatively, a detector can be placed perpendicular to the capillary for data collection. The first technique serves as an integrating waveguide. Fluorophores along the entire length of the capillary are being excited. A portion of the emissions from all the fluorophores is captured by the capillary and propagated down the waveguide. The signal accumulates as light propagates down the waveguide, thereby increasing signal.

In the second technique the detector is placed perpendicular to the capillary and data collection does not rely on light propagation down the capillary. The fluorophore emission from the capillary region directly in front of detector is collected. This signal is theoretically less than the integrated signal from the former method. Uncoated glass capillaries are not ideal waveguides because there is no lower index refraction medium to establish evanescent wave propagation and total internal reflections. Thus, excitation light and generated signal strays from the waveguide[6].

Several biosensors utilize the integrating waveguide capillary biosensor design with data collection at the ends of the capillary. Misiakos and Kakabakos used this method in making a multi-band immunosensor[29]. The immunoreactive regions of the patterned capillary are excited, the emission captured and transmitted down the capillary.

A four band disposable capillary to measure pesticides[18] and a multi-analyte sensor to detect hormones utilize similar techniques[24] have been reported using this method.

A biosensor to detect waterborne *E. coli* O157 has recently been reported by Zhu et al. in 2005[32]. This report is significant because it detects bacteria similar to *H. hepaticus* (discussed in section 1.3.1). Similar capillaries and excitation methods were used; however, this sensor utilized a sandwich technique that differs from the competitive technique used in this research. *E. coli* O157 capture antibodies are immobilized to the inner walls of the capillary. Samples are added to the capillary and any E. coli present should bind to the antibody. A fluorescently labeled secondary antibody is then added to the capillary and incubated. The capillary is then washed and the entire length of the capillary is excited perpendicularly. Data is collected at the end of the capillary tube. This method reported being able to detect 10 cells per 75 µl capillary. This sensitivity should be similar to that desired by a *H. hepaticus* system.

This project utilized a method in which both the excitation source and data collection occurred perpendicularly to the capillary, but in line with each other. Only a small region (approximately 3 mm) was illuminated. The method reported by Weigl and Wolfbeis was the similar to this, but measured absorption not fluorescence[26]. Dhadwal et al. reported a DNA biosensor using evanescent wave excitation and perpendicular collection[6]. This DNA capillary biosensor used an excitation source that was coupled to the capillary using optical fibers and transmitted down the capillaries. The immobilized DNA captures fluorescently labeled complimentary DNA strands. Data was collected using either a single 1 mm optical fiber or an optical fiber array coupled to the capillary.

The authors analyzed this configuration in depth and concluded that this was the optimal analysis method for a capillary biosensor.

Capillary biosensors represent a new field in biosensor development. Past research using capillaries show that it is a reliable technology capable of provide fast, sensitive results with low limits of detection. Using the capillaries as both an optical waveguide and fluid transport mechanism is conducive to a variety of biosensor platforms. Applying this technology to a *Helicobacter hepaticus* biosensor will advance the study in capillary based sensing.

1.3 *Helicobacter hepaticus*

1.3.1 *Helicobacter hepaticus* Overview

Helicobacter hepaticus is a bacterium known to cause chronic active hepatitis[33,34], inflammatory bowel disease (IBD)[35] and liver tumors in mice[36]. This bacterium was first discovered in 1992, at the National Cancer Institute –Frederick Cancer Research and Development Center (NCI-FCRDC), in Frederick, Maryland[36]. The effects of this bacterial infection were originally identified histologically in control mice of a long term toxicology study. Researchers noticed a distinct pattern of liver damage inconsistent with known disease patterns. Further studies were done to identify the cause of the disease.

A bacterium was isolated from the liver of the effected animals at the periphery of the present lesions. The liver tissue sampled was stained using Steiner's silver stain for histopathological examination. Analysis of these liver samples revealed a helical shaped microorganism. The bacteria were also found cecum and colon of the infected animals. After this bacterium was isolated, other mice were infected with this bacterium to

confirm that this was the causative agent. PCR analysis revealed that this bacterium contained the conserved 16S rRNA similar to other *Helicobacter* species. This study confirmed that the discovered bacterium was the infective agent and was classified as a novel helicobacter species named *Helicobacter hepaticus*.

A subsequent study by Fox et al. further defined the morphology of the bacterium[37]. The bacterium is helically shaped, 1.5-5 µm long, .2-.3 µm wide and gram negative (Figure 6[37]). There are bipolar, sheathed flagella on both ends of the organism. The bacterium is difficult to culture and stringent, microaerophillic or anaerobic environments at 37 °C growth conditions are required for proliferation[38]. The primary means of transmission is from the ingestion of feces from infected animals. Studies have shown that infection is spread from contact with contaminated bedding and direct contact with infected mice[39]. Mice housed in adjacent, open air cages are at risk for infection[40].

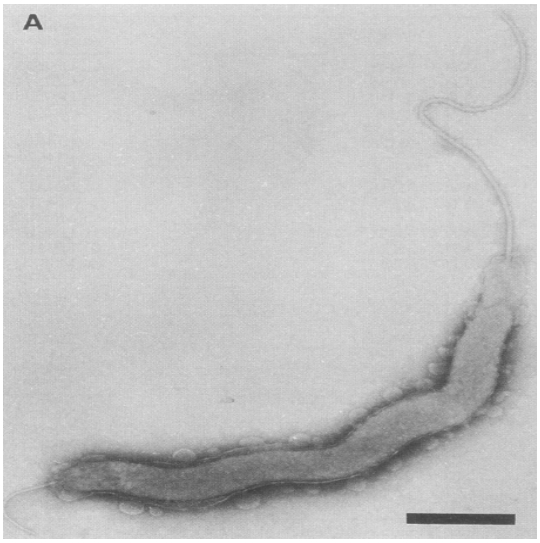


Figure 6: Micrograph image of *Helicobacter hepaticus*[37].

H. hepaticus infection is a major problem in mouse research facilities because it is easily transmitted and highly contagious. Presentation of the disease is often subclinical, making diagnosis difficult[38]. Infected mice have been unknowingly shipped to other rodent research facilities[36]. Subsequent testing of mouse colonies confirmed that *Helicobacter* infection is widespread[41]. The widespread nature of the disease poses a

severe threat to research studies involving rodents. Endemic infections are eminent unless preventative measures are taken. Bohr et al. in March 2006 documented that mice from commercial supply companies have tested positive for *H. hepaticus*. In one instance the mice had been certified as Helicobacter free by the company[40].

Identification and removal of infected animals is vital to curtailing the spread of the infection[41,42]. A rapid, inexpensive and user friendly test may facilitate prevention and ideally eradicate the disease from colonies in rodent research facilities. After a research facility has been cleared of *H. hepaticus*, steps can be taken to prevent reentry of the disease[40].

H. hepaticus infection is a major source of concern because of the potential impact on research [43]. Animal experiments using infected mice may be significantly influenced by the infection[44]. The unintentional use of infected mice in a study introduces an additional variable that will confound and possibly lead to invalidation of the study. Studies involving hepatic and intestinal organ systems are especially likely to encounter problems. Not using infected animals in experiments is important to fully preserving experimental integrity. An accurate, high throughput assay is needed to examine the large number of housed rodents for infection and to enable eradication of this bacterium from animal research facilities[45].

1.3.2 Current Detection Methods

A reliable test for *Helicobacter hepaticus* would be a valuable weapon in fighting the spread of infection. Cell culture, Polymerase Chain Reaction (PCR), histopatholigical examination and serological testing are the four primary diagnostics methods for

detection of *H. hepaticus* reported in literature[38,46]. Comparisons of these methods have been published in literature and these findings will be summarized here.

Histopathological examination involves analyzing tissue taken from infected animals. Trained personnel are required to complete these tests[42]. This method first identified *H. hepaticus* using silver stains of the tissue for analysis[36]. The staining allows the organisms to be seen in the tissue samples[38]. Tissues from the organs targeted by *H. hepaticus* are examined for pathogens and signs of the disease. It is relatively quick and inexpensive[46]. Euthanasia of the animal is required to obtain samples, making this method unsuitable for preventative screening. Additionally, histopathological examination lacks sensitivity because the bacterium may not be present at the time of analysis or lesions may not have been produced by the organs[42].

Cell culture involves recovering the bacterium from feces, cecum or liver samples[38]. This method is both specific and sensitive in nature. The culture process can take 3-7 days to complete. *H. hepaticus* is difficult to culture due to stringent growth requirements. These factors make cell culture both time consuming and expensive[42]. Additionally, there are often other bacteria similar to *H. hepaticus* in the cultured samples. Other bacteria in the sample may complicate or interfere with the results of the culture, making interpretation more difficult[41]. Cell culture is a reliable diagnostic method; however, the problems associated with it make it an undesirable test for primary screening. Culture has been mentioned as a quality method to confirm the results from other types of tests[46].

Polymerase Chain Reaction (PCR) analyzes samples for DNA or RNA from the bacterium and is the most widely used diagnostic method[46]. This method amplifies

genetic material present in fecal and cecal samples[38]. These samples are collected from live animals making PCR excellent for preventative diagnostics[47]. Assays for the *Helicobacter* genus and specifically for *Helicobacter hepaticus* have been developed. The general helicobacter assay amplifies a conserved 16S RNA sequence found in all known murine *Helicobacters*[47]. More specific PCR analysis for *Helicobacter hepaticus* uses different primers for three conserved urease structural genes[38]. The use of PCR is fast, sensitive and highly accurate[42]. Reports have shown that amplification can be done with sub-nanogram amounts of DNA in the sample[41,48]. Analysis can be completed within 24 hours. PCR is a highly sophisticated technique that requires trained personnel and expensive equipment[42]. Additionally, contaminants within the sample may interfere with PCR reagents, making contaminated data possible[47]. Steps can be taken to reduce the impact of these inhibitory substances[49]. PCR is the best method for screening live animals for *H. hepaticus* infection[46]. While this is the best method available, the drawbacks presented here show that a more user-friendly, less expensive assay would be useful for detecting *H. hepaticus*.

Serologic assays use immunological techniques to detect *Helicobacter hepaticus* antibodies. There are no commercially available serodiagnostic assays [38], but the development of these techniques has been presented in literature. These assays measure serum IgG or mucosal IgA in samples taken from possibly infected rodents using and an ELISA [38]. There are several techniques that have been reported using immunogenic membrane associated proteins[42] and recombinant antigens[45]. One method, presented by Livingston et al. in 1997 will be summarized[42].

H. hepaticus is adsorbed to 96-well plates. Serum from the animal is then added to each well. Specific antibodies in the samples will bind to the adsorbed antigen. The sample is then washed off and alkaline phosphatase labeled secondary antibody is added. This antibody will bind to any anti-*H. hepaticus* immunoglobulins adsorbed to the plate. A phosphatase substrate solution is added that will react with the alkaline phosphatase label, producing a colorimetric reaction. The amount of color present in each well reflects that amount of anti-*H. hepaticus* present. Animals whose serum resulted in an optical density result above a certain cutoff were considered infected.

This method shows potential, but required further refinement. Advantages of this method include ease of use, high throughput and relatively short assay time[46]. These assays would be inexpensive compared to PCR. However, PCR is more accurate then serodiagnosis by comparison[42]. It takes 5-10 days for the immune system to produce antibodies against pathogens[17]. Animals tested prior to the immune response would yield false negatives. Diagnosis of animals within the first two weeks of infection may not be possible due to low antibody titers[46] .

The four presented testing methods have all been used for the diagnosis of *H. hepaticus*. PCR is the recommended primary diagnostic test[46]. It is sensitive, reliable, and demonstrates relatively high throughput. Cell culture is the recommended method for secondary or confirmation tests[46]. All of the current assay systems have their benefits and drawbacks. There is not currently an ideal test that satisfies all of the researcher's needs in terms of time, finances, and ease of use. A novel sensor capable of detecting *H. hepaticus* infection would be a valuable addition to the field of laboratory animal research.

CHAPTER TWO
Introduction to Research

2.1 Research Objectives

The objective of this research was to develop a biosensor to detect *Helicobacter hepaticus* in infected animals. Current assays are capable of reliably detecting *H. hepaticus*; however, there are drawbacks associated with these methods, making an improved biosensor desirable. The proposed biosensor was designed to remedy these drawbacks and improve upon current detection capabilities. Several different sensing platforms have been attempted during this project. Optical fluorescent immunosensing techniques were utilized. Desired capabilities for the proposed biosensor include being fast, accurate, inexpensive and demonstrate high throughput. The capabilities of current detection methods will serve as suggested standards for this improved biosensor.

