RAPID LABEL-FREE DETECTION OF PATHOGENS BY LOCAL pH MODULATION

A Thesis

by

MATTHEW STEVEN LEWIS

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Chair of Committee,	Kung-Hui Chu
Committee Members,	Jason Gill
	Terry Gentry

Intercollegiate Faculty Chair, Ronald Kaiser

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ABSTRACT

Pathogenic bacteria present major issues for human health across the world. One of the ways to mitigate the negative impacts from contaminated food and water sources is to decrease the time required to test potentially contaminated sources. This study examined a new method of label free detection using local pH modulation to quantitatively detect bacteria. By tagging antibodies with a pH-sensitive fluorescent dye it was possible to detect the presence of bacteria bound to antibodies. Local pH can be effected by the presence of charged molecules because they attract counter ions. By utilizing the negatively charged surface of bacteria to attract counter ions in the form of hydrogen ions the local pH can be lowered, thereby lowering the fluorescence of fluorescein. By measuring fluorescence with respect to bacterial cell concentration a relationship between bacteria concentration and fluorescence can be established. It is also advantageous to know if the pathogens detected are active and alive or dead. Adding a rapidly uptaken carbon source (glucose) allows for differences between live and dead cells to be detected.

This approach was tested in microtiter plates and using immunomagnetic beads as the testing platforms. Using microtiter plates concentrations of ~10^6 *E. coli* cells could be detected although not to a statistically significant level. The addition of glucose showed that live cells could be distinguished from UV killed cells but cell numbers could not be established. Immunomagnetic beads displayed inconclusive results indicating the need for continued experiments.

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Soli Deo honor et gloria

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Contributors

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All work conducted for the thesis was completed by the student independently.

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1. INTRODUCTION AND OBJECTIVES

1.1 Introduction

Waterborne pathogens are a significant health risk for much of the world. It is estimated that four percent of all deaths are caused by diarrhea which is often related to poor sanitation in developing countries.[1] Worldwide one and a half million children die each year as a result of diarrheal disease, those hardest hit are children under five years old in Asian and African countries.[2] Even in the United States, each year five hundred thousand people suffer from severe waterborne diseases.[3] There are many potential diseases that can be caused by pathogen- contaminated water or food including bacteria such as *Escherichia coli* (O157:H7,O148, O124), *Salmonella* spp, *Vibrio cholera*, and *Shigella* spp, resulting in diseases such as gastroenteritis, typhoid fever, cholera and dysentery.[3]

In 2015, there were two high profile outbreaks associated with *E. coli*. After eating at Chipotle Mexican Grill locations a total of fifty five people in eleven states became ill with a Shiga toxin producing *E. coli* (STEC O26).[4] Although investigators were unable to discover the exact cause of the outbreak, Chipotle suffered its first sales decline in ten years and year-to-year profit for the fourth quarter of 2015 dropped forty four percent.[5] Costco Wholesale was also involved in a 2015 *E. coli* outbreak. Nineteen people in seven states were reported ill with a different Shiga toxin-producing *E. coli* (STEC O157:H7) after consuming a rotisserie chicken salad purchased from Costco. The store and producer (Taylor Farms Pacific, Inc.) issue a recall of the affected item and many other products that shared the celery ingredient.[6] These outbreaks in a country

with high standards of sanitation and food preparation show that these diseases can happen anywhere if proper precautions are not taken. While these outbreaks were related to food, most infections around the world are related to untreated wastewater entering the water supply.[2]

To ensure that water supplied is free of infectious pathogens, it is treated and tested. Because testing for all pathogens is impractical, samples are tested for either total coliforms, fecal coliforms or sometimes simply *E. coli*. There are many ways to test for *E. coli* in water samples, one of the most common is the EPA method 1603 " *E. coli* in water by membrane filtration using modified membrane thermotolerant *E. coli* Agar, (modified mTEC)".[7] While this method is sensitive, it is culture based and takes twenty four to thirty six hours before any results can be reported.[7] Many of the newer approaches to pathogen detection have faced a trade-off between speed and limit of detection. A brief description of detection methods will be given in the background section. In order to reduce infectious disease outbreaks, both rapid and sensitive testing methods for pathogens are needed.

In this work, a rapid fluorescence based method is presented for detecting *E. coli*. Fluorescent dyes are widely used in biological applications; the most common uses are to visualize specific bacteria or tissues. Fluorescence has the advantages of being simple to use, very sensitive, and of a visual nature. One of the most common dyes in use is fluorescein due to its low cost. The problems associated with fluorescein are its relatively rapid photobleaching and pH fluorescence intensity dependence. To combat these problems many new dyes have been developed that improve on the photobleaching

and pH dependence. The pH dependence of fluorescein is due to the protonation or deprotonation of a carboxylic side chain, which can also be used as a sensor. While the initial testing is being done with *E. coli,* the underlying principle of the method used in this study can be easily expanded for any pathogens.

1.2 Goal, Objective and Hypothesis

The goal of this research is to apply local pH modulation in conjunction with specific binding events in order to specifically, sensitively and rapidly detect live *E. coli* from water samples. To achieve this goal, four tasks are identified and outlined below.

Objective: Detect live *E. coli* using local pH modulation and fluorescence

Hypothesis: Rapid detection of *E. coli* can be done using antibodies tagged with a pH dependent fluorescent dye. By using the change in fluorescence, label free detection can be done with a single antibody in one-step in combination of utilizing of a rapidly uptaken substrate to differentiate live/dead cells.

Task 1: Attach fluorescent dye to antibody to create an antibody-dye complex.

Task 2: Create a titration curve of fluorescence vs pH to determine the most sensitive region to be used during detection.

Task 3: Detect *E. coli* using (a) immunomagnetic separation and by (b) a 96-well microtiter plate. Determine the limit of detection for and optimize for it sensitivity.

Task 4: Determine the response associated glucose being added to allow for live/dead testing.

2. LITERATURE REVIEW

2.1 General Background

In the history of human civilization, concern over pathogenic microorganisms is a relatively new problem; the first scientifically documented case of waterborne disease was in 1854. John Snow realized that it was a sewage contaminated well that lead to an outbreak of cholera in a London neighborhood.[8] In the nineteenth and twentieth centuries, sanitation practices have been implemented in the United States and other developed nations to improve the handling of drinking water and wastewater. Thanks to these developments, waterborne diseases have become a rare and newsworthy event in those parts of the world. Because it would be prohibitively expensive and difficult to monitor all potential pathogens, water treatment has historically relied on indicator species instead. The first indicator was coliforms adopted by the U.S. Public Health Service in 1914, later shifting to a subset known as fecal coliforms.[9] These organisms make good indicators because they are not naturally present in the environment, but are present in high concentrations in the feces of warm-blooded animals. Thus, the presence of these organisms indicates recent fecal contamination.[10]

Recent advances have made it clear that indicators are not universal predictors as was once thought. There are now alternative indicators for situations where fecal coliforms or *E. coli* do not serve as a good indicator of contamination, such as Enterococci for marine waters or tropical climates.[10] The Safe Drinking Water Act of 1974 empowered the EPA to enforce drinking water standards across the United States. Growing public concern over water pollution of all kinds led to the passing of

the Clean Water Act (CWA). The CWA gives the EPA broad authority to enforce water quality programs on effluent and ambient water quality standards. The CWA established a 303(d) list requiring states to define the pollutants and sources responsible for the degraded quality of each listed water body. The list also requires the establishment of total maximum daily loads (TMDL) necessary to achieve the appropriate standards.[10] The most common impairment of water bodies is an elevated level of bacterial indicators (coliforms or *E. coli*). Both drinking water and recreational waters are now tested for bacterial indicators routinely, leading to a demand for cheap, rapid testing methods. The realization that indicators can be misleading for certain pathogens has spurred new developments in immunology, nucleic acid testing, and biosensors for the detection of other pathogenic bacteria besides indicators.

2.2 Methods of Pathogen Detection

In order to decrease analysis time, lower detection limits or mitigate the weakness of a given method, it is becoming more common to incorporate more than one type of method into detection platforms. Some of the methods described below fall into more than one category. As different fields approach pathogen detection from different directions (food safety vs microbiology vs engineering) methods have been grouped by what is the primary mode of detection.

