TOWARDS THE DEVELOPMENT OF AN AUTOMATED, PORTABLE *ENTEROCOCCUS FAECALIS* BIOSENSOR FOR RECREATIONAL WATERS

by

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ABSTRACT

The quality of recreational waters is of great importance to the public. *Enterococcus faecalis* is one of the main indicators of recreational water quality. The standardized technology for quantifying bacterial concentrations require the transportation of water samples to a lab and measurement of the test by trained personnel. These actions delay the publishing of results from water quality tests. Thus, a portable, automated, and *in-situ* water quality solution can yield advantages towards the public welfare. In this project, we design and build a novel biosensor that is portable and sensitive to various bacterial concentrations. We use rapid prototyping techniques to build and automate an enzyme-based water quality assay using a millifluidic biosensor. We use a Raspberry Pi micro-computer, a CMOS image sensor, an Arduino micro-controller and Python programing for control and data acquisition. Fluorescence from the enzyme-based assay is indicative of bacterial growth and is measured over a 24-hour period. Our sensor has detected a bacterial concentration as low as 23 Colony Forming Units (CFU) per 100 milliliters. The CMOS camera and RGB analysis is used to measure the increase of fluorescence from the assay as a function of time. Blue light is indicative of fluorescence from the assay and of a positive contamination result.

RESUMEN

La calidad de las aguas recreativas es de gran importancia para el público. *Enterococcus faecalis* es uno de los principales indicadores de la calidad del agua. La tecnología estandarizada para cuantificar las concentraciones bacterianas requiere la transportación de muestras de agua a un laboratorio y la medida del ensayo por personal entrenado. Estas acciones retrasan la publicación de resultados de pruebas de calidad del agua. Por lo tanto, una solución para cuantificar la calidad de agua que sea portátil, automatizada e *in situ* puede ofrecer ventajas para el bienestar del público. En este proyecto, diseñamos y construimos un novedoso biosensor que es portátil y sensitivo a diversas concentraciones bacterianas. Utilizamos técnicas de prototipado rápido para construir y automatizar un ensayo de calidad del agua basado en enzimas usando un biosensor milifluídico. Utilizamos una microcomputadora Raspberry Pi, un sensor de imágenes CMOS (CMOS por sus siglas en inglés), un microcontrolador Arduino y la programación de Python para control y adquisición de datos. La fluorescencia del ensayo basado en enzimas es un indicativo de crecimiento bacteriano y se mide durante un período de 24 horas. Nuestro sensor ha detectado concentraciones de bacterias en muestras de agua con una sensibilidad tan baja como 23 unidades formadoras de colonias (CFU por sus siglas en inglés) por cada 100 mililitros. La cámara CMOS y el análisis RGB (RGB por sus siglas en inglés) fue utilizado para medir el incremento de fluorescencia del ensayo en función del tiempo. Luz azul es indicativo de fluorescencia de los experimentos con lecturas positivas de resultados con contaminación.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1 – INTRODUCTION

1.1 Motivation

Clean water is quickly becoming a scarce natural resource given the rapid increase in human population and the high amount of clean water required by industry. Clean water is important for human consumption and for recreational coastal activities. Thus, effective supervision of water quality has become vital for water resource management and improvement [1]. Water quality for drinking and recreation is related to public welfare. Therefore, accurate, efficient facilities for water quality testing is of great importance.

Water quality regulation is enacted by U.S. Environmental Protection Agency (EPA), an independent institution from US federal government. Its main goal is to protect humans and the environment by enacting and enforcing the national standard and method based on US environmental laws. All water quality measurements need to be performed using methods approved by the EPA. One of the official standards for Water Quality is Method 1600 issued in 2012. This method involves *E. coli* and *enterococci* as reliable indicators related to swimming-associated gastrointestinal illness and provides the criteria recommendations for bacteria [2]. The bacterial level for recreational waters can be measured by the concentration of the *enterococci* [3]. The positive test result is calibrated by using the Most Probable Number (MPN) method. The MPN method applies probability and statistics to estimate the amount of bacteria present in a large volume by separating this large volume into smaller independent volumes and counting the presence or absence of contamination in these smaller volumes [4].