The goal of the research was to investigate the development of a *H. hepaticus* biosensor that:

- Detects *H. hepaticus* at a minimum detection level, at least in the nanogram range.
- Produces results within minutes.
- Provides a high throughput system to test many animals in a short period time.
- Is easy to use and require minimal training.
- Provides both equipment and reagents that are inexpensive, allowing for widespread access and use of this technology.

A cage mounted, fully automated biosensor is the end goal of this project. The research presented provides a foundation to reach this ultimate goal. The efforts in design have focus on developing novel biosensor platforms that may be incorporated into an eventual automated system.

2.2 Outline of Methods Attempted

There were many different methods attempted during the course of this project. Two different optical waveguides, optical fibers and capillary tubes, were investigated. An optical fiber biosensor was explored first. The optical fibers showed a great deal of variation that made working with them difficult. Attempts at using optical fibers as a waveguide were unsuccessful and thus the capillary tubes were explored.

Capillary tube optical waveguides were tried with promising success. The capillary tube sensors were not difficult to make, but the physical construct to analyze the capillaries proved challenging. Early tests were done using the Jobin Yvon Fluoromax-3 Spectrofluorometer (SPEX) to analyze the capillary tube, which produced some accurate results. But, the sample volumes of the samples required augmentation for SPEX reading, thereby inducing variability. A technique to read small volumes to prevent dilutions was necessary.

A series of explorations to fabricate a platform to analyze the samples while still in the capillary ensued. There were several techniques attempted that ultimately led to a successful biosensor platform capable of analyzing both small volumes and the capillary tubes themselves. Not all of the biosensor platform designs examined in this project are presented as many of them were unsuccessful. The unsuccessful platforms provided the ideas that launched sensor platform improvements which were incorporated into future designs. The research presented is a conglomeration of a learning process characterized by unsuccessful attempts but ultimately led to a successful capillary based biosensor platform.

CHAPTER THREE
Materials and Methods

3.1 Materials

A polyclonal *Helicobacter hepaticus* antibody and a protein digest of *Helicobacter hepaticus* bacteria was produced and provided by Kristen Correll and Dr. Lela Riley of the MU Department of Veterinary Pathobiology. MTS ((3-Mercaptopropyl) trimethoxysilane) and Dimethyl Formamide (DMF) were purchased from Fluka (St. Louis, MO). GMBS (N-Succinimidyl 4-maleimidobutyrate) was purchased from Pierce Biotechnology, Inc (Rockford, IL). Absolute ethanol, phosphate buffered saline (PBS), bovine serum albumin (BSA) and toluene were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). FluoReporter® Mini-biotin-XX Protein Labeling kits, Alexa Fluor 546 (AF 546) antibody labeling kits, streptavidin and biocytin were purchased from Invitrogen Company (Carlsbad, CA). Antibodies were conjugated following the protocols provided with the kits. Cortisol-3 BSA antibody from U.S. Biological (Swampscott, MA) was purchased for another project was used as a biotin carrier. Additionally, silica core optical fibers with a 600µm diameter, step indexed, and a 0.48 numerical aperture from Thor Labs (Newton, NJ) were used. Microhematocrit capillary tubes 7.5 cm in length, inner diameter of 1.1-1.2 mm, outer diameter of 1.5-1.6 mm and 70 µl volume capacity were purchased from Fisher Scientific (Pittsburgh, PA). Kendall Healthcare Critocaps to seal the capillary were also purchased from Fisher. Thin wall glass single capillaries with an inner diameter of 1.12 mm, outer diameter of 1.5 mm and 7.5 mm in length were purchased from World Precision Instruments (Sarasota, FL). Opitcal components were purchased from Thor Labs (Newton, NJ). A 2 meter, 600µm UV-Vis optical fiber was purchased from Ocean

Optics (Dunedin, FL). A 5 mW 532 nm green laser module was purchased from Roithnek Lasertechnik (Vienna, Austria) and was the first laser used. A second 5 mW 532 nm green laser module was purchased from Apinex (Montreal, Canada).

3.2 Optical Fiber Preparation

The Thor Labs optical fibers originally came in 2 m lengths with SMA connectors at both ends of the fiber. These fibers were cut into two, approximately 1 meter segments prior to use. Fibers were initially prepared by removing the Kevlar supporting jacket from the distal end of the fiber. A blunt edge was made on the silica core with a razor blade. Subsequently, the fiber tips were immersed in 48% hydrofluoric acid (HF) for approximately 4 hours to taper the silica core of the fiber. The HF eroded the silica core, thereby tapering the distal end of the fiber. Capillary action drew the HF between the silica core and cladding, advancing the HF toward the proximal end of the fiber. The fibers were then washed in distilled water to remove any possible HF residue. Approximately 1 cm of the cladding was removed from the fiber to expose the tapered optical fiber tip.

A cleaning and immobilization process described by Bhatia et al[19] was then performed to prep the silica surface with hydroxyl groups to facilitate silanization. The optical fiber tips were placed into a 1:1 solution of methanol: hydrochloric acid for 30 minutes. The optical fiber tips were then rinsed in distilled water and air dried. They were then placed in concentrated sulfuric acid for 30 minutes, rinsed in distilled water and air dried. The final step involved boiling the fiber tips in distilled water for 30 minutes. After air drying, the fiber tips were ready for silanization and immobilization.

The fiber tips were immersed in a 2% volume/volume solution of MTS in toluene to add a silane film to the silica core. They were incubated in this solution inside a nitrogen filled bag for 90 minutes. Next, the fiber tips were removed from the MTS-toluene and rinsed in toluene for five minutes, and then air dried. The cross-linking step followed silanization. The tips were placed in a 2mM GMBS in ethanol solution. GMBS for the solution was first dissolved in a minimal amount of DMF prior to adding it to the ethanol. A one hour incubation at room temperature was used. The fiber tips were rinsed in PBS. Next 100 µl of a 0.01 µg/ µl *H. hepaticus* in PBS solution was added to each fiber tip and incubated over night at 4ºC. After the incubation, the fiber tips were washed in PBS. They were then placed in a 3% BSA blocking buffer solution and incubated for 2 hours at room temperature. The fiber tips were then washed in PBS for five minutes and were ready for scanning. For storage, the fiber tips were placed in PBS and stored at 4°C until use.

3.3 Capillary Tube Immobilization

Borosilicate capillary tubes from Fisher Scientific and World Precision Instruments were utilized for this project. The Fisher capillary tubes were initially used and had diameter variations up to 0.3 mm. These variations could potentially alter the optical properties of the capillary, hindering inter-capillary comparisons. Less variable, higher quality capillaries with the same diameter and length specifications were purchased from World Precision Instruments. These capillaries were used in the final part of this research when the most successful experiments were performed. The capillaries came in 7.5 mm lengths. Shorter length capillaries were made by cutting the

capillaries to smaller sizes using a diamond tipped etcher. Capillary length does not affect the immobilization protocol other than the volumes used to fill the capillaries.

The capillaries were first cleaned by boiling them in distilled water for 30 minutes. A layer of hollow glass beads was placed on the bottom of a beaker to prevent breakage of the capillary tubes. Capillaries were removed from the boiling water and air dried first in ambient air and then under nitrogen stream. Silanization followed these cleaning steps.

The capillary tubes were placed into a 2% volume/volume solution of MTS in toluene and incubated in a nitrogen bag for 90 minutes. Next the capillaries were removed from the MTS-Toluene and rinsed in Toluene. A transfer pipette with a 1-10 µl pipette tip was used to force the toluene through each capillary three times. The capillaries were submerged in toluene until all the capillaries had been rinsed (approximately 5 minutes), then dried under a nitrogen stream.

A cross-linking step followed silanization. The capillaries were placed in a 2mM GMBS in ethanol solution. GMBS for the solution was first dissolved in a minimal amount of DMF prior to adding it to the ethanol. The capillaries were incubated in the GMBS solution for one hour at room temperature. Capillaries were then rinsed in PBS using the same transfer pipette injection method as the toluene. Any protein with free amine groups added to the capillary will immobilize to the inner surface of the capillary. The capillary tubes were individually filled with the either *H. hepaticus* or biotinylated cortisol antibody solutions, capped with critocaps and incubated at least overnight at 4 °C. The reagent added in this step changed depending on the experiment and details will be provided when necessary.

The immobilized reagent solution was removed and the capillaries were washed in PBS. The capillaries were filled with a 3% BSA blocking solution and incubated at room temperature for 1-3 hours (times varied between experiments). The capillaries were then washed in PBS. Finally, capillaries were individually filled with PBS, capped with critocaps and stored at 4 °C until use. Prior to use, the PBS was removed from the capillary tube.

3.4 Statistical Analysis Methods

This biosensor was designed to detect *H. hepaticus*. For diagnostic purposes, the biosensor must distinguish between infected and non-infected samples. Samples containing *H. hepaticus* were considered infected animals, regardless of amount in the samples. Conversely, samples made without *H. hepaticus* represented non-infected animals. Statistical analysis methods that distinguished between samples with and without *H. hepaticus* was implemented. Quantification of the amount of *H. hepaticus* in the samples was not as significant as achieving a minimum detection level. GraphPad Prism software was used for data analysis and curve fitting.

The most important aspect of this biosensor was to distinguish between the samples with and without *H. hepaticus*. A one-way ANOVA with a Dunnett's post test assessed whether the observed differences between the samples with and without *H. hepaticus* were statistically significant. The Dunnett's post test calculates a p-value resulting from a comparison of all sample groups to the control group. A zero *H. hepaticus* sample was the uninfected standard for comparison (control group). Each sample group of *H. hepaticus* was compared to the non-*H. hepaticus* sample group using

a Dunnett's post test of the ANOVA results. Sample groups with p-values less than 0.05 (the 95% confidence interval) indicated infection. This analytical method determined the minimum level of detection for each experiment.

Data from each experiment was also fitted with an equation that added a trend-line for each experiment. The trend line helped compare samples of different concentrations to one another in the same experiment and allowed for the determination of unknown sample concentrations. Some of the experiments were difficult to fit because the immunological principles used did not have known equation models. The immunological theories used in this project were unique to biosensor research. In some cases, user-defined equations were used for lack of better analytical program defined equations. These factors were taken into account when analyzing the curve fitting results from this research.

CHAPTER FOUR
Optical Fiber Biosensor

4.1 Overview

4.1.1 Immunological Principles

Principles of a competitive immunoassay were adapted for use on optical fibers, as shown in Figure 7. The first step involved covalently immobilizing via silanization of *H. Hepaticus* to the tapered cores of 600 μm optical fibers. The second step involved adding a fixed amount of anti- *H. hepaticus*-AF 546 to samples containing different amounts of *H. hepaticus,* forming the sample mixture. Any *H. hepaticus* present in the sample mixture would bind to the antibodies in solution during a set incubation period. The tip of the optical fiber with the antigen immobilized was then placed into the sample mixture and incubated. Labeled antibodies with free binding sites attached to the immobilized antigen on the optical fiber. More labeled antibodies attached to the optical fiber in samples not containing *H. hepaticus*. The fibers were then removed from the sample mixture and washed in PBS, which removed any non-bound antibodies from the fiber tip. Each fiber was then scanned using the SPEX to excite the fluorophores bound to the optical fibers. The optical fiber collected more fluorescence from samples *not* containing *H. hepaticus* than samples containing *H. hepaticus*, translating into a higher recorded signal.