2.2.1 Culture Based Methods

Culture based methods are still the most sensitive and offer potentially the most specific detection depending on the number of confirmatory tests run for that particular pathogen. Although the ability to reliably detect a pathogen can vary widely depending

on the specific strain being studied. Culturing is commonly able to detect a single colony-forming unit (CFU) in a sample. However, one major downside of culture based methods is the time it takes to perform them and an inability to detect viable but non-culturable (VBNC) cells.[11]

2.2.1.1 Membrane Filtration

A cultural method (EPA method 1603) developed by the EPA and commonly used by commercial labs provides a direct count of *E. coli* in ambient water or wastewater based on colony growth. A sample is filtered through a membrane, which is then placed on a selective media and then incubated at 35°C for two hours and 44.5C for 22 hours. The media contains a chromogen that is catabolized by *E. coli* with the β-D-glucuronidase enzyme to a red colored compound for easy colony identification.[7]

2.2.1.2 Presence/Absence

Colilert tests come prepared with two substrates, o-nitrophenyl-β-D-galactopyranoside (ONPG) and 4-methylumbelliferone glucuronide (MUG). ONPG is hydrolyzed by coliforms to produce a yellow compound and, MUG after interacting with the β-glucuronidase enzyme found in *E. coli* produces a fluorescent product. Incubated 24 hours these tests allow for simultaneous detection of coliforms and *E. coli* from a sample to with a detection limit of one CFU.[12]

The commercial Colilert-18 kit uses a chromogenic substrate for presence/absence tests but can also be used in its Quanti-Tray method to get MPN results. Budnick et al. found that there was a strong linear correlation and no statistically significant difference between 109 freshwater samples analyzed by the Colilert-18

Quanti-Tray method and the mTEC agar method (EPA method 1103.1 now 1603) for the detection of *E. coli*. They note that the Quanti-Tray method requires less preparation and expertise to get results in 18 hours instead of 24-26 hours with fewer quality control procedures required.[13]

2.2.1.3 Most Probable Number

The most probable number (MPN) test is one of the most established methods for enumerating coliforms, fecal coliforms and *E. coli*. In short, it relies on dilutions and replicates in the dilution series to determine a quantitative estimate of the starting concentration. Each positive test must be confirmed through further culture tests and when a final tally of positives and negatives is known published tables may be used to quantify a most probable number.[14] This test has been largely replaced for routine monitoring by the membrane filter test due to the development of selective media, time constraints, and ease of use. Some modern solutions such as the Colilert-18 Quanti-Tray mentioned above have combined selective media and traditional MPN to speed up the process from 24 to 18 hours and reduce the amount of bench time required to set up and analyze the samples.[9]

2.2.2 Nucleic Acid Based Methods

The nucleic acid based methods offer rapid results with little need for sample enrichment and low limits of detection. One of the weaknesses of this method is it is difficult to distinguish between live and dead cells and contaminating free DNA can also pose problems.

2.2.2.1 PCR (Polymerase Chain Reaction)

Hong et al. developed probes in a PCR-enzyme-linked immunosorbent assay (ELISA) for the rapid detection of *Campylobacter jejuni*, *Campylobacter coli*, *Salmonella enterica* from poultry samples. By linking PCR and ELISA, they were able to increase the sensitivity of the conventional PCR method by one hundred times. In PCR-ELISA a chemically tagged sequence in incorporated into the PCR amplicon, which is detected via ELISA. In this study they designed a PCR primer set based on the *Salmonella* virulence gene *invA* and the *Campylobacter ceuE* gene in order to quickly screen poultry carcass wash samples for these two food borne pathogens.[15]

2.2.2.2 QPCR (Real-Time/Quantitative PCR)

Quantitative or real time PCR is a popular method for detection of pathogens with results being produced while the procedure is running and eliminate the need to run a gel to analyze the PCR products. Many groups have demonstrated quantitative detection using different genes for the detection. The shiga toxin genes (*stx1* and *stx2*)[16, 17] the *sltI* gene,[18] and the *rfbE* locus[19] as just a handful of examples. QPCR is based on the creation of a signal incorporated into the newly made amplicon (molecular beacon) or in the release of a dye away from a quencher as in the various Taqman assays or using the Sybr green dye which fluoresces strongly when bound to double stranded DNA.[20]

2.2.2.3 Phage Based Detection

A new and expanding field focuses on the utilizing genetic engineering of phages towards beneficial uses. One of those uses is pathogen detection, Oda et al. used a T-

even-type PP01 bacteriophage to produce recombinant phages containing green fluorescent protein (GFP). The fusion did not change the specificity of the phage towards its *E. coli* O157:H7 host. Adsorption of the phage onto host cells allowed both cultivable *E. coli* and viable but non-culturable cells with specificity for the O157:H7 strain. [21] *E. coli* detection by genetically engineering a phage to inject lux genes has been reported to be capable of detecting one CFU/mL in tap water in approximately 12 hours or less depending on pre-incubation steps.[22, 23]

2.2.3 Immunology Based Methods

Immunologic methods are the most widespread in commercial testing due to the ease of automation and consistent results with the use of standard curves. The basis of all immunology-based methods is some kind of biologically relevant recognition molecule followed by signal amplification.

2.2.3.1 Enzyme Linked Immunosorbent Assay (ELISA)

ELISA comes in many varieties, direct, indirect, competitive, and sandwich. The basics of the method are similar regardless of the specific type. A recognition element binds to antigen and a signal is then produced by a chemiluminescent procedure. While the most common recognition element is antibodies, there have been increasing numbers of attempts to use phages as the recognition and binding element to reduce cost and increase specificity over polyclonal antibodies. Galikowska et al. were able to use bacteriophages instead of antibodies to detect *E. coli* and *Salmonella* with similar sensitivity as standard ELISA methods.[24] Park et al. created one of the early ELISA tests for *E. coli* using a sandwich ELISA but did not attempt to quantify the limit of detection.

The assay created functioned as a presumptive positive test for further analysis with 91.2% sensitivity, which was higher than the 82.4% sensitivity of the culture based comparison method. [25]

Indirect ELISA has also been used by Wang et al. it was found that is method developed had a limit of detection of 104 CFU/L in detecting samples directly from the environment with no enrichment step.[26] ELISA has shown promise for very rapid detection but can suffer from a lack of specificity and higher detection limits than other methods. To increase sensitivity preconcentration or enrichment steps are sometimes used, Mushin et al. used immunomagnetic beads to isolate and concentrate *E. coli* O157:H7 from milk and ground beef samples before detection with horseradish peroxidase and Tyramide to lower the detection limit to fifty CFU/mL, dropping to five CFU/mL with an enrichment step. [27]

2.2.3.2 Lateral Flow Immunoassay

Lateral flow immunoassays are typically simple to use and have simple results. Park et al. developed a paper microfluidic assay for detecting *Salmonella* using a smartphone. By preloading the channels with anti-Salmonella conjugated submicro particles, the assay time was 1 minute. When dipped in a sample antibody conjugated particles still confined in the paper will immunoagglutinate, the degree of immunagglutination could be quantified by Mie scattering using a smartphone application developed allowing for single cell detection.[28]

2.2.3.3 Magnetic Bead Linked Immunology

While immunomagnetic separation is widely used, it is still being innovated for increased specificity and capture efficiency and new uses are being found. To help recover higher yields from meat samples, Balarkrishnan et al. coated beads with protein A before attaching antibodies and were able to detect 103 CFU/mL of *E. coli* in one hour. [29] A novel use of nanoparticles was put forth by Jung et al. to detect rotavirus. Rotavirus was captured by antibodies on a photoluminescent graphene oxide array, then a second antibody with a gold nanoparticle we be added. The gold nanoparticles were responsible for a fluorescence resonance energy transfer (FRET) with the graphene oxide array resulting in fluorescence quenching. Using this platform a limit of detection of 105 PFU/mL was achieved.[30] Chen et al. used dye-doped silica nanoparticles as a secondary detection bead after initial separation with antibody to generate higher signal compared to traditional fluorescent dyes. The silica dye-doped also displayed a resistance towards photo-bleaching compared to traditional fluorescent dyes.[31] Another example of secondary beads being used as signal enhancers was investigated by Jayamohan et al. The secondary beads were functionalized with polyG, an oligonucleotide. After complexing the beads together differential pulse voltammetry is used to detect *E. coli* down to 3 CFU/100 mL.[32]

Immunomagnetic separation can be used as a preconcentration step before detection by other methods, two groups used immunomagnetic bead separation to isolate bacteria before performing whole cell matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-MS) to confirm the identify of *E. coli* in about one

hour. MALDI-MS works by comparing a fingerprint mass spectrum with known spectrums of biomarkers. Samples were taken from river water [33] and ground beef. [34] Immunomagnetic beads are also used for non-fluorescent detection methods. Alocilja et al. used antibody labeled magnetic/polyaniline nanoparticles to separate out *E. coli*. The beads are positioned on a screen-printed carbon electrode and the presence of cells impedes the flow of electricity between the nanoparticles and the electrode. Using this method six CFU/mL of *E. coli* were detected in 1 hour. [35]

2.2.4 Biosensors

Biosensors operate on a simple principle. Some form of sensing platform detects a change, which is coupled to some kind of transducer to make the change observable by an instrument or scientist. Both the sensing platform and type of transducer can take many forms; some of the most common setup are examined below.