The concentration of *Enterococci* in recreational water samples has positive correlation with gastroenteritis relating to swimming in marine and fresh water bathing beaches [3]. To detect the concentration of *Enterococci* bacteria, the operation usually requires a trained professional to transfer sample of water from the original location to a testing facility, then mix the sample with reagents, put inside an incubation chamber for a complete incubation cycle (24-48 hours), and then measure the fluorescent result under a

UV light. These tests require much hands-on work. In addition, these types of procedures normally take a long time to produce results, which ranges from 24 hours to 48 hours [5].

In this project, we plan to build an automated biosensor to detect the presence of *Enterococcus faecalis* in inoculated samples of water. This sensor can be portable and carry out the water quality assay automatically without hands-on work and water sample transportation. The water-quality assay chosen is the enzyme-based Enterolert® test, which is approved by US EPA.

The Enterolert® system from IDEXX (*IDEXX* Laboratories, Inc., Westbrook, Maine) is an innovative method for assessing water quality. Enterolert is easy to learn and simple to use. Results are available in 24 hours, which is important for reducing the time for notifying results of recreational water quality to the public while still providing accurate and consistent test result compared to the method of membrane filtration [6]. The Enterolert method does not require the use of expensive biological instrumentation and reduces the need for highly-skilled workers. Unlike the traditional membrane filtration technique, this assay does not need degassing equipment, membrane filters, or sterilization equipment for cleaning after bacterial culture in order to implement the water quality test. Instead, Enterolert needs only five easy steps. The detailed procedure are as follows.

1. The water sample is obtained, transferred, and then poured into a sterile bottle (100mL).
2. The Enterolert reagent is added into the sample and fully shaken to total dissolution.
3. The sample is poured into the Quanti-Tray which is sealed by the Quanti-Tray Sealer machine.
4. The sample is in 41±0.5°C incubator for 24 hours [7].
5. The fluorescent data is acquired under UV light with 361nm wavelength.
6. The fluorescent data is used together with the MPN table to know the original bacterial concentration [8].

The Quanti-Tray 2000 is the innovative component of Enterolert methodology, which is based on the same statistical model as the traditional 15-tube serial dilution [9]. The Quanti-Tray 2000 distributes a 100-milliliter water sample into 97 wells of 2 different sizes, 49 big wells and 48 small wells. This method does not need hands-on work of test tubes and can achieve a counting range of 1–2,419 CFU/100mL with a 95% confidence limit.

Considering the goals and objectives from our project, we will combine microfluidic techniques with this enzymatic water quality assay to build a portable and automated biosensor with potential to carry the *in-situ* water quality measurements.

1.2 Challenges

The main challenges of this project is to miniaturize the Enterolert Quanti-Tray 2000 PVC plate and to automate liquid handling and data acquisition. The miniaturization of the Quanti-Tray implies manufacturing a device where milliliter-sized volumes can be loaded, separated into independent chambers, incubated, analyzed and discarded using a platform compatible with pumps and valves. Water quality tests require this device to be cleaned after each experiment. The material has to be inert, non-porous, non-biofouling, non-fluorescent, manufacturable, transparent and resistant to disinfectants. This device needs to be resistant to UV-light since water samples have to be excited with UV light. The results from this device need to be detected by a CMOS camera. We chose acrylic as the material from which to build our device since it satisfies all of the above criteria.

After choosing the material, the second challenge is to convert a sheet of cast acrylic into a millifluidic device where sample is introduced, divided into small volumes, incubated for 24-hours, excited with UV light, and discarded. Two-dimensional layers of cut acrylic need to be bonded into a three-dimensional device using an inert and transparent adhesive. One major challenge involves the difficulty of cutting acrylic layers into the desired shape.

The third challenge includes the building of a mechatronic system with components for sample incubation, fluorescent detection and analysis. Finally, the last challenge is to

use Python programming language to create a program that controls the data collection, the mechatronic system, and the analysis of data for fully autonomous operation. This challenge involves the integration of every component in order to follow the standard procedures of the Enterolert biological assay in an automated format.

1.3 Goal and Objectives

The goal of this project is to build a miniaturized, portable and automated water quality biosensor. This biosensor will carry out the Enterolert assay, which uses fluorescence as the readout, inside a custom made acrylic milli-fluidic device. The biosensor will have automated incubation and data analysis systems. The size of this sensor will be small and compact enough for it to be portable. This biosensor would be able to provide bacterial contamination data using inoculated bacterial water samples and clean water (distilled water) samples. The biosensor will measure contamination based on the fluorescent results after a 24-hour incubation. In order to reach this goal, the following objectives have been defined.