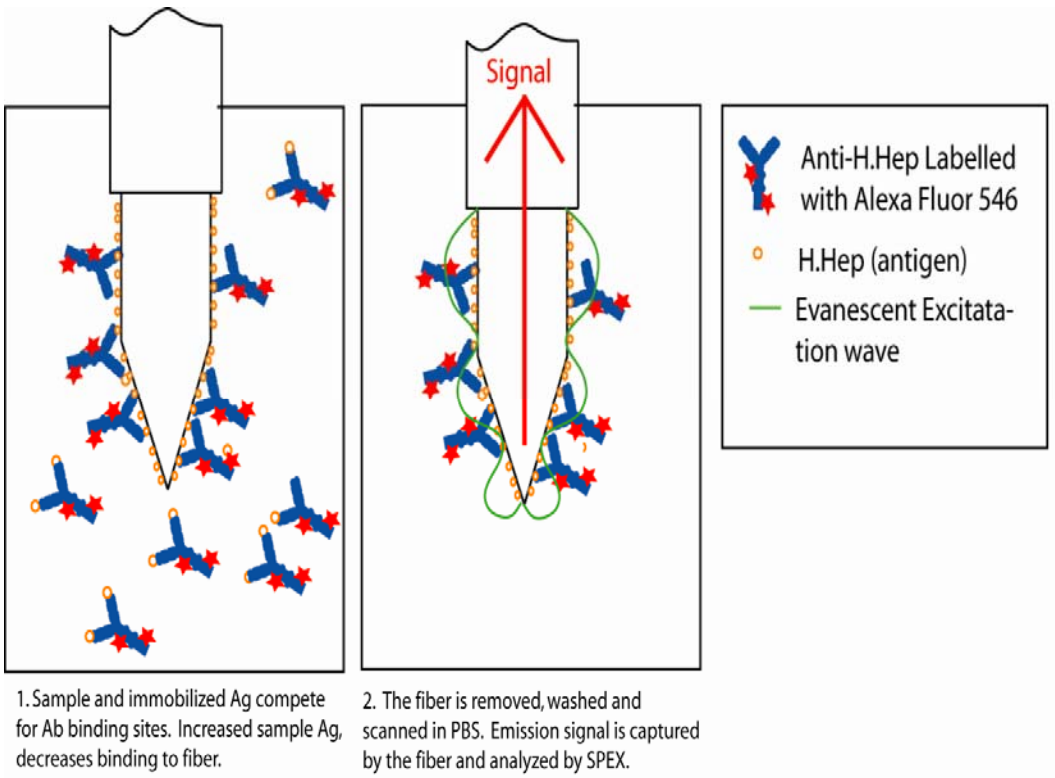


Figure 7: Representation of the methods employed for the optical fiber biosensor.

4.1.2 Optical Principles

The *H. hepaticus* antigens were used to capture the labeled antibodies. Antibody binding resulted in an antigen-antibody complex attached to the core of the optical fiber. The labeled antibodies were within the penetration depth of the evanescent wave. Evanescent wave excitation was used to excite the AF 546 molecules conjugated to the antibodies. The resulting fluorophore emission was collected by the fiber and returned to the SPEX for data collection.

A fiber stage, shown in Figure 8, was fabricated for the SPEX that allowed the scanning of the optical fibers. The fiber stage consisted of a series of filters that helped direct the light for both excitation and emission collection. An SMA connector on the proximal end of fiber attached to the fiber stage inside the SPEX. A 560 nm dichroic

short pass filter was positioned at 45-degree angle inside the stage. The dichroic allowed the 546 nm excitation light to pass through and reflected the longer wavelength emission light returning through the optical fiber towards the SPEX photomultiplier tube (PMT) for collection. A 530nm, 30 nm bandpass pass filter was used to filter the excitation light and a 560 long pass filter was used to filter the emission. The excitation and emission filters reduced scattering and noise inside the SPEX during measurement.

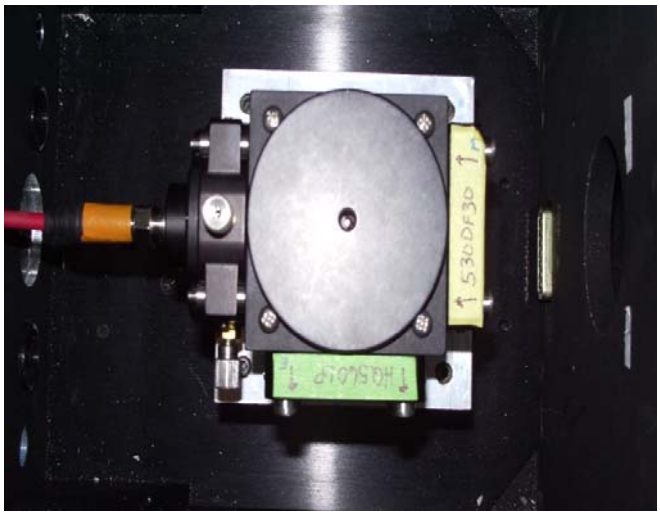


Figure 8: Picture of the fiber stage used in the SPEX for optical fiber analysis.

4.2 Antigen Immobilized Optical Fiber Experiments

4.2.1 Sample Preparation and Data Collection

The purpose of the initial experiments was to determine the feasibility of the optical fiber biosensor method. The fiber was attached to the fiber stage and an initial scan was performed to obtain the fiber background intensity. Sample mixtures containing only 250 ng of anti-*H. hepaticus*-AF 546 were prepared in 100 µl of PBS. The sample was added to the fiber tip and incubated for 20 minutes. A PBS wash was performed to remove non-bound antibodies from the fiber tip. The fiber was then

scanned to collect the emission light from the antibodies attached to the optical fiber. This process was repeated for four additional optical fibers.

4.2.2 Discussion and Results

Each optical fiber had an inherent and unique background fluorescence, thus making inter-fiber comparisons difficult. A scan of the antigen immobilized fiber was taken prior to antibody addition measured the background fluorescence for each fiber. The fiber background was subtracted from the scans taken after the fiber was exposed to the antibody. These intensity values after antibody exposure (minus fiber background) were plotted against the corresponding wavelengths.

As shown in Figure 9, the AF 546 emission peak from the bound antibodies was seen in all five fibers, demonstrating that the antibody did bind to the immobilized antigen. However, all five fibers had considerably different peak intensity values. The results from the five fibers should have been much more similar since the fibers were all exposed to the same amount of antibody. The variation was from 5,000 to 30,000 counts per second (cps). The antibody only samples rendered a variable signal, making comparisons between samples with and without *H. hepaticus* difficult. Additionally, the difference between the fluorescence signal and fiber background was small, meaning minor variations in fiber background would have large impacts on the fluorescent signal. This wide variation suggested that fiber optics may not be a viable platform.

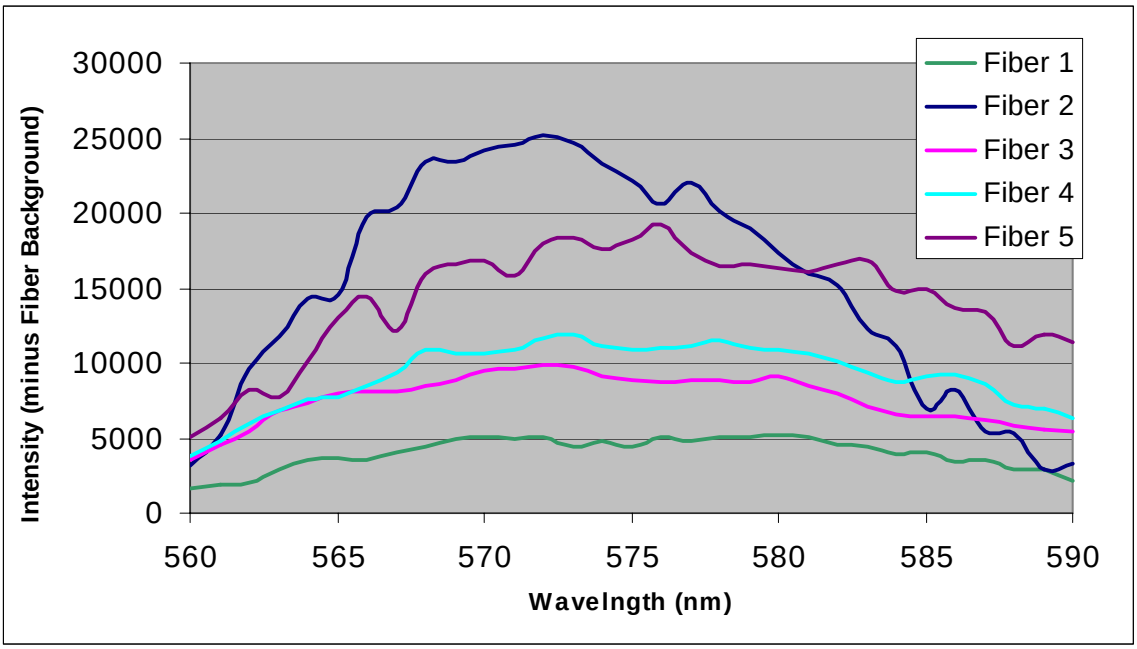


Figure 9: Results of the optical fiber scans from the immobilized antigen optical fiber experiment.

4.2.3 Conclusions

The results from this experiment indicated that the anti-*H. hepaticus*-AF 546 binds to the immobilized *H. hepaticus*. This showed that an antigen immobilized biosensor was a feasible approach. However, the optical fiber experiments were discontinued after this initial experiment due to the optical fiber variations. Optical fiber variation was difficult to account for and eliminate, thus leading to complications in data analysis. Also, variations in optical fibers made it unfeasible to perform calibration studies. For these reasons, it was decided to discontinue development of optical fiber based biosensors and alternative waveguides for the *H. hepaticus* biosensor were considered.

CHAPTER FIVE
Capillary Based Biosensor

5.1 Immunological Principle

This biosensor was based on a competitive immunoassay approach. *H. hepaticus* was immobilized to inner surface of the capillary walls as described above. Anti- *H. hepaticus* was conjugated to AF 546 to form a fluorescent tracer that produced a signal upon excitation. The sample was the antigen in solution. A fixed amount of anti- *H. hepaticus*-AF 546 was then added to each sample containing known amount of *H. hepaticus* (analyte). The sample became the sample mixture once the labeled antibody was added. An incubation step allowed all *H. hepaticus* present in the sample to bind to the anti-*H. hepaticus*-AF 546 in the sample mixture. If larger amounts of *H. hepaticus* were present in the sample, then more anti-*H. hepaticus* binding sites were occupied. This results in less antibodies binding to the immobilized *H. hepaticus*.

As shown in Figure 10, the sample mixture was then added to the capillary tube and incubated. Antibodies with free binding sites attached to the antigens immobilized to the capillary walls. Antibodies with occupied binding sites will remained in solution inside the capillary. This provided two methods of analysis. The sample mixture can be removed from the capillary for analysis of the non-bound components. This method was referred to as sample mixture analysis. Alternatively, the immobilized capillaries themselves could be analyzed for the amount of bound anti-*H. hepaticus*-AF546. This method was called immobilized capillary analysis. Both methods resulted in an inverse relationship between signal and analyte concentration.

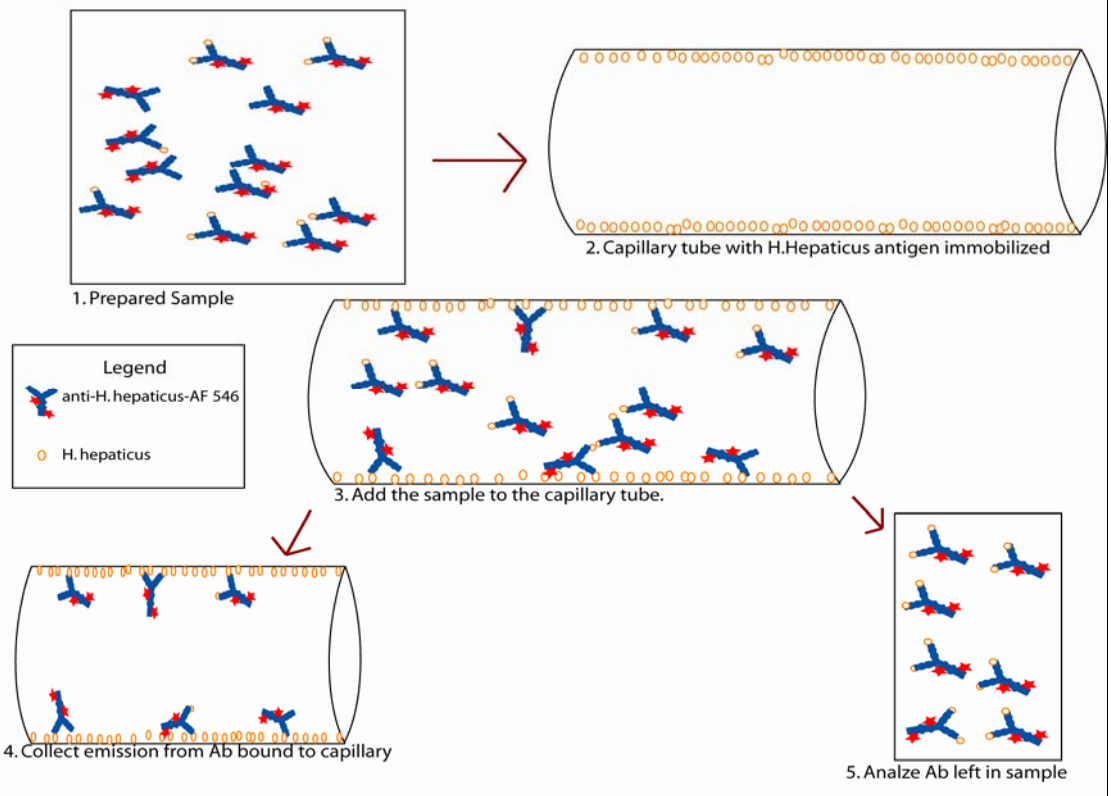


Figure 10: Representation of the principles used for the capillary biosensor.