2.2.4.1 Optical

Optical methods of pathogen detection take advantage of the visible, UV, and infrared parts of the electromagnetic spectrum. Microfluidics have exploded in their capabilities since the introduction of polydimethylsiloxane (PDMS). PDMS is for the most part non-reactive, optically clear, gas permeable, and can be molded into any shape to a very fine level of detail. Golberg used immunomagnetic separation to isolate *E. coli* and a microfluidic device to encapsulate the captured bacteria with fluorescently labeled antibodies in a small area to allow for improved tagging.[36] After a six hour enrichment the limit of detection falls to 3 and a half CFU/mL with only 520 nL of reagents used.[36] An optofluidic imaging system uses refractive indices and morphologies based on

immersion refractometry to uniquely identify different bacteria. Distinctive signals were created for *E. coli*, *Shigella flexneri* and *Vibrio cholera*, this type of detection has potential to be integrated into real time data collection.[37]

Besides antibodies and phages, another common biological recognition element is aptamers, which are oligonucleotides (single stranded RNA or DNA) that bind with high levels of specificity. *Lactobacillus acidophilus* was detected using an aptamer based porous silicon biosensor. The bacteria were captured on oxidized Psi Fabry-Perot thin films and changes in the reflectivity spectrum allowed for live/dead and direct capture measurements to a limit of detection of 106 cells/mL. [38] A new formulation of available enzymatic substrates in a hydrogel has been developed to allow for rapid detection of *E. coli* down to 400 CFU/mL within one hour using a very simple plunger-tube assembly and a smart phone application. The enzymatic substrates are suspended in the hydrogel and only react when *E. coli* enter the same area and produce a color change. [39] Other microfluidic devices have been developed concentration of cells for later analysis by surface enhanced Raman spectroscopy (SERS) or other methods. The device concentrates one hundred μL of sample to five hundred nL droplets in fifteen minutes. Initial tests showed one hundred times signal enhancement for SERS tests using *Staphylococcus aureus*.[40]

Surface Plasmon Resonance is a popular method for non-destructive real-time monitoring. Subramanian et. al. were able to detect *E. coli* O157:H7 using a sandwich assay with a limit of detection of 10^3 CFU/mL using antibodies as the biological recognition element.[41] As with other sensors phages have increased in popularity as

recognition elements in optical sensors. A *Salmonella* bacteriophage was used in a SPR based biosensor with low cross-reactivity with limits of detection of 10^7 CFU/mL.[42]

2.2.4.2 Electrochemical

The three most common types of electrochemical biosensors are based on voltammetry, amperometry, and electrochemical impedance spectroscopy. Xu et al. have reported a device capable of detecting 10^2 CFU/mL of *E. coli* in ten minutes based on a bifunctional polydopamine-polymeric nanocomposite. Streptavidin coated microbeads are coated with glucose oxidase by a streptavidin-biotin interaction, then the beads are coated with dopamine before gold nanoparticles are reacted on the surface of the bead. Antibodies and external glucose oxidase are bound to this surface to bind target cells and to block non-specific binding. The beads are mixed with cells, filtered, and analyzed by cyclic voltammetry. [43]

Electrochemical sensors can use as many detection elements as are available, Nikkhoo et al. used a T6 phage specific to *E. coli* as the biological detection agent. When a phage injects its DNA into a cell there is an efflux of ions from inside of the cell to the outside, mostly potassium. A sensor was built to take the transient flux of potassium ions and convert it to an electrical signal that can be measured. With this method, a positive/negative result could be obtained in thirty minutes.[44]

2.2.4.3 Mass Based

Mass based sensors utilize some sort of sensitive binding element that is functionalized and some way to measure how additional mass loading changes the properties of the binding element. Cheng et al. developed a magnetostrictive resonator

capable of using antibodies or bacteriophage as the bioprobe. These biosensors were placed directly on the surface of lettuce and tomatoes, and it was found that the limit of detection is approximately fifty to one hundred CFU/mL for *E. coli*.[45, 46] Magnetostrictive mill/micro-cantilevers have also been developed to detect *Bacillus anthracis* spores in water. These are similarly made with phages adsorbed onto the surface and the resonance change in a magnetic field is a result of additional mass loading onto the cantilevers.

Fu et al. found that the smaller the cantilever, the lower the detection went with a cantilever with dimensions 1.4mm x 0.8mm x 35 μm having a detection limit of 104 spores/mL.[47, 48] Using a magnetoelastic biosensor functionalized with phage and an alternating magnetic current, detection limits were lowered to $5x10^3$ CFU/mL for *E. coli*.[49]

This technology promises single cell detection and high specificity, Poshtiban et al. reported a device that could detect an additional mass of 52 fg, lighter than a single cell, and confirmed the device was specific to *Campylobacter jejuni* cells due to functionalization with phage.[50, 51] Mass based sensors continue to develop rapidly, Wang et al. created an array of micro cantilevers capable of distinguishing among eight *Salmonella* serovars using phage-derived peptides for *Salmonella* showing that multiplexing these cantilevers can be done easily.[52]

2.2.5 Review Papers for Further Reading

There is such a wide range of approaches to detect pathogenic bacteria that only a small sampling has been examined here. For further background, several review

papers from different fields have been published recently. For a general perspective on recent developments, see references 53 and 54.[53, 54] For a foodborne perspective, see references 11 and 20.[11, 20] For a water based perspective see references 55 and 56[55, 56], and for a primer on immunological methods see reference 57.[57]

2.3 Theory of Local pH Modulation for Detection

Fluorescence microscopy has used fluorescent dyes to image all kinds of cells and tissues. The earlier generation of dyes has been replaced with more photostable dyes as well. One of the difficulties with using a very common dye fluorescein is its rapid photo-bleaching and pH sensitivity. Fluorescein does have the advantage of being among the least expensive dyes and continues to see use for this reason. While pH sensitivity is often seen as a weakness in the applications of imaging, such characteristics can be utilized as a transducer in a biosensor.

Jung et al. developed a microfluidic device coated with a lipid bilayer. The bilayer contained a pH sensitive dye (ortho-Texas Red) and anti-biotin.[58] Using this device, it was possible to detect the binding of biotin to anti-biotin because the negatively charged protein recruited hydrogen atoms to the surface of the bilayer resulting in a change in the local interfacial pH. The dye used (o-Texas Red) fluoresces strongly when it is protonated. When the recruited hydrogen atoms protonated the dye an increase in fluorescence signal was detected.[58] The same group has demonstrated similar binding events using different dyes in the same platform.[59-61]

Bacterial cells are generally accepted to be negatively charged, although this can change depending on the environment surrounding the cells.[62] Assuming the cells are

negatively charged, it is expected that as they bind to pH sensitive fluorescently tagged antibodies there will be a change in the monitored fluorescence. In the case of fluorescein, a decrease in pH will result in a decrease in fluorescence making a sensor that turns off as the detected bacteria increases in concentration. As metabolically active bacteria can produce acids upon uptaking substrates, the local pH is expected to be dropped due to the produced acid and thus leading to further decrease in fluorescence.

This method could be used to differentiate live from dead cells during the detection. This binding event is created using antibodies in this work, but any biological recognition element that can have a pH sensitive dye integrated could be used such as phages or aptamers. One of the advantages of this method is its simplicity after the initial set up, all that needs to be done is add the sample, incubate, wash and measure. It also has the advantage of being label free, fast, adaptable to many sensor platforms, and requires no custom built or expensive instrumentation.

3. DETECTION OF LIVE *E.COLI* BY LOCAL pH MODULATION

3.1 Introduction

The need to rapidly detect pathogens is important to protect people from food or waterborne illness. The method proposed would reduce the time required in order to make the determination of pathogen presence. This method proposes to answer two questions, first, is there a specific pathogen present, and second, is that pathogen alive.

3.2 Materials and Methods

3.2.1 Chemicals

The following supplies were obtained from Thermo Scientific, 96 well untreated black 'NUNC' microtiter plates (#437112), NHS-fluorescein (#46410), and two *E. coli* (O+K) antibodies, biotin labeled antibody (PA1-73035) and unlabeled antibody (PA1-73032).

Streptavidin coated magnetic beads "Dynabeads" were purchased from Invitrogen (#65305). Neodymium magnets were purchased from CMS magnetics (NB035-45NM). A 15 W, 254 nm UV light was purchased from GE (#G15T8). Microcentrifuge tubes were purchased from Neptune (#3765.X), R2A was purchased from Teknova (#R005), and gas permeable membranes for titer plates were purchased from USA Scientific (9123-6100). From Sigma-Aldrich BSA was purchased as a lyophilized powder (232-936-2), also obtained from Sigma-Aldrich was glucose (G-5400) and DMSO (#D5879-500ML).

3.2.2 Bacterial Strains and Growth Conditions

E. coli K12 was purchased from American Type Culture Collection (ATCC #10798) and was grown in R2A media at 37° C and 150 rpm until reaching an OD_{600} of 0.6-0.7. The cells were then harvested by centrifuging for 5 minutes at 10,000xg and washed twice with 0.1 M pH 7.3 phosphate buffer. The pellet was then resuspended in 25mM saline water. *P. butanovora* provided by Dr. Daniel J. Arp, Oregon State University and prepared in the same way as *E. coli*.