1. To manufacture a millifluidic device capable of automating the liquid handling steps of the EPA approved enzymatic water quality test.

2. To build an incubation platform capable of incubating the water quality test over a 24-hour period.

3. To build a fluorescent excitation and emission platform capable of exciting the assay and obtaining fluorescent data every hour for a 24-hour period.

4. To develop software running on a microcomputer to achieve automatic control of temperature, fluorescence emission, and data collection.

5. To integrate the millifluidic device, the incubation platform, the excitation and emission platform, with the microcomputer and obtain fluorescent measurements every hour for the duration of the 24-hour water quality assay incubation period.

6. To study the potential of this biosensor to obtain qualitative and quantitative data from the enzyme-based Enterolert assay.

1.4 Approach

The novelty of this automated biosensor is the development of a millifluidic device capable of automating the liquid handling steps of an EPA approved enzyme-based water quality assay and the integration of the millifluidic device with hardware and software for automated sample incubation, temperature control, UV excitation and fluorescent emission detection, and software to acquire and analyze the data. To manufacture this biosensor, several approaches are as following (more details shown in Chapter 3).

We selected acrylic as the building material to build a three-well millifluidic device. The channels and incubation chambers are designed by CAD software and cut using a laser cutter. The millifluidic device is made by bonding three laser-cut acrylic layers with medical grade double sided tape; this makes the device reusable and leakproof. This device will be used for handling and incubating bacteria inoculated samples pre-mixed with the Enterolert reagent.

For temperature incubation and control, we use a hotplate from a 3D printer with on/off control. To build the fluorescent excitation and emission system, 3D printing technology was used to build a 361nm LED array holder and an ambient-light blocking chamber in order to achieve automated fluorescence measurements and to avoid external light source.

To develop the automation software, we use Python programming language to develop a Graphical User Interface (GUI) that enables users to interact with the electronic devices by graphical and visual indicators. Finally, we perform the water quality measurements using our biosensor and the Enterolert assay. Fluorescent data is acquired and analyzed by measuring the intensity of Red, Green and Blue (RGB) components of the captured light. Histograms and average intensity values from the RGB analysis are used to determine contamination and compared to the Quanti-Tray data acquired by the approach of the Most Probable Number (MPN) Table.

1.5 Thesis Outline

Chapter 1 is a general explanation of the motivation, challenges and goals for

toward the development of automated an automated *enterococcus faecalis* bacterial biosensor.

Chapter 2 is a description of previous water quality methods that supports this work. The technologies used throughout this research are found in Chapter 3. The description of the procedure to obtain data and the Most Probable Number (MPN) Table information is detailed in Chapter 4. Chapter 4 also shows how to acquire and analyze each set of data.

Finally, in Chapter 5, each set of data is introduced in detail including temperature data, RGB analysis from the millifluidic device, MPN analysis for the Quanti-Tray, and linear regression analysis for the hourly RGB data from the millifluidic device.

CHAPTER 2 - LITERATURE REVIEW

2.1 Membrane Filtration Method for Water Quality Detection

The Membrane Filtration (MF) technique is a gold-standard for obtaining water quality measurements. The MF method is a direct and visual counting of bacteria in water based on counting the number of colonies trapped in a the membrane filter after a volume of water is flowed through it [11]. The method concerns ambient water quality monitoring and is a revision of EPA's previous *enterococci* method, which is used since 1986. It shortens analytical time into 24 hours with enhanced analytical results.

The general procedure is to make the water sample filter through the membrane which allows the water to pass through but retain the bacteria. After filtration, the membrane containing the bacterial cells is placed on a selective medium (mEI agar). In order to prepare mEI Medium, 71.2g of dehydrated basal medium plus 0.75g of indoxyl β-D glucoside is added to 1L of reagent grade water in a flask. This mixture in the flask is heated to the boiling temperature to dissolve ingredients, and autoclaved at 121°C (15 lb pressure) for 15 min and then cooled in a 50°C water bath [2].

The incubation period for mEI agar culture is 24 hours. The incubated bacteria colony with blue halo is counted as presence of *enterococci*. Then for accurate counting of colonies, a fluorescent lamp is used to visualize colonies.