Patterned capillaries were also made for this project. As shown in Figure 11, a patterned capillary had distinct regions with and without antigen immobilized. For this research, capillaries were made in which half the capillary had *H. hepaticus* immobilized and other half was only blocked with BSA. The patterned capillaries were made by adding *H. hepaticus* to only a certain region of the capillary tubes. This method allowed for the immunoreaction and signal collection to be performed in the same capillary tube. Antigen immobilized regions served as the site for the immunoreaction and non-immobilized regions served as the reading chamber where the sample mixture was analyzed. The immunological principles were the same as described above, except instead of removing the sample mixture from the capillary for analysis it was forced into

the reading chamber. A fluid pumping system must be incorporated into the biosensor to effectively utilize the patterned capillaries.

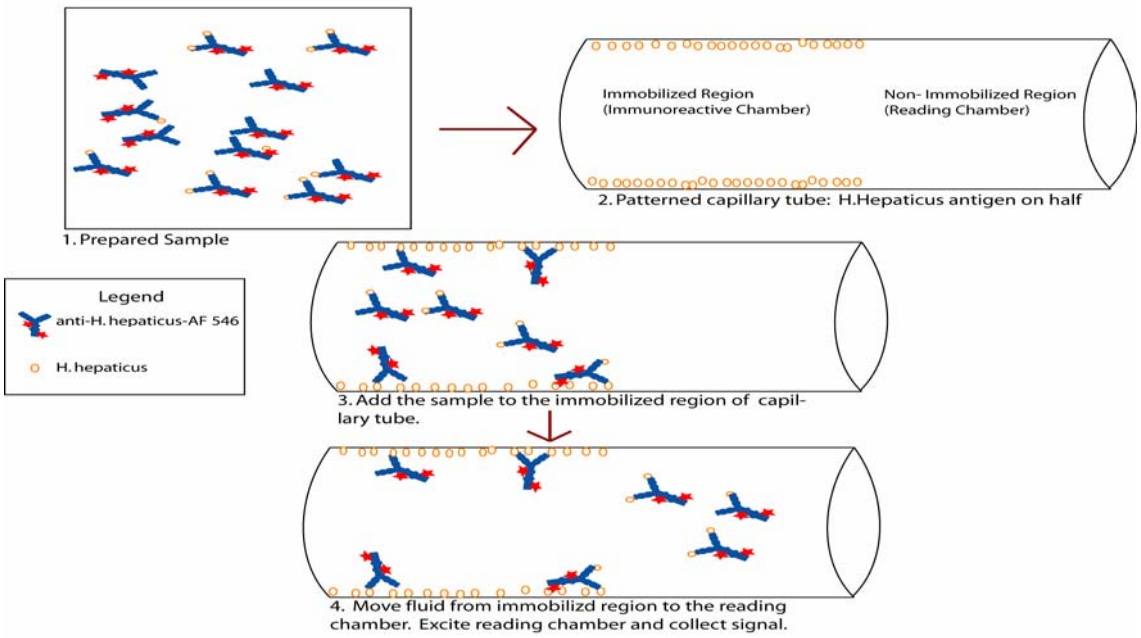


Figure 11: Representation of methods used for a patterned capillary biosensor.

5.2 Early Capillary Biosensor Trials: SPEX analysis

The purpose of these experiments was to determine the feasibility of a capillary based biosensor. Several unsuccessful attempts to measure the antibodies bound to capillary walls were attempted. For example, an image of the capillary was taken using a CCD camera, but the fluorescent intensity from the capillary was too low to produce an image. The capillary tubes were also filled with PBS and placed into the SPEX for scanning. This was unsuccessful as well because the capillaries produced unexplained optical noise that prevented collection of the AF 546 emission light. However, there was one set of promising results from earlier experiments involving the removal of the sample

from the capillary and scanning it in the SPEX to compare the fluorescent intensities before and after capillary exposure.

5.2.1 Sample Preparation and Data Collection

Two experiments were performed as a part of this trial. The first experiment only used samples with or without antigen (Experiment 1) and the second used samples with varying amounts of antigen in the samples (Experiment 2). They are discussed together because the procedure for both experiments was identical.

Experiment One utilized sample groups containing 0 and 500 ng of *H. hepaticus* in triplicate. The samples were made and 250 ng of anti-*H. hepaticus* was added to each sample. The total sample mixture volume was 140 µl and they were incubated for one hour at room temperature. After one hour, 70 µl of each sample mixture was added to a *H. hepaticus* immobilized capillary tube. The remaining 70 µl of the sample mixture was saved and was called the "before capillary exposure sample mixture". The sample mixture was allowed to incubate inside the capillary tube for ten minutes, after which the sample was removed from the capillary using a pipette and placed into a clean micro-centrifuge tube. These samples were then called "after capillary exposure sample mixtures".

Twenty five µl of each sample mixture, both before and after capillary exposure, was placed in cuvettes, and was diluted by adding 475 µl of PBS. The dilution was necessary in order to sufficiently raise the volume for analysis in the SPEX. (Approximately 0.5 ml was the minimum volume required for SPEX sample analysis.) The fluorescent intensity for each sample mixture was collected using the SPEX. The

scanning parameters were: excitation wavelength = 546 nm, emission slit = 9 nm, excitation slit = 9 nm, integration time = 0.5 sec. Experiment Two used this same process but with variable concentrations of *H. hepaticus*. Sample groups of 0, 10, 50, 100 and 500 ng of *H. hepaticus* in triplicate were used.

5.2.2 Discussion and Results

Experiment One

The AF 546 intensity was calculated by averaging the intensity values from 570-574 nm. The before and after capillary exposure fluorescent intensities from each capillary were compared to analyze the data. This was done by calculating the change in intensity using the equation:

$$\text{Change in Intensity} = (\text{Intensity}_{\text{before}} - \text{Intensity}_{\text{after}}) / \text{Intensity}_{\text{before}} \quad \text{Eqn. (3)}$$

The average intensity changes from the three sample of the same type were averaged and plotted in Figure 12. The sample standard deviation was calculated and is represented by the error bars. Samples containing no *H. hepaticus* had a higher intensity change than the 500 ng *H. hepaticus* samples, as was expected. The results showed that more labeled antibodies attached to the capillary walls and were removed from the sample mixture in the 0 ng samples than in the 500 ng samples. Since more antibodies were removed from the solution, the differences between the before and after capillary exposure intensities were greater. This resulted in a greater intensity change in the 0 ng *H. hepaticus* samples.

There was considerably more variability between the 0 ng samples compared to the 500 ng samples. The standard deviations of the 0 and 500 ng samples were .08 and .002, respectively. A t-test was done to see if there was a statistical difference between

the samples with and without antigen. The p-value was .05, which matched the confidence limit. These results were promising and warranted further exploration of this capillary biosensor system.

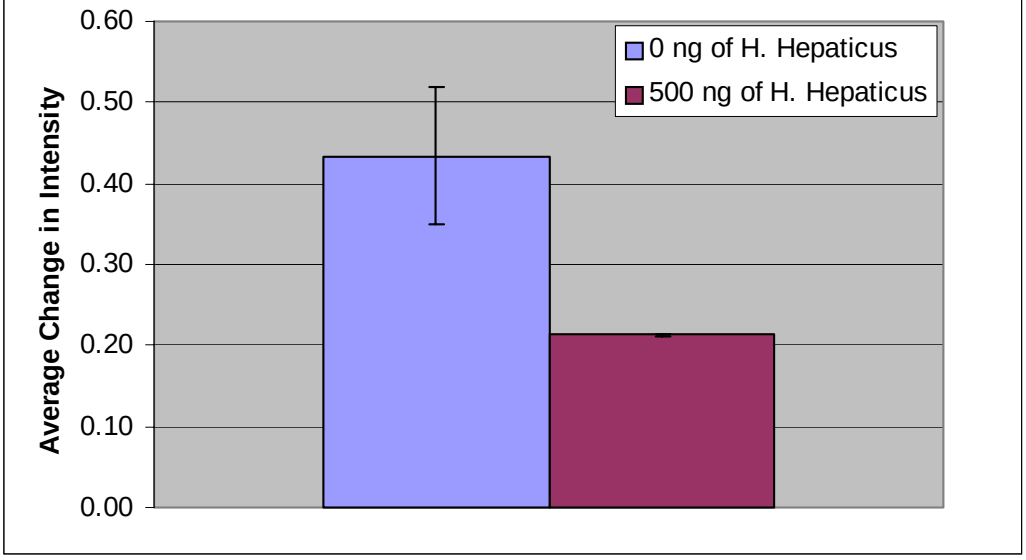


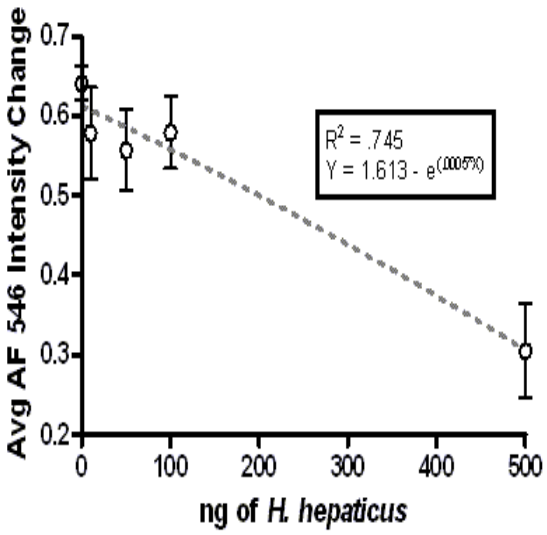
Figure 12: Results from experiment one of the preliminary *H. hepaticus* capillary experiments.

Experiment Two

The results from the second experiment resembled the first and are shown in Figure 13. Additional samples containing varying amounts of *H. hepaticus* allowed sufficient points to form a dose response curve. Additionally, the table in Figure 13 shows the average, standard deviations and p-value results from the analysis. The presented p-values shown resulted from the comparison of the three samples at each concentration of *H. hepaticus* to the three 0 ng samples.

The results from this experiment showed there was a dose response associated with varying concentrations of *H. hepaticus*. As *H. hepaticus* concentration increased,

the average percent change decreased. This response was expected based on the immunological theory employed with the capillary biosensor. The dose response curve was fitted with a user defined equation that is more fully explained later in this paper (section 6.1). The resulting R-squared value was 0.745, indicating that the equation does not perfectly fit the data.



ng of H. hepaticus	Average	Std Dev	P-Value
0	0.64	0.04	N/A
10	0.58	0.10	P > 0.05
50	0.56	0.08	P > 0.05
100	0.58	0.09	P > 0.05
500	0.30	0.08	P < 0.01

Figure 13: Results from experiment two of the preliminary *H. hepaticus* capillary experiments.

The data showed there was significant variability in the system as indicated by the high standard deviations. Standard deviations between the 0 ng samples and each of the 10, 50 and 100 ng samples overlapped. This indicated that the biosensor was not capable of accurately distinguishing between these categories. In an actual system, this would be read as a false negative and fail to properly diagnose the animal. The Dunnett's test showed that there was not a statistical difference between the 10, 50 and 100 ng data points and the 0 ng data points. There was a statistically significant difference between the 0 and 500 ng data points.

5.2.3 Conclusions

The results from both experiments showed that the capillary biosensor system was able to significantly distinguish between samples with (500 ng) and without *H. hepaticus* (0 ng). However, the lower concentrations did not show significant differences from the 0 ng sample. There was large variability between identical samples, which was attributed to human error in making the samples, because considerable pipetting and manual fluid transport was required. Additionally, the diluted samples required wide slit settings for scanning in the SPEX. Widening the slits increased noise and decreased the accuracy of the SPEX measurements. Despite these drawbacks, these initial capillary biosensor experiments yielded promising results. Perhaps the most encouraging aspect of these experiments was the difference between samples with and without *H. hepaticus*. This proved that immunological components were functioning as expected. The next step involved developing a better mechanism for scanning small volumes without dilution in order to reduce variability and provide greater system control.