3.2.3 Antibody-Dye Complex Preparation

One mg of NHS-fluorescein was dissolved in one hundred μL of DMSO immediately before use to prevent loss of activity. The NHS moiety reacts with primary amino groups (NH_2) on the antibody. The antibody is an *E. coli* polyclonal antibody. After receipt and prior to any use the antibody (four - five mg/mL) is aliquoted in twenty five μL aliquots and kept at - 20° C. For every twenty five μL of antibody to be labeled one μL of NHS-fluorescein solution is added to the reaction mixture. For ideal labeling to occur the pH of the reaction mixture should be ~eight and amine containing buffers should be avoided. A pH eight borate buffered saline solution (BBS) was used as the buffer in the reaction mixture. The reaction mixture is made up of five hundred μL of (BBS), twenty five μL of four to five mg/mL antibody and one μL of NHS-fluorescein solution. All components of the reaction solution are added to a two mL microcentrifuge tube and placed on a rotator for one hour at room temperature. The results of this process can be seen in Figure 1.

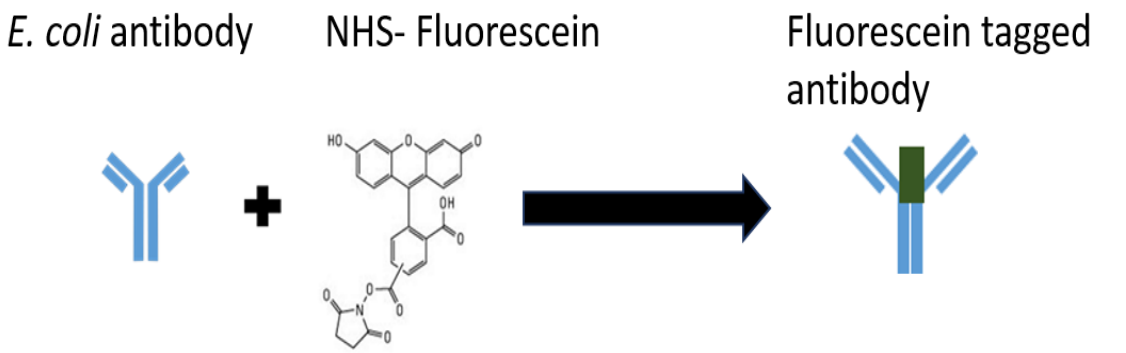

Figure 1. Schematic of tagging antibody with fluorescent dye. Fluorescein a green dye is represented in the "ON" state as dark green and in the "OFF" state with light green

3.2.4 Microtiter Plate Preparation

A "Nunc Polysorb" black flat bottomed ninety six well microtiter plate is first sterilized by exposure to UV light for thirty minutes.[63] One hundred µL of the antibody-dye complex is added to each well that is to be functionalized by hydrophobic interactions. This results in approximately 5 µg of antibody per well. The plate is sealed with a gas permeable membrane and incubated at 4° C for eighteen to twenty two hours. The wells are then washed with PBS three times and tapped dry between each wash. To reduce nonspecific binding the plate is blocked using 100 µl of 1% BSA per well for 30 minutes. The plate is then washed with PBS three times and stored at 4° C until ready for use.

3.2.5 Magnetic Bead Preparation

Streptavidin coated magnetic beads (Dynabeads) are used for both immunomagnetic separation and as a detection tool. One hundred µL of beads (per 25 µL antibody) is washed in pH 7.4 PBS three times and then suspended in five hundred µL of PBS. After the dye-antibody reaction is complete, the reaction mixture is added

directly to the bead solution and placed on a rotator for thirty minutes at room temperature. The beads are then washed four times in PBS with 0.1 percent BSA before resuspension in 1 mL PBS. An example of the final product can be seen in Figure 2.

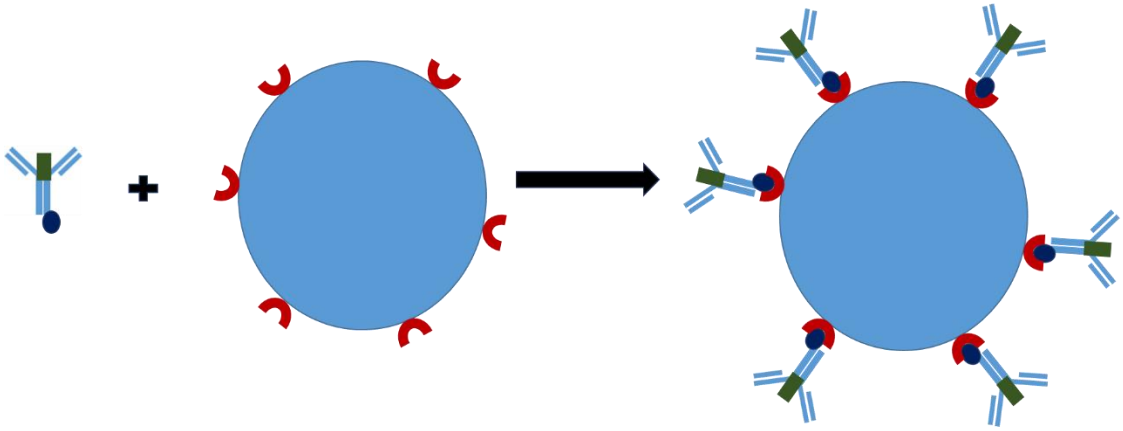

Figure 2. Attachment of biotin tagged antibodies to a streptavidin coated magnetic bead

3.2.6 Live/Dead Cell Preparation

Live cell counts were determined using OD_{600} to approximate the number of bacteria. In order to use this method all cells were freshly harvested from exponential growth phase cultures. 10x dilutions were used to create the dilution series. UV killed cells are prepared by exposing the cells in a shallow dish to a lethal dose of UV radiation confirmed by plating (see appendix).

3.2.7 Analysis by Genios Plate Reader

A Genios Tecan plate reader (F129021) is equipped with band gap filters for fluorescein (excitation 485 nm, 20 nm band gap ; emission 535, 25 nm band gap) and can read microtiter plates. The instrument is controlled by software (Xflour4) provided by the manufacturer and installed on a computer with a windows operating environment.

The precision, cross-talk, and linearity performance of the plate reader was confirmed using the procedures recommended in the user's manual. A new image of the specific plate being used was created to ensure accuracy according to the procedure in the manual. The settings used for analysis were a gain of one hundred, and twenty flashes, three independent measurements were made of three replicates. The reported results are averages and error bars are standard deviations.

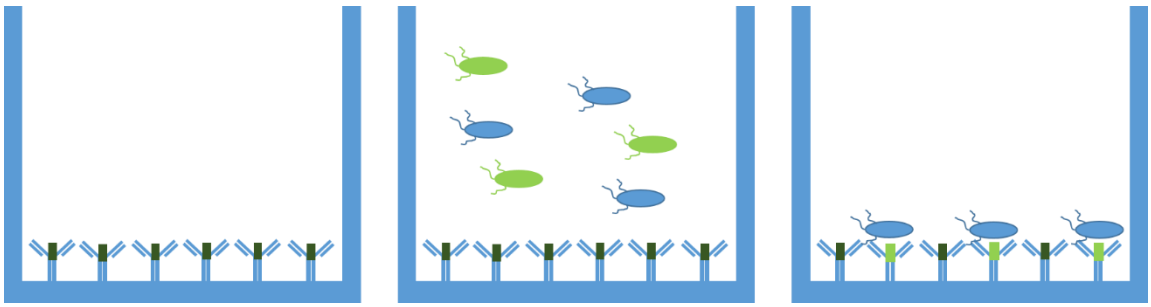

Figure 3. Pictorial representation of the plate analysis procedure

The plates prepared have 100 µL of cell suspension incubated at room for one hour. The plate is then washed three times with 5 mM PBS before 100 µL of 25 mM NaCl pH 7.3 saline water is added. The plate is then incubated for an additional 30 minutes at room temperature before measurements are taken. A visual representation of the expected results of this procedure can be seen in Figure 3.

Beads prepared above can be added in different amounts to varying amounts of cell suspensions or samples. In this experiment 30 µL of bead solution was added to 100 µL of cell suspension in a microcentrifuge tube. The tube was rotated/shaken for 30-40 minutes before being placed on a magnet for five minutes in order to concentrate the

beads. The liquid is then removed from the tube by pipet and 100 µL of pH 7.3 25 mM NaCl saline water is added to the tube. The beads are resuspended by pipetting up and down gently several times before 100 µL is added to a well in an untreated microtiter plate and analyzed.

3.3 Results and Discussion

3.3.1 Detection of Live E.coli by microtiter plates

Using the microtiter plate it is possible to detect *E. coli* in the range of 10^5 - 10^8 CFU/mL. This number is similar to other ELISA and immunobased methods, but has the advantage of using fewer expensive antibodies and has the ability to be more easily miniaturized to a portable device for point of care or field diagnostics.

As seen in Figure 4 there is approximately a 15 percent decrease in relative fluorescence for wells containing 10^7 *E. coli* cells. A noticeable decrease of 8 and 5 percent is also observed for concentrations of 10^6 and 10^5 respectively.

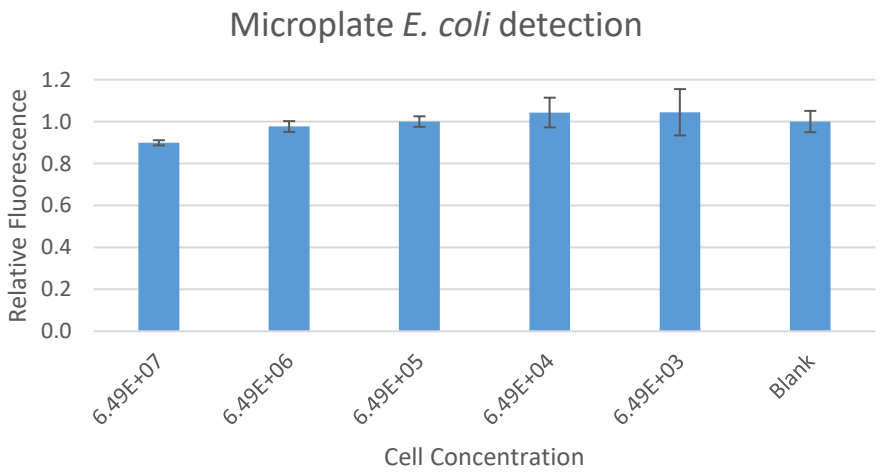

Figure 4. Fluorescence response of microtiter plate to different concentration of *E. coli*

At concentrations below this (10^4, 10^3) the range of measurements becomes very large making it difficult to say with much confidence how fluorescence is impacted. Repetitions of this experiment showed similar results as can be seen in Figure 5.

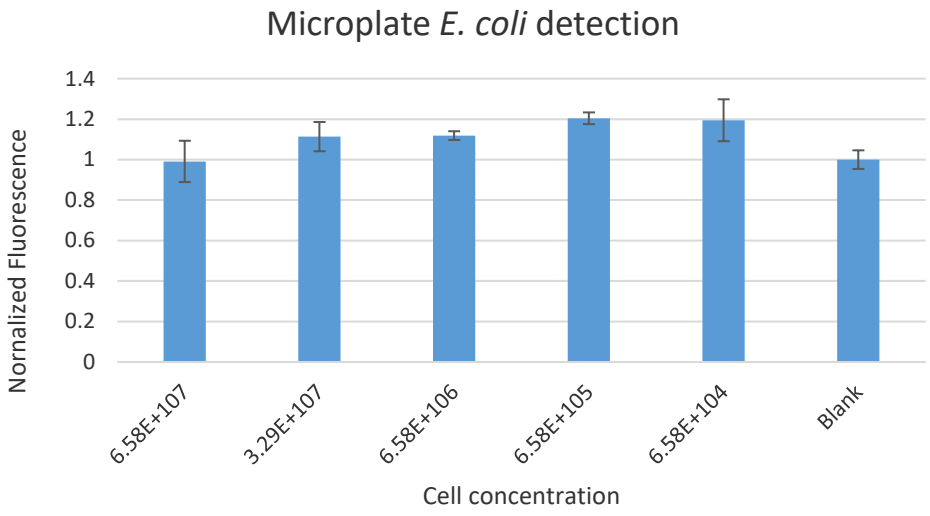

Figure 5. Repeated experiment for microplate showing similar results as before but with larger standard deviations

There is was significant amount of variation between replicates, which resulted in large standard deviations. Using microplates under these conditions allows for a small but quantifiable difference in measured fluorescence in the range of 10^6 cells. Statistical analysis (paired mean t-test) however showed that the difference between the highest concentration of cells and the blank could not be statistically significantly identified using an alpha of 0.05 (figure 4, $p = 0.06$, figure 5, $p = 0.25$). In these experiments, that involves 100 µL of a cell suspension with a concentration of 10^7 CFU/mL. For the purposes of analyzing unknown samples some sort of pre enrichment step and a method

for concentration would be employed to bring the numbers up and concentrated in a smaller volume suitable for testing.

As a control the same procedures were followed using the same *E. coli* antibody, but using a different bacteria. The control strain chosen was *Pseudomonas butanovora*. The expected results for the control strain were that the fluorescence would be flat across the different concentrations since the cells are expected to all be washed out during the washing phase.

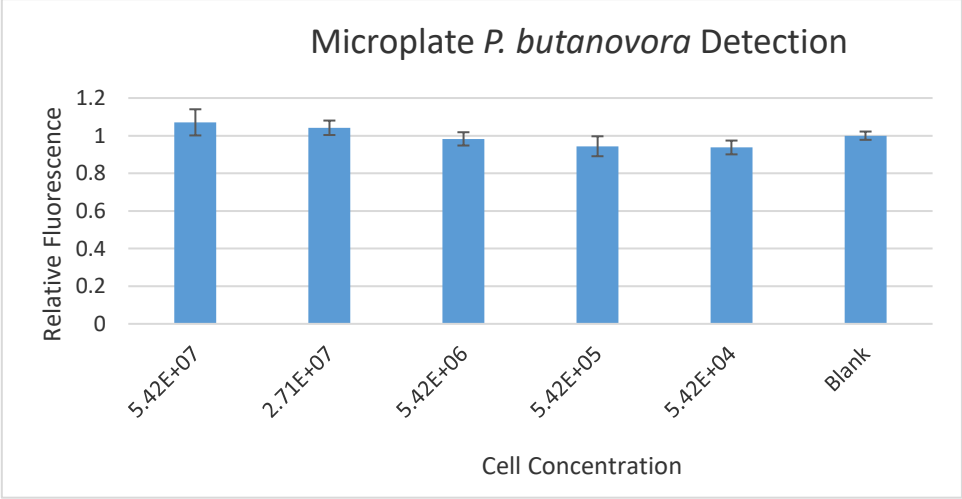

Figure 6. Fluorescence response of microtiter plate to different concentrations of *P. butanovora*

However, as seen in Figure 6 there is a clear downward sloping trend as the concentration of cells that were applied decreases. There are a few possible explanations for this; it is possible that the wells needed to be washed more vigorously to dislodge cells that may have become attached to the walls of the plate through hydrophobic or electrostatic forces. However, it should be noted that the differences observed were not

statistically significant at the alpha = 0.05 level. It is unlikely that the cells became attached to the antibodies and did not impact the fluorescence of the dye molecules attached to antibodies. It was noticed during preliminary experiments that pre washing wells with high concentrations of cells had higher recorded fluorescence values. After washing this trend vanished when analyzing *E. coli* but has stayed with *P. butanovora*, indicating that it may be possible that there is some mechanism that is resulting in fluorescence unrelated to the dye molecules. To test this antibody with no dye was placed into wells and fluorescence was measured for *E. coli* post washing.

The results are shown in Figure 7 and indicate that there is a mechanism that is unrelated to the dye molecules the effects the fluorescence values read by the plate reader. For the highest concentration of cells, there was a 5 percent increase in fluorescence reading compared to the background, while for all other there was an increasing decrease in observed fluorescence compared to the background. While this mechanism is currently unclear, it clearly plays a role in the analysis of this data. The effect of this mechanism is opposite behavior compared to what is expected to happen to fluorescence upon the binding of *E.coli* to antibodies. As a result, this can decrease the observed results. However if an alternate dye was used that turned on at lower pH's then this effect would increase the observed signal and perhaps increase the sensitivity.

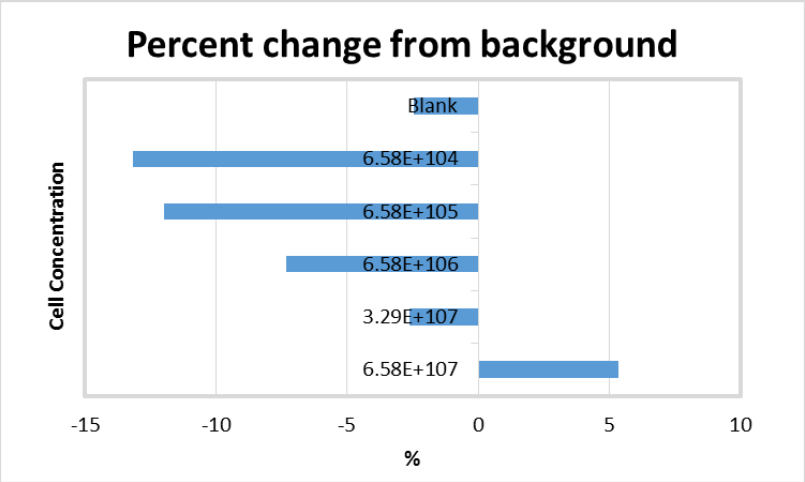

Figure 7. Percent change from the background because of cells attached to antibodies with no dye molecules

To test the ability to identify live vs dead cells the same plates used to test presence are used. After presence testing is complete, 50 µL of glucose (1g/L final concentration) is added to each well and the fluorescence response is monitored over time. There is a difference in how UV killed cells responded to glucose. The addition of glucose itself reduced the pH and fluorescence in each well, but to a greater degree in the live cell cases regardless of cell concentration.