5.3 Early Capillary Trials: Oil Reservoir Capillary Stage

The purpose of these experiments was to read small volumes inside the capillary tube without dilutions. Eliminating dilutions should decrease human error and variability by limiting fluid transport. Also, the resulting signal should be higher since all of the fluorophores are confined to a smaller volume. To save reagent, *H. hepaticus* immobilized capillaries were not used in this portion of the experiment. Instead, samples containing only AF546 dye conjugated to protein A were utilized to construct and refine this system.

5.3.1 Oil Reservoir Capillary Reading Stage SPEX Analysis

The capillary stage incorporating elements for both fluid transport and signal collection is shown in Figure 14. Fluid transport was performed using an oil pumping system. The capillaries were inserted into an oil reservoir fitted with a syringe pump. The syringe pumped the oil into the capillary, thereby moving the sample within the capillary. PBS based samples did not mix with the vegetable oil used in this experiment. Additionally, the index of refraction for vegetable oil (n = 1.49) is almost identical to borosilicate glass (n = 1.5). The matching indices of refraction helped aid light transmission down the capillary and into the collector.

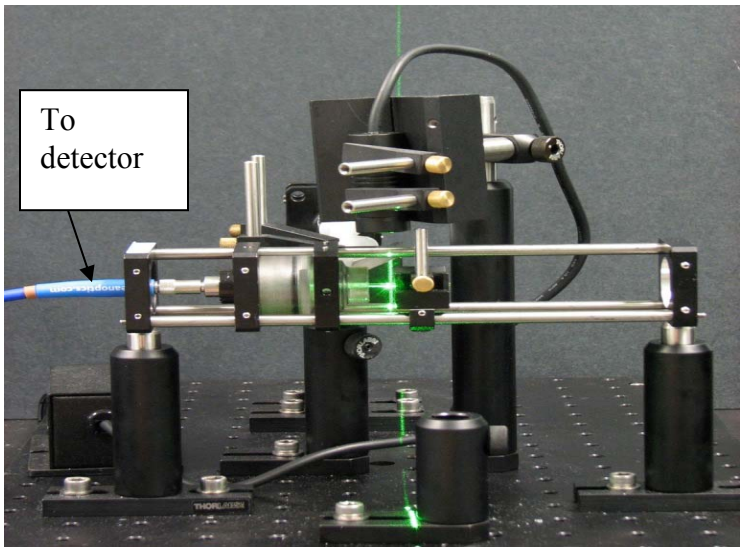


Figure 14: Picture of capillary platform with oil reservoir capillary holder.

A schematic of the detection system is shown in Figure 15. An acrylic cylinder was milled that served as both the capillary holder and oil reservoir. A 1.54 mm diameter hole was drilled through the center of the proximal end of acrylic cylinder. This served as the capillary holder. The end of the hole opened into a one inch diameter oil

reservoir. A one inch lens with 53.4 mm focal length was on the distal side of the oil reservoir. The lens both enclosed the oil reservoir and helped focus the light for signal collection. A collimating lens with an SMA optical fiber attachment was distal to the lens. The collimator collected the fluorescence from the oil reservoir and funneled it into the optical fiber. The distal end of the optical fiber was connected to either the USB 2000 Oceans Optics Spectrometer or the SPEX with fiber stage for data collection (refer to section 4.1.2). This system was designed on rails to aid in alignment. A sliding stage with a clamp guided the capillary tubes into the oil reservoir insertion hole.

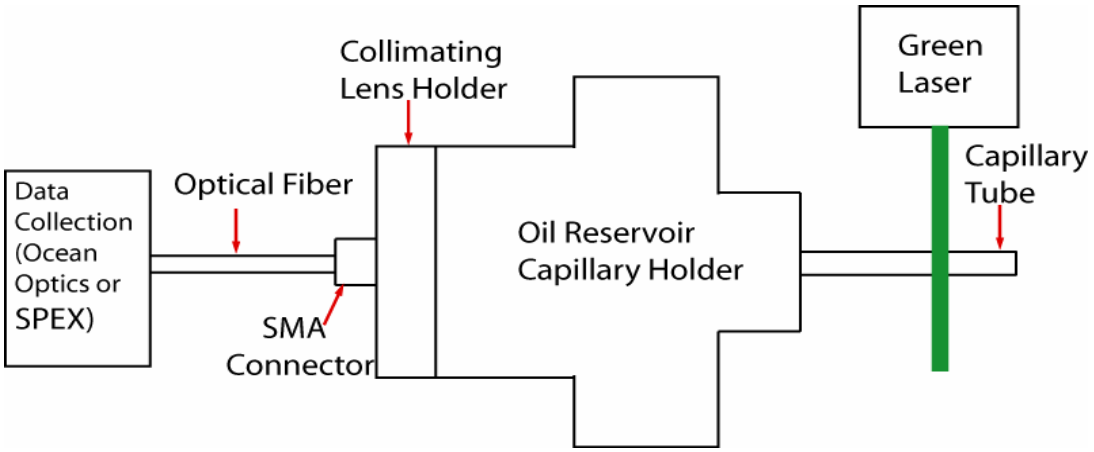


Figure 15: Schematic of capillary platform with oil reservoir capillary holder.

This stage was designed for patterned capillary analysis (refer to section 5.1). The oil pumping system was used to move the sample from the immunoreactive region into the reading chamber. A 532 nm green laser was positioned directly above the reading chamber for excitation of the sample mixture. Resulting fluorophore emissions was transported down the capillary and through the oil reservoir. The lens focused the emission light onto the collimator lens and into the optical fiber.

Testing was performed with non-immobilized capillaries to determine if a stable signal could be obtained from this design. A 1:5 dilution of stock protein A-AF546 was

used as the test sample. Five µl of the protein A-AF 546 dilution was inserted into a capillary. Subsequently, 20 µl of vegetable oil was added, pushing the sample further into the capillary. The oil added prior to insertion into the reader ensured that no air bubbles formed at the oil sample interface, as air bubbles scatter light and reduce the amount fluorophore emission collected. The air bubbles were likely to form between the sample and oil if the capillary was inserted into the reader without the 20 µl of oil. Air bubbles formed between the oil in the capillary and oil in the reservoir were easily removed by reversing the syringe pump. Once the capillary had been inserted into the reader and all air bubbles were removed, the syringe pump was used to position the samples beneath the laser. Data collection was done using the SPEX with the fiber adapter stage. Multiple scans were taken without moving the capillary to determine the stability of the signal. This procedure was repeated for four capillaries. The first experiment using the SPEX was completed and variability was noted. A second data collection device, USB 2000 Ocean Optics Spectrometer, was used to determine if the SPEX was the source of variability.

5.3.1.1 Discussion and Results

The data was analyzed by examining the intensities of the AF 546 peak. Peaks from each scan were averaged from 570-574 nm (Table 1). Each capillary scanned did not yield a consistent fluorescent intensity. The intensity values from each capillary varied significantly without the capillary being touched in between scans. Since the capillaries were not moved in between scans, alignment could not have changed between scans. This caused concern because all scans of the same capillary should have provided

identical AF 546 fluorescent intensity values. This variability in the same capillary made it impossible to compare capillaries to one another.

Capillary	Scan	AF 546 Peak Avg	Average	Standard Deviation
1	1	245154	282258	30661
	2	258906		
	3	297248		
	4	322023		
	5	287957		
2	1	150955	195935	41494
	2	204133		
	3	232719		
3	1	344834	234507	83000
	2	242313		
	3	148456		
	4	202424		
4	1	243168	N/A	N/A
Average		244638		
Standard Dev		57161		

Table 1: Data from the oil reservoir reader SPEX analysis.

5.3.1.2 Conclusions

There was considerable variability in this capillary reader platform design. A method to reduce variability was required. Possible sources of the variability included noise, excitation source fluctuations, data collection and capillary tube heterogeneity. These factors were explored in subsequent experiments described below.

5.3.2 Oil Reservoir Capillary Reading Stage Troubleshooting

The purpose of these experiments was to account for the variation seen in the previous experiment. The capillary platform design and sample preparation methods were identical to the ones used in 5.3.1.1, with the only difference being in data collection methods. An Ocean Optics Spectrometer was used to determine if the SPEX collection was creating variability. Laser source fluctuations were tested using a Newport Optical Power Meter prior to testing the capillaries. The power meter showed laser intensity fluctuations of approximately 0.2 mW. Two capillaries were then tested to examine laser variations and the resulting fluorescent intensities. Data was collected at the 532 nm green laser and 569 nm AF 546 peaks over a two minute interval for capillary 1 and a five minute interval for capillary 2.

5.3.2.1 Discussion and Results

Both the green laser and AF 546 peak intensities varied in a sinusoidal pattern over time as shown in Figure 16. The AF 546 peak intensity was directly related to the laser peak intensity. These results showed that the laser fluctuation may account for the large variability seen in previous experiments, and thus had a major impact on the fluorescent intensity. This also explains why some scans from the same capillary would be similar while others differ considerably. A second 5mW 532 nm green laser purchased from Apinex that was available in the laboratory was tested with the power meter to determine source fluctuations. This laser intensity fluctuated initially, but stabilized over time. As a result, the Apinex laser was used for the remaining experiments and was turned on at least 30 minutes prior to data collection.

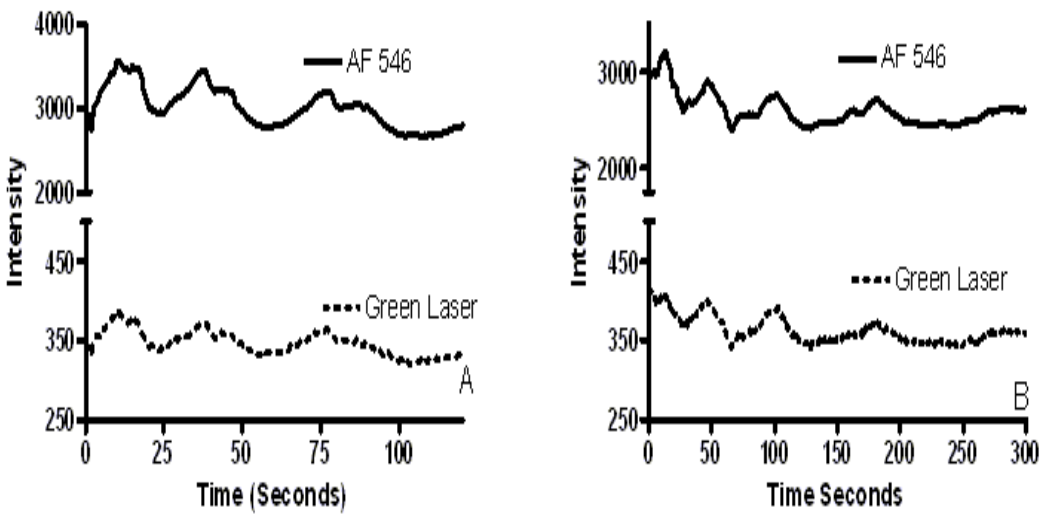


Figure 16: Plotting of laser and AF 546 intensities recorded over time. A is from capillary 1 and B is from capillary 2.

5.3.2.2 Conclusions

The laser used for the previous experiments had wide intensity fluctuations. Thus, the second laser with less fluctuation was utilized. The Ocean Optics Spectrometer was also used for data collection for the remainder of the experiments because it is able to collect multiple wavelengths over time to track the laser fluctuations. Capillaries from a different supplier (World Precision Inc) were purchased that were better quality and more homogenous. These steps were undertaken to reduce variability and create a more accurate biosensor.

CHAPTER SIX
Final Capillary Based Biosensor

Figures 17 and 18 display a picture and schematic of the final capillary biosensor platform. This design was based on the information acquired from previous designs of the capillary platform. The platform was utilized to analyze both the fluid inside a capillary (sample mixture analysis) and the antibodies bound to the capillary walls (immobilized capillary analysis). This versatility made it an advantageous design.

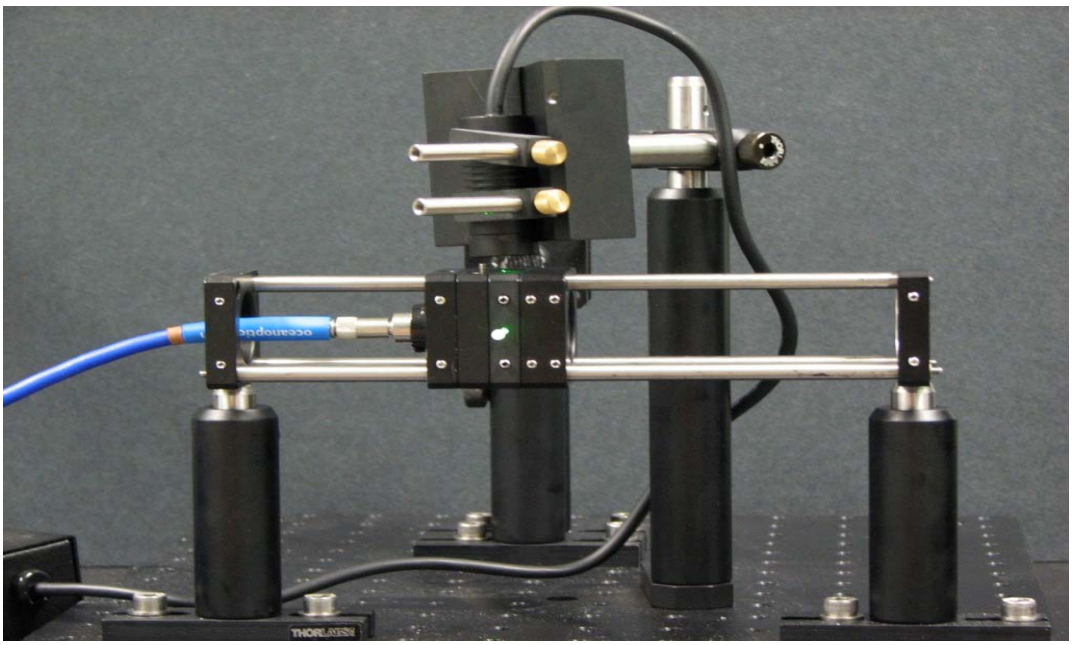


Figure 17: Photograph of the final capillary biosensor platform.

The system was designed using a Thor Labs cage plate system mounted on rails to facilitate alignment. The laser and detector were mounted perpendicular to the capillary holder. A 1.54 mm hole was drilled horizontally directly through the center of the long axis of a cage plate to hold the capillary. Another 3 mm diameter hole was drilled through the vertical axis to serve as an excitation window. The 532 nm green laser was mounted above the excitation hole on a separate angled post. A third 7 mm hole was drilled through the short axis of the capillary to serve as the emission window. The

collimating lens was mounted distal to the capillary holder and was in line with the emission window. A 560 nm long pass filter was placed between the collimating lens and the capillary holder. There was also a silver mirror mounted on the proximal side of the capillary holder that blocked ambient light and reflected emission light towards the collimating lens for collection. A black box was built around the capillary stage to further remove stray ambient light.

The green laser excited the fluorophores inside the capillary tubes. Emission light was then collected by the collimating lens perpendicular to the capillary. Perpendicular collection reduced the impact of capillary heterogeneity on light collection and positioned the detector closer to fluorophore emission site. The results obtained with this design are outlined below.

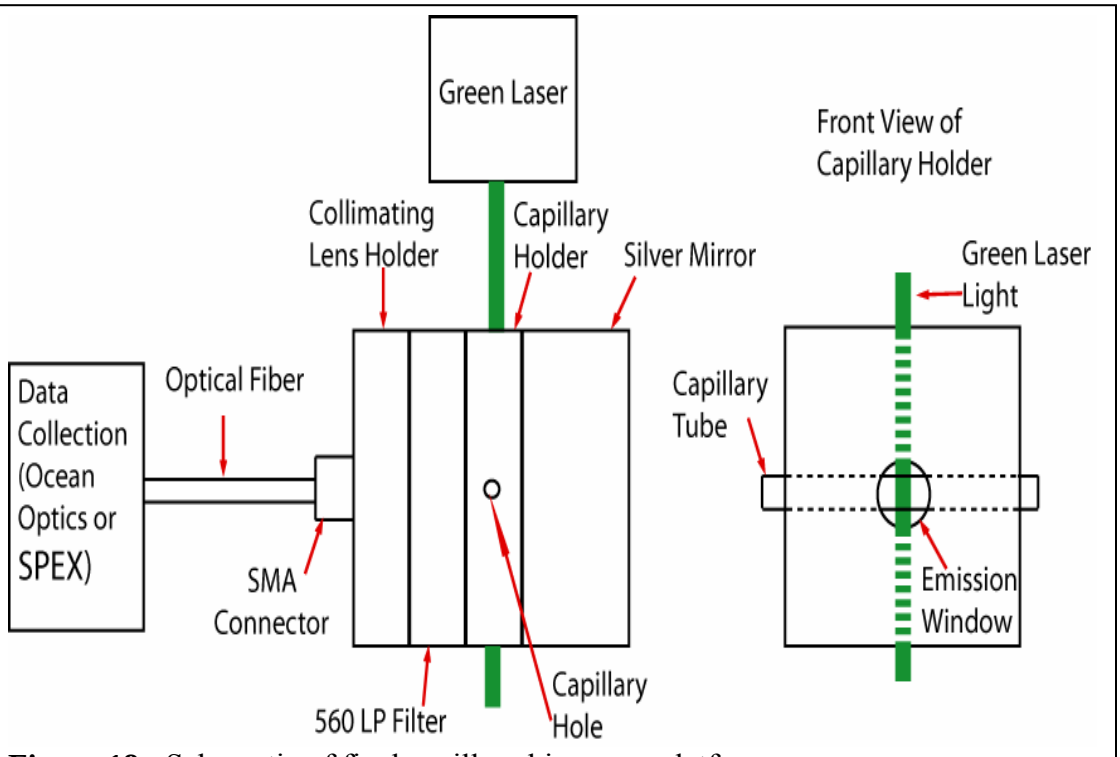


Figure 18: Schematic of final capillary biosensor platform.

6.1 Avidin-Biotin Capillary Trials

An avidin-biotin test system was used to initially evaluate this capillary biosensor platform. Avidin is a tetrameric protein that has a high affinity for biotin (dissociation constant of 10^{-15}) in which one avidin binds four biotin molecules[50]. Avidin and biotin are often used as test reagents because of their high affinity. This system was designed to mimic the *H. hepaticus* system. The avidin and biotin were viewed as the antibody and antigen, respectively, and served as an ideal binding system to assess the data collection method at less cost.

6.1.1 Sample Preparation and Data Collection

The purpose of this experiment was to further explore the feasibility of measuring the bound fluorescent emission. World Precision capillaries were utilized in this experiment as well as subsequent experiments. They were divided in half using a diamond tip etcher. A biotinylated cortisol antibody (due to its availability) was immobilized to the inner walls of the capillary. Avidin was conjugated to AF 546 according to the instructions provided with the antibody labeling kits.

Samples containing molar ratio of 0:1, 1:1 and 1:20 avidin to biotin were made in triplicate. The molar concentration of avidin-AF 546 was 8.5 picomoles. Corresponding biotin concentrations were 0, 8.5 and 170 picomoles. Total sample volume was 30 µl. The samples were mixed and incubated at room temperature for 15 minutes. Each sample was then added to individual capillaries and incubated at room temperature for 30 minutes. Subsequently, the samples were removed and the capillaries were washed with PBS. The capillaries were then filled with PBS and scanned using the capillary stage.

The capillaries were scanned in five minute intervals, using an integration time of 500 msec. Both the 532 nm green laser and 569 nm AF 546 peaks were collected. The emission signal from the AF 546 peak would sometimes increase for the first five minute scan, and then remained constant for the second five minute scan. Data from the second five minutes were averaged and a graph of molar ratio to intensity was developed.

6.1.2 Discussion and Results

A ratiometric analysis was used to account for laser variability. The AF546 intensity was divided by the green laser intensity. This ratio helps correct laser source variation. The molar ratio biotin: avidin was plotted versus the AF546/Green laser ratio as shown in Figure 19.

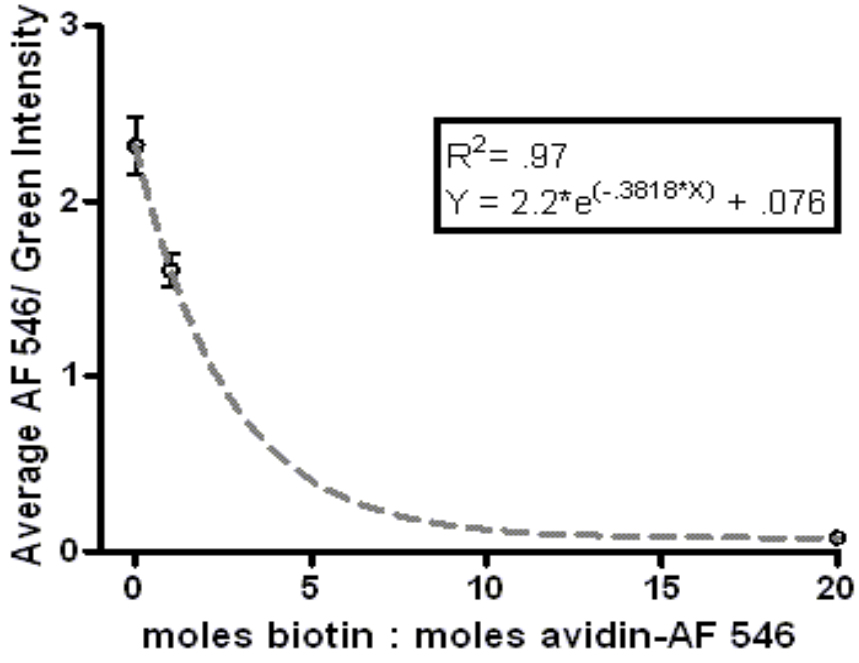


Figure 19: Results from the avidin- biotin capillary test system.

Standard deviations of the triplicate samples were low. This graph was fitted with an exponential decay equation from GraphPad Prism more fully explained is section 6.3.2. The r-squared value was 0.97 which indicates that the curve fit the data well. An exponential decay curve was selected because as the avidin-AF 546 becomes saturated in solution, the binding to the capillaries walls should plateau. Saturation occurs at increased concentrations. The fluorescent intensity response should be steeper at lower concentrations. An exponential decay describes this theory and results indicated a good correlation. These results confirmed the proposed theory described in section 5.1. There was an excellent response to increasing biotin concentration as expected.

One concern with this experiment was that the signal increased then stabilized after five minutes of data collection. This problem was not seen with the protein-A-AF-546 or unconjugated AF 546 tested in solution. The avidin had a high fluorophore to protein ratio which implied that the avidin was over conjugated. This may have made the fluorescent dyes unstable and caused the initial instability. Since this problem was only seen with the avidin-AF 546, it was not explored further because this was only a test system.

6.1.3 Conclusions

This experiment provided evidence that the capillary platform was appropriately designed to accurately analyze the capillary tubes. Avidin and biotin have an extremely high affinity for one another compared to affinity of antibodies and antigens. The characteristics of an immunological system are different than the avidin-biotin system used here. High avidin affinity may result in increased binding that translates to higher

signals. Based on the success from this experiment, it was decided to try this capillary biosensor platform with *H. hepaticus* reagents.

6.2 *H. hepaticus* Sample Mixture Analysis

6.2.1 Sample Preparation and Data Collection

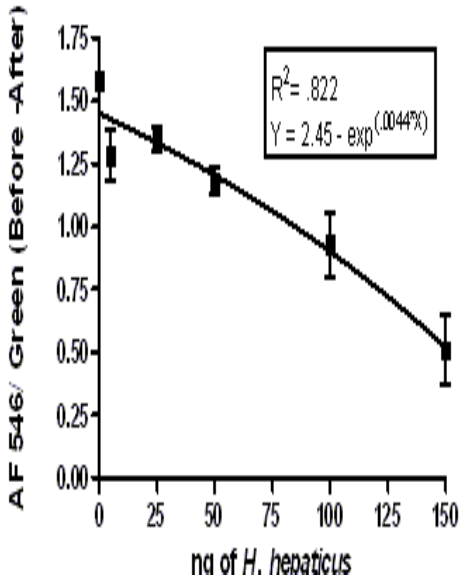
H. hepaticus immobilized capillaries were prepared using the method described previously. The amount of *H. hepaticus* added during the immobilization step was 20 µl of a 0.01 µg/µl solution and the blocking time was one hour. Samples containing 0, 5, 25, 50, 100 and 150 ng of *H. hepaticus* were made in triplicate for experiment 1. Experiment 2 used samples with lower concentrations at 0, 0.1, 1, 5 and 10 ng of *H. hepaticus* in triplicate. The sample volume was approximately 30 µl. Sufficient amounts of *H. hepaticus* for three samples were added to 90 µl of PBS. Then 1.5 µl of anti-*H. hepaticus*, or 0.5 µl per sample, was added to the solutions (approx. 55 ng of antibody/ per sample). Antibody amounts were changed in experiment 2 to 0.75 µl / sample (approx. 82.5 ng/sample) in an attempt to increase the fluorescent signal. The samples were then mixed and incubated at room temperature for one hour. Twenty µl of each sample mixture was added to a *H. hepaticus* immobilized capillary. Part of the solution was saved to serve as the "before capillary exposure sample mixture" for comparisons. The sample mixtures were incubated inside the capillary for 30 minutes, then removed and stored in a micro-centrifuge tube. The capillaries themselves were washed and filled with PBS for scanning. Results from the immobilized capillary are presented in the next section. The saved sample mixtures, both before and after capillary addition, were individually placed into new capillaries for scanning. Sample mixture analysis used an

integration time of 700 msec. Both the 569 nm AF 546 peak and 532 nm green laser peaks were collected for 60 seconds. Prior to scanning, each capillary was cleaned with acetone, which removed residues from the capillaries.