The degree to which the well fluorescence decreased did not have a correlation to the actual concentration of cells but rather was an indicator that the cells were alive. In Figure 8, it is shown that the blank well response is the largest in the UV killed case and the smallest in the living cell case. The numbers can be more clearly seen in Table A1 in the appendix. The difference is small but holds for all cell concentrations, suggesting that the living cells are capable of utilizing the glucose to produce more acidic compounds and lowering the bulk pH of those wells. The time frame in which the

response is seen is rapid and can be seen in the first ten minutes; increasing times simply confirm the trend holds. Interestingly for the UV killed cells there was a statistically significant difference between the means of the highest (10^7) number of cells and the blank ($p = 0.05$) at the alpha = 0.05 level. This was not the case for the live cells

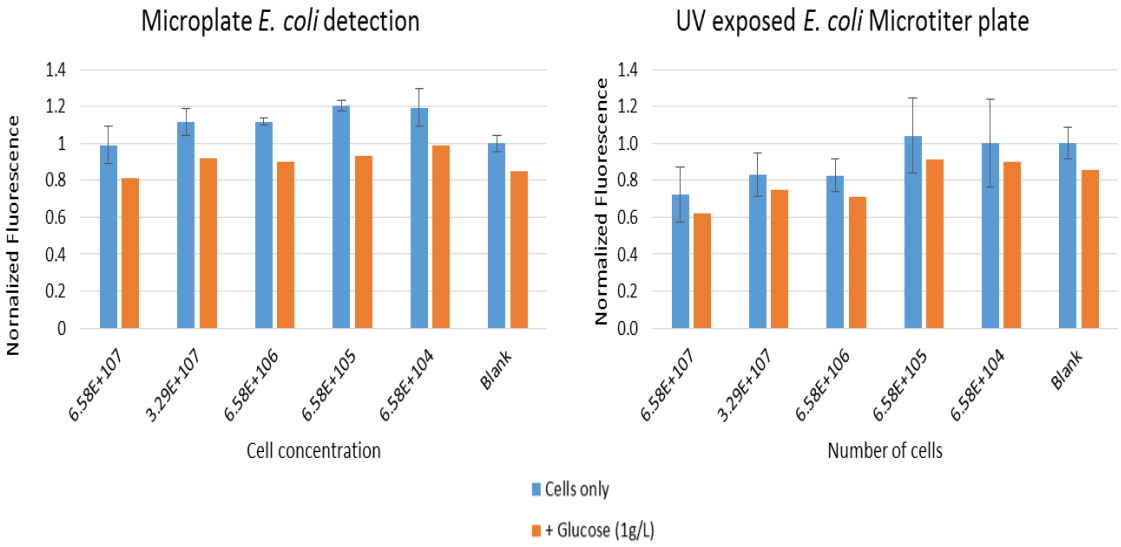

Figure 8. Fluorescence response to the addition of glucose of live cells and UV exposed cells (*E. coli*)

One option to increase the sensitivity of the plate method would be to use plates that have wells pre-coated in streptavidin or protein A and use biotin labeled antibodies (or non-labeled antibodies with protein A). Doing so would change the functionalization of the plates from depending on hydrophobic interactions between the polystyrene antibodies to a protein-antigen binding event, which is stronger and would allow correct orientation of the antibody so that the binding availability is consistent as shown in Figure 9. Using this method would remove a potential source randomness among wells.

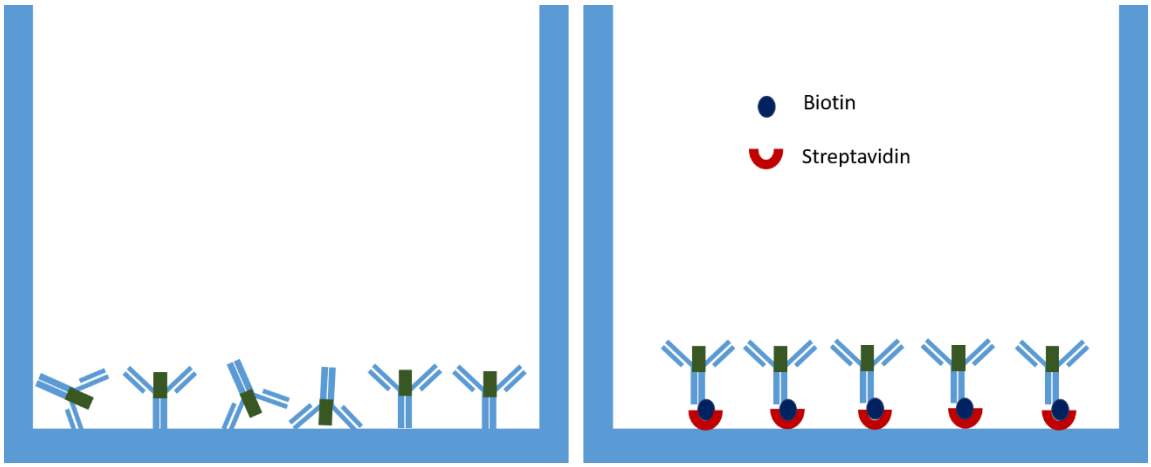

Figure 9. Schematic representing how using streptavidin (or protein A) coated plates could allow for a higher percentage of antibodies having the desired positioning

3.3.2 Detection of Live E. coli by magnetic beads

Using magnetic beads for separation and concentration is such a common technique there are instruments capable of automating the process. Using this common method to not only selectively capture bacteria but also to enumerate them through pH-modulated fluorescence is a new development. The basic principles of using magnetic beads and microtiter plates are the same. One of the potential benefits of the magnetic beads are that there is a preexisting pipeline for the purpose of isolating bacteria with the beads by attaching antibodies making it easy to adapt the procedure. Another advantage is that the number and concentration of beads needed can easily be scaled up or down depending on the needs of a particular analysis. However, on the downside the bead method is highly sensitive to errors in pipetting, both in the initial application and in removing the solution after magnetic concentration. This major downside can be

mitigated by automated systems that currently exist for isolating all kinds of biological items of interest.

The experiments done to support this method show that the current challenges may outweigh any advantages this method has over microtiter plates. For detecting *E. coli* there was only the slightest decrease in fluorescence because of bacteria binding at the highest concentration of 10^7. The decrease is so slight and unpredictable that it is unlikely to be presented with any confidence as seen in Figure 10a. The possible explanations for this outcome include errors in pipetting, insufficient dye binding, insufficient mixing to ensure beads and bacteria interact, and clumping of beads that results in no binding of bacteria. The simplest solution to overcoming these challenges is to automate some of the processes susceptible to human error, such as pipetting.

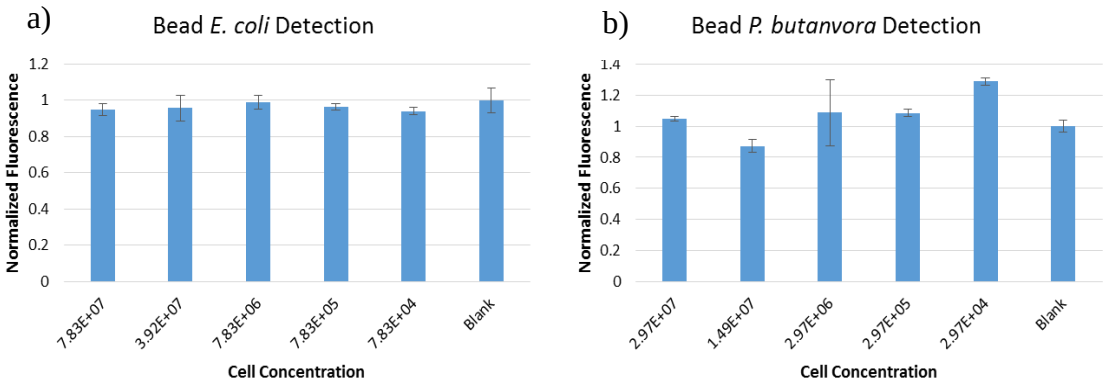

Figure 10. Fluorescence responses of a) *E. coli* and b) *P. butanovora* as they are subjected to the magnetic bead procedure described in section 3.2.5

For the magnetic beads, the results expected for the control strain *P. butanovora* were the same as expected for the microtiter plate, that there would be a relatively flat response across the different concentrations. However, this was not the case, in fact

there were 20 percent differences between samples while the *E. coli* beads saw only a 5 percent difference. The low reading on the blank and the large standard deviations indicate that there may have been some problems with the experiment controls that resulted in these results. It is also possible this is the result of pipetting or human error or even beads becoming stuck on the side of the tube. To really understand what is happening with the bead experiments additional control experiments should be devised and ideally some sort of automation should take place of manual pipetting.

The results of the tests for *E. coli* and *P. butanovora* were inconclusive as to if the method was working as designed. In the same way the results of the UV exposed cells and the living cells response to glucose was also inconclusive. Figure 11 shows the results of the glucose addition on fluorescence observed over 36 minutes.

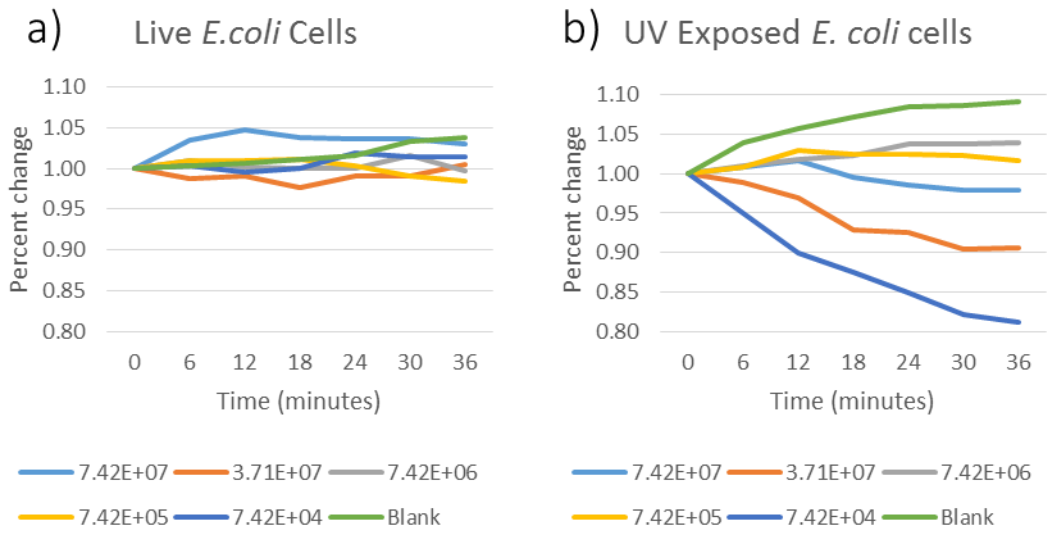

Figure 11. Percentage fluorescence response after the addition of glucose to beads expected to have live *E. coli* and UV exposed *E. coli*

There is little change in the live cells in terms of total fluorescence, the only samples that have any noticeable change is the highest concentration of cells and the blank and those changes were an increase in fluorescence, not a decrease as would be expected if acids were being produced by glucose metabolism. The UV killed cells on the other hand show no discernable pattern in the changes in fluorescence. With half rising and half falling. There is little to take away from the bead experiments other than they are more difficult to execute and may have a lower sensitivity unless large numbers of beads are used.

4. SUMMARY, CONCLUSIONS, AND FUTURE STUDIES

4.1 Summary and Conclusions

This is the first attempt to quantitatively measure bacteria using local pH modulation and the project met with some success. There was an approximately 20 percent decrease in fluorescence when using microtiter plates as the testing medium at a bacteria number in the range of 10^7. There was a measurably decrease in fluorescence for bacteria in the range of 10^5-10^6, which is similar to the limits of detection for ELISA measurements for *E. coli*. However these measurements were not statistically significant at the alpha = 0.05 level, meaning that if the observed effect is possibly due to random error and further testing must be done.

The speed at which the plate measurements can be completed is approximately 2 hours, much faster than culture based methods and even PCR based methods. Biosensors continue to develop better capabilities and pathogen sensing will continue to be a priority for new devices and approaches.

Using magnetic beads as a platform for both separation of target bacteria and a sensor to this point has not been proven as a viable platform for pH modulation. With the introduction of automation it is hopeful that the use of the beads will become more consistent. Further experiments are needed to confirm the issues effecting the limited fluorescence changes detected when the beads were mixed with *E. coli*.

The results presented support the hypothesis proposed with some caveats for future studies to examine some of the unexpected results.

4.2 Future Studies

One of the goals of this work was to reduce the cost of rapid testing. Two major strategies can be employed in order to significantly decrease the cost. The first strategy would be to replace antibodies as the recognition element. Antibodies are expensive and delicate requiring minimal time to elapse between preparation and use. In addition, antibodies do not last long under non-ideal conditions. To combat these issues alternate recognition elements should be explored, some of which are already used in the literature. The most prominent replacements are aptamers and phages (whole or tail fibers). Both are just as or more sensitive and can be mass-produced cheaper than antibodies. Phages especially have the advantage of being especially hardy under non-ideal conditions.[64]

The second major strategy that should be focused on to reduce cost is the miniaturization of the device. Using a microfluidic platform would decrease the required reagents to nL scale. An additional advantage of microfluidics is the surface phenomenon being utilized would be focused to a higher degree and would likely increase sensitivity.

Sandwich ELISA has already been done inside a microfluidic utilizing the same chemistry that would be used to set up this device, showing that the execution of such a device is a current possibility.[65] An additional method that could reduce the errors associated with instantaneous fluorescence readings and difficulties in pipetting beads accurately is to use time resolved fluorescence. Fluorescein has a different lifetime depending on if it is protonated or not (3 ns vs 4 ns) which would allow for a ratio to be

developed as opposed to instant measurements. Using this time resolved technique would be more accurate and remove some of the issues related to human error. This technique does require a more advanced set up than a simple plate reader, but as instruments become more and more powerful a nanosecond difference is detectable by more affordable instruments.

The advantages of fluorescein lie in its low price and ubiquitous filter sets. Additional experiments to explore a dye that turns on at lower pH values would be advisable and more intuitive and potentially easier to analyze.

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APPENDIX

Additional Figures and preliminary experiments will be expanded on in this appendix. To determine at what pH the initial solution should be set a curve relating pH to fluorescence was created for the exact conditions that existed in the microtiter plates. AS the dye was attached to the antibody and the antibody to the well the curve referenced in the materials included with fluorescein could not be relied on to be accurate. Figure A1 shows the titration curve generated by and why a pH of 7.3 was chosen.

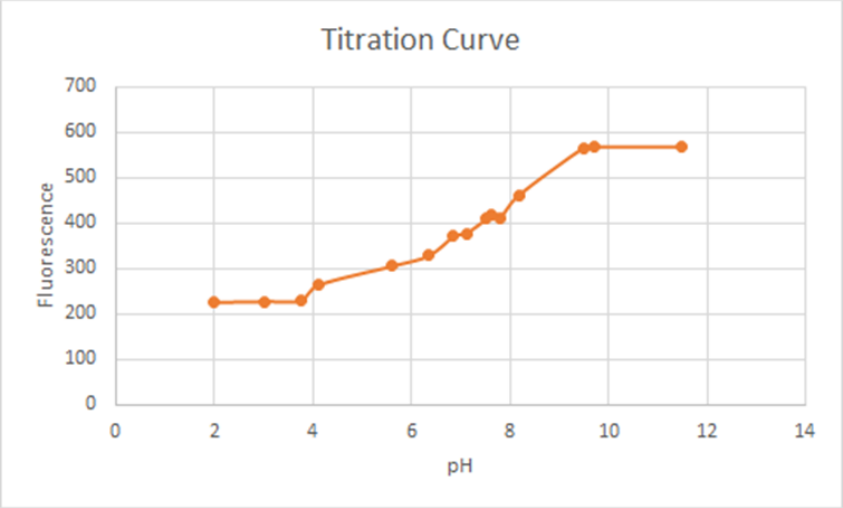

Figure A1. Titration curve for conditions

The salt concentration of the initial solution also impacts the titration curve. Solutions with low ionic strength have a lower apparent pKa as shown in figure A2 and A3.