6.2.2 Discussion and Results

Experiment One

A ratiometric analysis method was used to reduce the impact of any laser intensity fluctuations on the data. The AF 546 and green laser peaks were averaged over the 60 second collection period. The AF546 average was divided by the green laser average to help account for variation. Next, the ratio of the after capillary exposure mixture was subtracted from the before capillary exposure sample mixture. Comparing the before and after sample exposure accounted for the change in fluorescence between different sample types. The difference in fluorescence was plotted versus the amount of *H. hepaticus* in the sample as shown in Figure 20.



ng of H. hepaticus	Mean	Standard Dev	P-Value
0	1.58	0.05	N/A
5	1.28	0.18	P > 0.05
25	1.35	0.07	P > 0.05
50	1.18	0.09	P < 0.05
100	0.93	0.22	P < 0.01
150	0.51	0.24	P < 0.01

Figure 20: Results from sample mixture analysis experiment 1

The results showed an inverse relationship between *H. hepaticus* concentration and intensity. There were no standard equations to fit this data found in Prism. The expected trend is that the data should plateau at lower concentration and decrease rapidly at higher concentrations. A user defined equation was derived to approximate this trend:

$$\text{Intensity Ratio} = \text{Intensity Ratio}_{max} - e^{(k*[H.hep])} \qquad \text{Eqn. (4)}$$

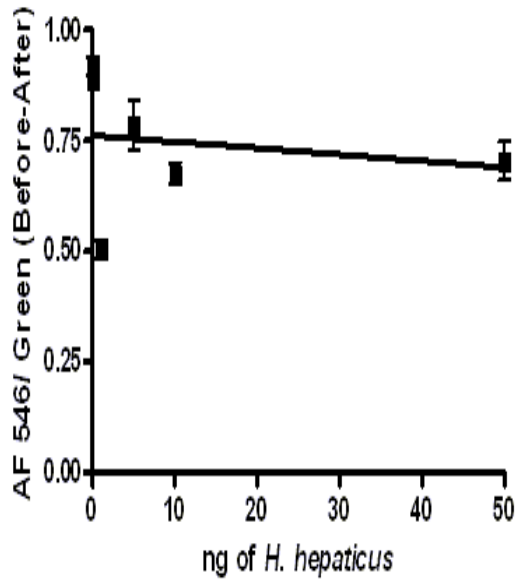
where K is the rate constant. More data points were needed in order to obtain a more reliable fit curve.

An ANOVA analysis with a Dunnett's post test was also performed. Samples containing *H. hepaticus* were different from the 0 ng samples and should result in a low P-value (Figure 20). The samples containing greater than 25 ng of *H. hepaticus* had a p-value less than .05. Sample groups with less than 25 ng of *H. hepaticus* did not yield statically significant results and the minimum detection limit from this experiment was between 25 and 50 nanograms. The results from this experiment showed that this biosensor method reliably distinguishes between samples that have greater than 25 ng of *H. hepaticus* from non-*H. hepaticus* samples. Since the level of infectivity for *H. hepaticus* is unknown, a lower limit of detection was attempted and a repeat experiment was performed with lower *H. hepaticus* sample concentrations.

Experiment Two:

The purpose of this experiment was to utilize lower concentration samples containing 0, 0.1, 1, 5, 10 and 50 ng of *H. hepaticus*. The amount of antibody added to

each sample was increased from 55 to 83 ng per sample to increase signal. The same data analysis techniques were used and the results are shown in Figure 21.



ng H. hepaticus	Mean	STD Dev	P-Value
0	0.919	0.037	N/A
0.1	0.886	0.017	P > 0.05
1	0.505	0.037	P < 0.01
5	0.787	0.099	P > 0.05
10	0.676	0.043	P < 0.01
50	0.705	0.073	P < 0.01

Figure 21: Results from sample mixture analysis experiment 2.

The data from this experiment was inconsistent. Curve fitting the data could not be accomplished because of the small data range and inconsistent trends. However, Equation 4 was utilized to outline the differences between the two experiments. The R-squared value was 0.03, indicating there was essentially no correlation between the plotted data and the fitted trend line.

The Dunnett's test showed that samples containing greater than 5 ng of *H. hepaticus* were statistically different from the non- *H. hepaticus* samples. The P-value for the 1 ng samples was less than .05, but these points were considered outliers since they were much less then the surrounding data points.

Overall, the sample mixture method appeared promising and useful with the higher concentrations of *H. hepaticus*. However, it is desirable to achieve an even lower

limit of detection. Thus immobilized capillary analysis was also performed under similar experimental conditions as the sample mixture method as described in Section 6.3.

6.2.3 Conclusions

Sample mixture analysis was a reliable analysis method for concentrations greater than 5 ng of *H. hepaticus*. But, the results were inconsistent at lower concentrations. The method required micro-volume fluid transport that resulted in human error. An automated system would reduce some of the variability evident in these experiments.

6.3 Immobilized Capillary Analysis

The purpose of the following experiments was to explore the immobilized capillary method in order to achieve lower detection limits of the *H. hepaticus*. All the experiments were performed using the final capillary stage design shown previously in Figure 17 and 18.

6.3.1 Sample Preparation

All samples were prepared using the same method described in the sample mixture analysis, Section 6.2.1.1. Four experiments were conducted using these techniques. The first two experiments were performed in conjunction with the sample mixture analysis experiments. Experiments three and four were performed after the sample mixture analysis experiments were discontinued.

Other experiments were performed to determine if the incubation times could be reduced. In experiment 5, samples were made containing *H. hepaticus* concentrations of

0, 1, and 10 nanograms. They were mixed with the antibody and incubated for 30 minutes instead of 60 minutes. The sample mixture was then added to the capillary tube for an incubation of 15 minutes incubation (instead of 30 minutes) after which the samples were removed and the capillaries washed in PBS. The capillaries were then scanned using the same procedure as previous experiments. Incubation times were further reduced to 20 and 10 minutes for the second part of this experiment.

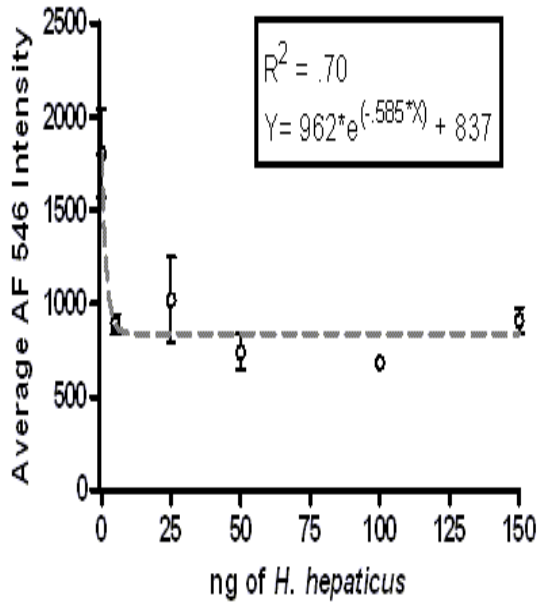
The immobilized capillary experiments required additional steps in order to that scan the capillaries. After the final incubation, the sample mixtures were removed and the capillaries washed in PBS. The capillaries were filled with PBS and capped. Prior to scanning, each capillary was wiped with acetone that removed residues. The capillary was then inserted into the capillary stage and excited with the green laser. An integration time of 20 seconds was used due to the weak fluorescent intensities. The long integration time introduced characteristic noise peaks, but these were removed by subtracting the background from each spectrum. The entire spectrum was saved and analyzed. AF 546 intensity data was obtained by averaging from 568.25 – 572.17 nm. Average peak intensities from each sample were graphed versus *H. hepaticus* concentration.

6.3.2 Discussion and Results

Experiment One

This experiment was performed in conjunction with experiment one from the sample mixture analysis section. Samples containing 0, 5, 10, 25, 50, 100 and 150 nanograms of *H. hepaticus* were made in triplicate. The results from this experiment showed a significant difference between samples with and without *H. hepaticus* as shown

in Figure 22. As expected, there was a decrease in AF 546 intensity with increasing *H. hepaticus* concentration. All samples containing *H. hepaticus* had a mean significantly lower then the 0 ng samples. Most had low standard deviations as well. The p-values from each data set were less than 0.01.



ng of H. hepaticus	Mean	STD Dex	P-Value
0	1800	408	N/A
5	889	90	P < 0.01
25	1019	397	P < 0.01
50	741	169	P < 0.01
100	682	21	P < 0.01
150	908	116	P < 0.01

Figure 22: Results from immobilized capillary analysis from experiment one.

The immunological method used in this research is not common in biosensor research. As a result, there was no standard equation found in literature to use in curve fitting this type of data. A text by Moltulsky and Chrisopolus was used to find an equation that would best match the data[51]. An exponential decay equation was selected to fit this data set because it was the most consistent with the immunological theory. This graph was fitted with an exponential decay equation from GraphPad Prism:

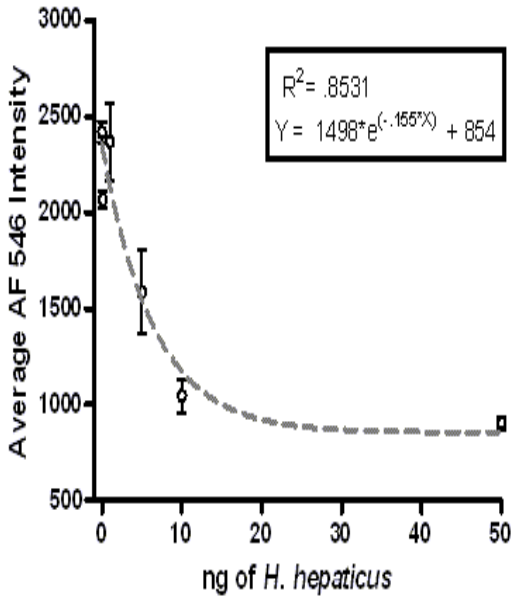
$$\text{Intensity} = \text{Span}*e^{(-K*[H.Hepaticus])} + \text{Plateau} \qquad \text{Eqn. (5)}$$

Where K was the rate calculated rate constant and plateau was the saturated intensity reached at high concentrations. This equation provided a good approximation for the data obtained. The exponential decay curve decreased sharply at lower

concentrations and plateaued once a saturation threshold was reached. AF 546 intensity appears to level off at high concentrations, indicating saturation. Another experiment using lower *H. hepaticus* concentrations was performed to prove that this relationship was correct.

Experiment Two

This experiment was conducted in conjunction with experiment one from the sample mixture analysis section. Samples containing 0, 0.1, 1, 5, 10, and 50 nanograms of *H. hepaticus* were made in triplicate. The average AF 546 intensities for each sample set were calculated and plotted against *H. hepaticus* concentration as shown in Figure 23.



ng of H. hepaticus	Mean	STD Dev	P-Value
0	2419	95	N/A
0.1	2076	109	P > 0.05
1	2369	346	P > 0.05
5	1585	376	P < 0.01
10	1045	154	P < 0.01
50	903	54	P < 0.01

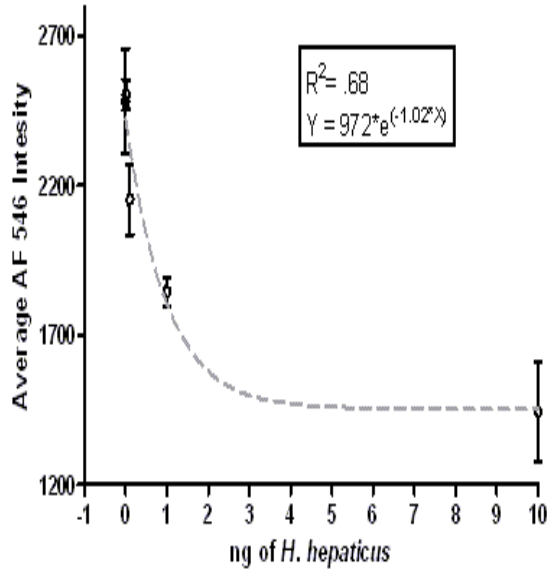
Figure 23: Results from immobilized capillary analysis experiment two.

The same data analysis methods from experiment one were used. There was a sharp decrease in AF 546 intensity followed by a plateau as *H. hepaticus* concentration increased. This observed trend matched an exponential decay equation. The R-squared value was 0.85, which was acceptable considering the triplicate points and variability of

the system. Dunnett's test results showed there was a statistically significant difference between the 0 ng sample set and sample groups greater than the 5 ng of *H. hepaticus*. The 1 ng sample set was anomalous to the trend as it was nearly the same intensity as the 0 ng samples. Based on this experiment, the limit of detection for this biosensor was approximately 5 ng. Additional experiments were performed to ensure the observed relationship was real and to determine if a lower limit of detection was possible.

Experiment Three

The purpose of experiment three was to explore the biosensor functionality at lower concentrations in order to determine the minimum detection limit. The concentrations used were 0, 0.01, 0.1, 1, and 10 nanograms of *H. hepaticus.* Each sample type had five replicates. The same analysis methods as the previous experiments were used. The results from this experiment were plotted in Figure 24.



ng of H.Hepaticus	Average	Stdev	P-Value
0	2482	395	N/A
0.01	2504	115	P > 0.05
0.1	2154	264	P > 0.05
1	1846	105	P < 0.01
10	1443	376	P < 0.01

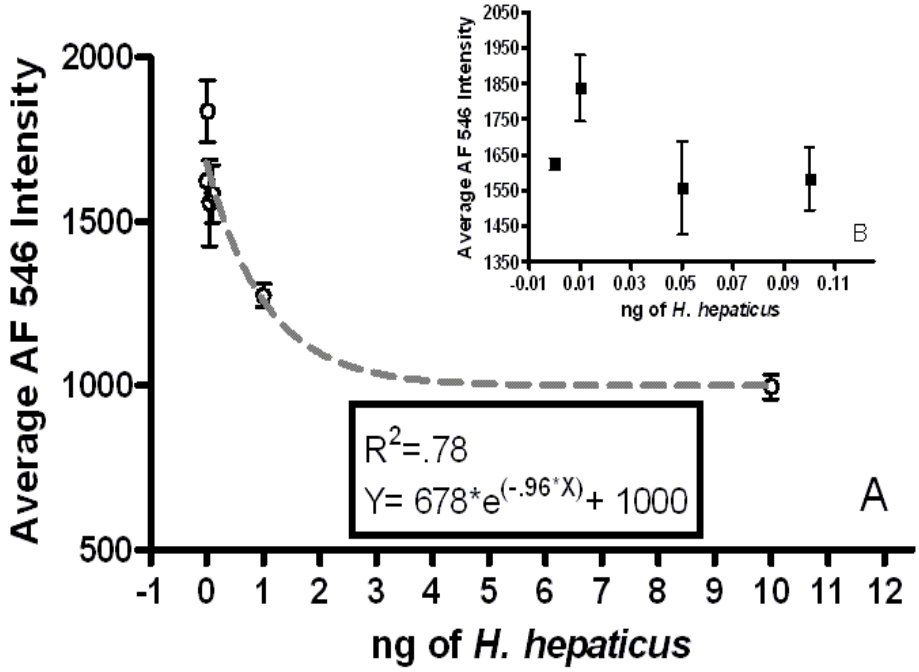
Figure 24: Results from immobilized capillary analysis three.

The data followed similar trends as demonstrated in previous experiments. Saturation of the system was reached at 10 ng *H. hepaticus*. There was a good dynamic range between 0 and approximately 2 ng of *H. hepaticus*. This indicates a highly sensitive region that may be utilized for quantitative measurements. The increased number of replicates also increased the variation in the sample sets. Variations remained relatively low and did not adversely affect the results. Results from the Dunnett's test showed that only the 1 and 10 ng samples sets were statistically different. The limit of detection from this experiment was 1 nanogram of *H. hepaticus*. A lower limit of detection is likely possible because of the steep response observed at low concentrations.

Experiment Four:

Experiment Four used a sample set containing similar concentrations compared to the previous experiment. Samples containing 0, 0.01, 0.05, 0.1, 1 and 10 ng of *H. hepaticus* were made in triplicate. The same analysis methods as the previous experiments were used. The results from this experiment were plotted in Figure 25.

The results from the experiment were consistent with those seen in experiment three. Data from samples containing less then .1 ng of *H. hepaticus* were erratic. The Dunnett's test results from this experiment again showed that the limit of detection for the immobilized capillary analysis technique was 1 ng of *H. hepaticus.*



ng of H.Hepaticus	Average	Stdev	P-Value
0	1624	27	N/A
0.01	1837	163	P > 0.05
0.05	1557	227	P > 0.05
0.1	1584	153	P > 0.05
1	1274	62	P < 0.05
10	997	65	P < 0.01

C

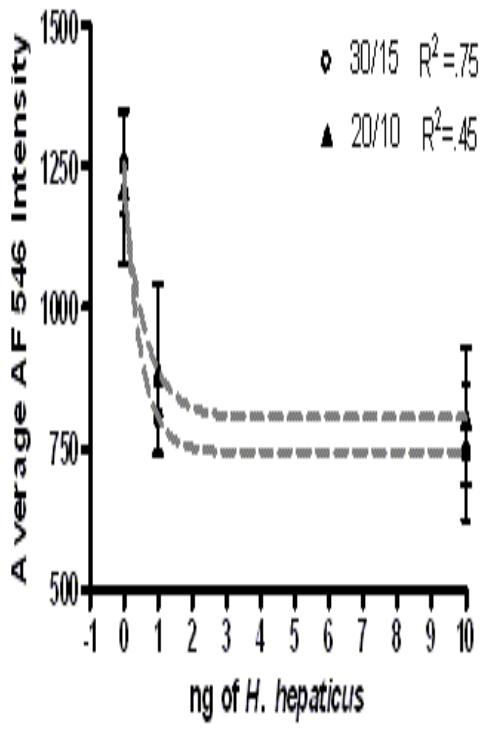
Figure 25: Results from immobilized capillary analysis experiment four. A: All data plotted. B: Graph of 0, .01, .05, and .1 ng data points C: Data Table.

Experiment Five

Experiment Five reduced the incubation times. Reducing incubation time shortened sample analysis time. Samples containing 0, 1 and 10 ng of *H. hepaticus* were made in triplicate. Two sets of incubations times were used. The first incubation time set used was 30 minutes prior to capillary addition and 15 minutes inside capillary (abbreviated 30/15). The second incubation time set used 20 minutes prior to capillary

addition and 10 minutes inside the capillary (abbreviated 20/10). The same data analysis methods as previous experiments were used.

Figure 26 showed results of this experiment. The 30/15 incubation set showed results similar to those seen in previous experiments that used longer incubation times. Both the 1 and 10 ng sample sets had p-values less than 0.05 and low variability. The R-squared value from the curve fit was consistent with those observed in previous experiments. A 1 nanogram limit of detection was possible using the 30/15 minutes incubation times. Data from this experiments indicated that shortening incubation times to 30/15 minutes will yield the same results as using 60/30 minute incubations used in previous experiment.



	30/15 Incubation		
ng H. hepaticus	Average	STDEV	P-Value
0	1254.42	152.92	N/A
1	805.25	104.00	P < 0.05
10	743.86	208.83	P < 0.05
	20/10 Incubation		
ng H. hepaticus	Average	STDEV	P-Value
0	1214.94	234.84	N/A
1	889.69	261.21	P > 0.05
10	805.47	212.95	P > 0.05

Figure 26: Results from immobilized capillary analysis incubation time experiment.

Figure 26 shows the results of the 20/10 incubation times as well. The results using these incubation times were considerably different than previous experiments. Statistical analysis showed both the 1 and 10 ng samples had p-values greater than 0.05. The R-squared value was much less then previous experiments as well. Shortening incubation below 30/15 minutes will need further study before a conclusion can be made.

6.3.3 Conclusions

Immobilized capillary analysis was shown to be a sensitive, accurate and reliable method of detection *H. hepaticus* at low concentrations. The minimum detection limit was shown to be 1 ng. A lower limit of detection is possible by decreasing sample variability. Incubation times can be decreased to 30/15 and accurate results are obtained. This reduced analysis time to less than one hour, which is a considerable improvement over current assay time requirements. The project has shown the feasibility of producing accurate and reliable disposable capillary biosensor.

CHAPTER SEVEN
Future Work

Much work remains to thoroughly develop this capillary biosensor technology. This project has successfully developed the disposable capillary component of the biosensor, thereby establishing a foundation for future investigations. Future work can now build off this success. A platform that combines sample preparation, mixing and analysis in a portable and automated system is the next step of this project. This will be performed by incorporating microfluidics and micropump systems for fluid transport through the capillaries. Figure 27 is a schematic of a possible capillary biosensor platform. A sample from an animal is loaded into the sample well. A pump will transport the sample into a separate well where antibody is added. The sample mixture is pumped through a meandering mixer to thoroughly mix the reagents. An incubation chamber can be included at the end of the mixer if additional incubation time required. The sample mixture is then pumped into a disposable immunocartridge where the immunoreaction takes place. After incubation, the sample mixture can be pumped into a reading chamber for analysis. Additionally, the immunocartridge can be analyzed for immobilized antigen analysis. The biosensor is then washed after analysis occurs and is ready for the next sample. This is one of many ideas that could be implemented into future capillary biosensor systems. Hopefully the knowledge acquired from this project will contribute to the development of a fully functional capillary biosensor system.

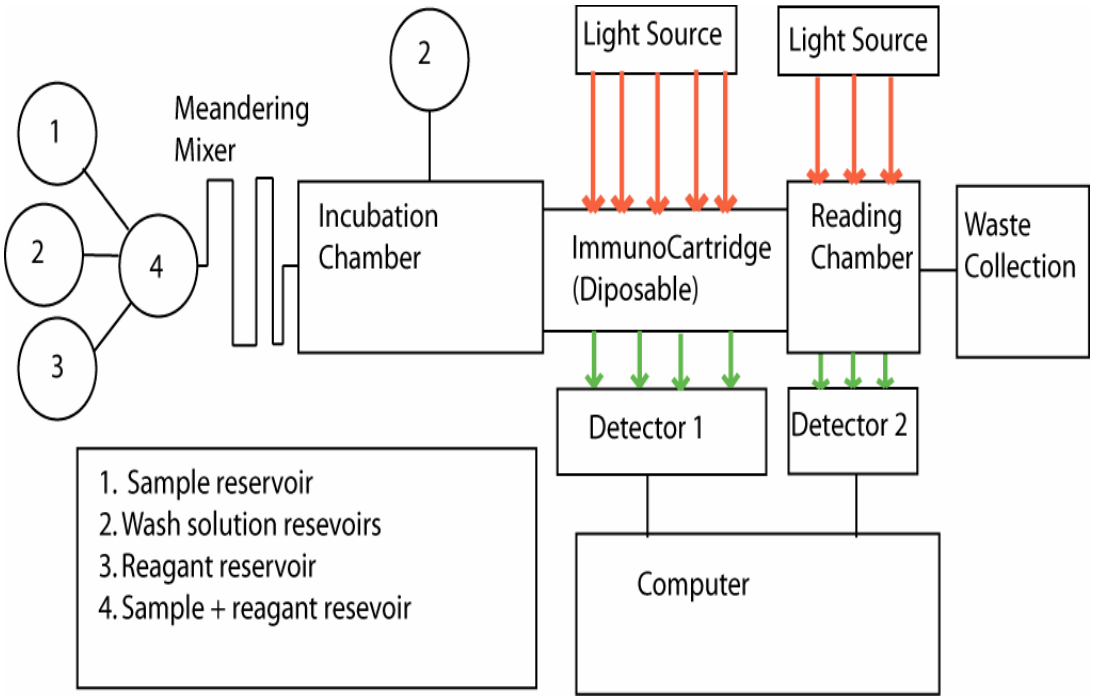


Figure 27: Schematic of a possible future automated capillary biosensor platform.

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