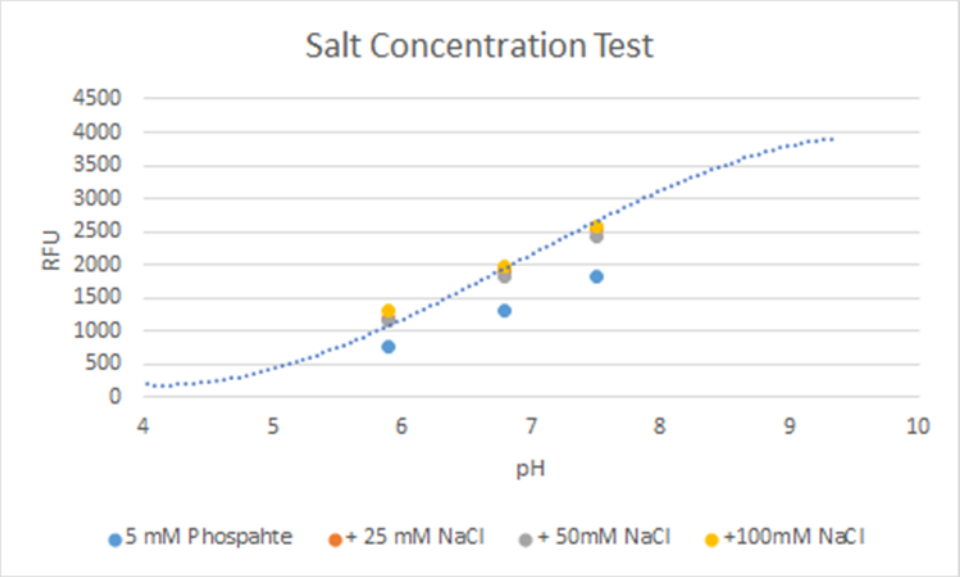

Figure A2. Related fluorescence for solutions of the same pH but different ionic strengths

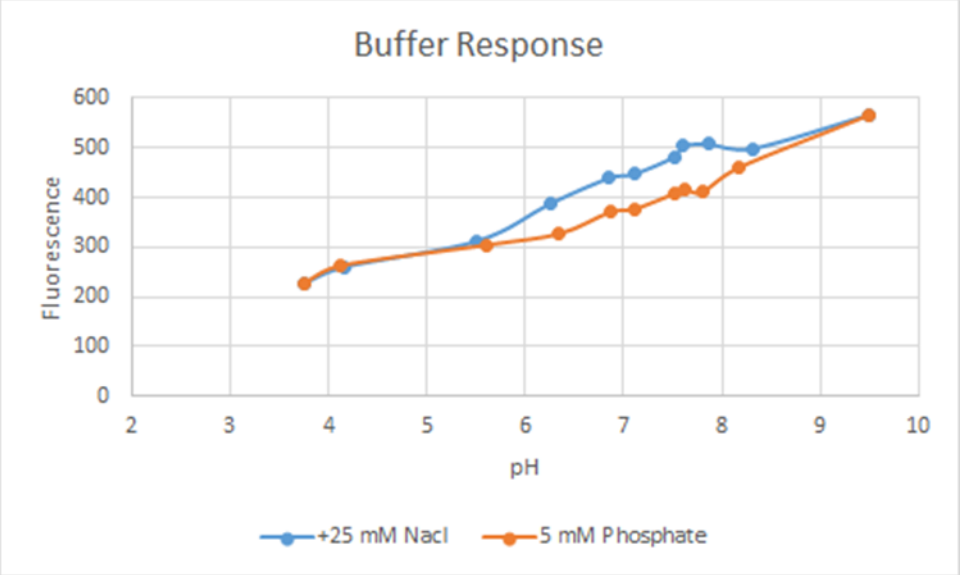

Figure A3. Buffer response over a wide range for two different ionic strength solutions. The solution with an additional 25 mM NaCl has a higher apparent fluorescence at the same pH

To ensure UV exposed cells were truly killed under the experimental conditions a 10 µL aliquot was plated and allowed to incubate overnight at 30° C.

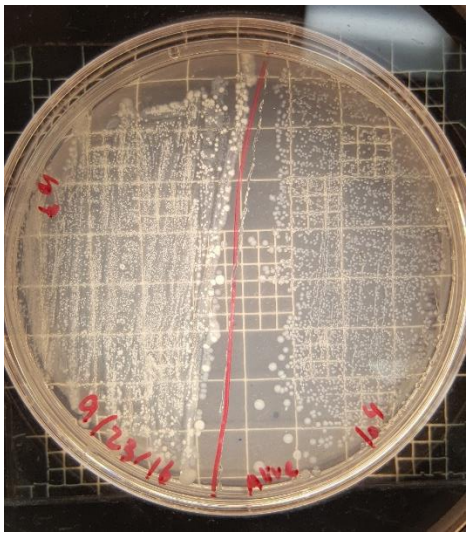

Figure A4. Live cells show clear growth at concentrations 10^4 cells/mL and 10^7 cells/mL

The first attempt to kill the cells by UV exposure was insufficient at high cell concentrations at both 30 minutes and 1 hour at a distance of 18.5 inches from the 15 W 285 nm lamp. As can be seen in Figure A5.

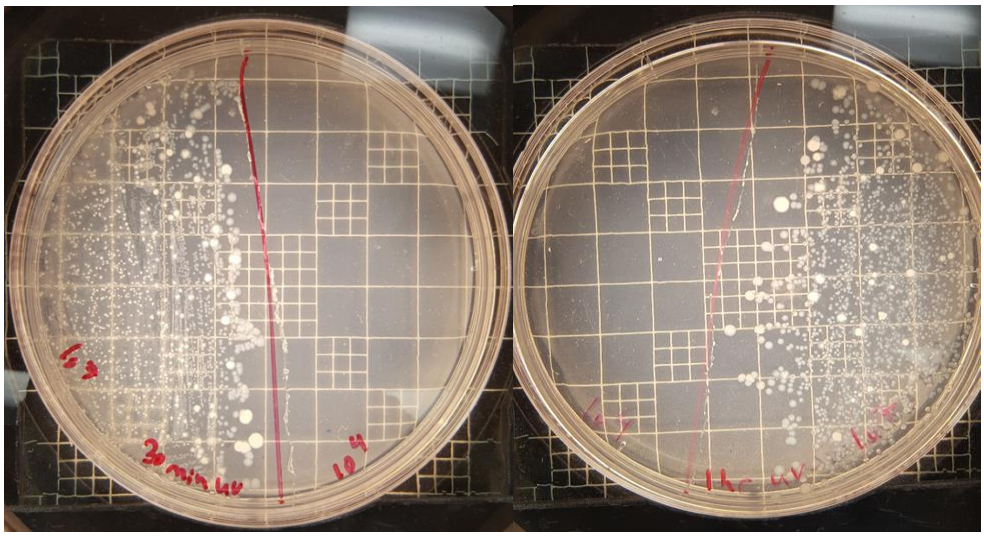

Figure A5. Left: cells were exposed to UV light for 30 minutes which was sufficient to kill the low concentration but not the high at a height of 18.5 inches Right: The same result is seen for 1 hour exposure time.

When the distance was decreased to 7.5 inches all cell concentrations were killed at both 30 minutes and 1 hour. These were the conditions utilized in all experiments for live/dead cell testing.

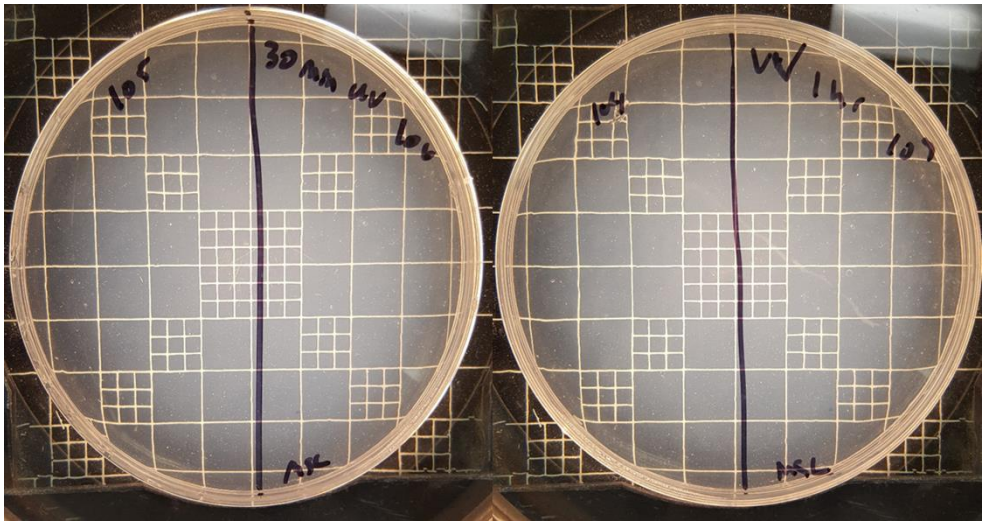

Figure A6. Left: cells exposed to UV light for 30 minutes at 2 concentrations Right: cells exposed to UV light for 1 hour at 2 different concentrations.

Table A1. Percent change from time 0-30 minutes and 0-60 minutes for cells in microtiter plates.

E. coli	%0-30	% 0-60		UV	%0-30	% 0-60
6.6E+107	-18.4	-20.2		6.6E+107	-14.4	-15.4
3.3E+107	-17.5	-19.4		3.3E+107	-9.7	-11.6
6.6E+106	-19.7	-21.5		6.6E+106	-13.9	-16.5
6.6E+105	-22.7	-22.5		6.6E+105	-12.5	-13.5
6.6E+104	-17.4	-18.8		6.6E+104	-10.1	-11.5
Blank	-14.9	-15.6		Blank	-14.5	-16.5