The advantage for membrane filtration using indoxyl-β-D-glucoside Agar (mEI) is that it applies the fluorescence technique to quantify the number of *enterococci*. But the shortage is that the whole experiment needs microbiological environment to do experiment, which make in-situ experiment hard to achieve. In addition, the test involves implementation of boiling, autoclaving, cooling process, which is time consuming, and needs much hands-on work.

2.2 Enterolert Water Quality Testing Kit

The Enterolert® method is an EPA-approved water quality method. EPA criterion for recreational waters uses unit of CFU which is Colony-Forming Units; based on the counting of individual colonies growing in an Agar plate using the MF technique. The EPA standard limit for recreational water contamination is 35 CFU out of 100mL of water sample tested. In practice, the EPA also recommends a Beach Action Value to estimate water quality, which suggests that the bacterial concentration does not exceed 70 CFU/100mL *enterococci* (Beach Action Value) [12]. Enterolert simplifies the procedure of membrane filtration and it is widely used in testing recreational water quality. The Enterolert system uses defined substrate technology with an enzymatic indicator substrate, 4-methylumbelliferyl-b-D-glucoside. In the presence of *enterococci*, 4-methylumbelliferyl-b-D-glucoside is divided into b-D-glucoside and a fluorescent product, 4-methylumbelliferone [10]. This substrate technology makes the assay viable without the need for expensive instruments and a trained expert. Meanwhile, the ease of operation is fulfilled by simplifying the test procedure into several steps with much less hands-on work.

To begin with, 100mL standard sample volume is required. The reason of using 100mL water sample is due to the EPA test requirement and 100mL is the required volume for the Enterolert test. A Most Probable Number (MPN) Table then estimates the bacterial level in unit of CFU per 100mL. After sample acquisition, the water sample needs to be

transported to the test laboratory within 30 hours. The ideal condition for water transportation is below 10°C.

To implement the Enterolert lab test with water sample, 10 mL is taken from the 100mL sample and added to 90 mL of sterile deionized water and combined with Enterolert reagents. This dilution is used to expand the range of detection of the Quanti-Tray and MPN table method as well as reducing salinity in the sample and providing a proper environment for bacterial growth. After full mixture with reagent powder, the mixed dilution is to be poured into the Quanti-Tray. Then Quanti-tray with distributed sample in each well is mechanically sealed and is incubated for 24 hours at 41°C. After 24 hours, Fluorescing (positive) wells are observed under a 365nm UV light. The wells with greater color density indicate that the wells are counted as *enterococci* positive. We can indirectly estimate most probable number of colonies of *enterococci* via referring to MPN Table.

Enterolert technology is more efficient than other EPA approved methods since it reduces manual labor, needs no culture media preparation and requires only a UV light and an incubator to be performed. In addition, the procedure of reagent adding is easy to implement. But for the method of Enterolert, human operation of water acquisition and transportation must be implemented, which make real-time results hard to obtain.

2.3 Biosensor Approach for Water Quality Measurement

Microfluidic biosensors emerged in the 1980's. This technology is a multidisciplinary field of biology, chemistry, physics, nanotechnology, and biotechnology. The volume scales of microfluidics are not compatible with the higher volumes required for water quality testing, however, a larger millifluidic device design can bridge the gap between the benefits of microfluidics and the volume requirements of water quality testing. A millifluidic biosensor approach has three advantages. 1) Laminar flow can be mathematically modeled, making quantitative predictions of the biological assay, especially important for diffusion of bacteria within the device under no-flow conditions [13]. 2) Based on the miniaturized scale of device, one can integrate it with other portable components such as the 3D printer incubator, a CMOS camera and microcontrollers. 3)

Millifluidic devices consume less reagent and analyte compared with traditional test, which could in theory lower the costs of water quality testing, and allow for a water quality system to work in a remote location without needing continuous reagent replacements. The automated biosensor of this project can have the advantages mentioned and can be *in-situ*, reusable, and automated.

There are many types of biosensors that can be found in the literature. Usually, the material of these biosensor is polydimethylsiloxane (PDMS). PDMS is a transparent, gas permeable, flexible silicone rubber and has advantages for micromanufacturing in that it is easily moldable and is the prime material used in soft lithography [14]. PDMS suffers from some disadvantages such as being porous and prone to small molecule adsorption [15]. PDMS is hydrophobic and prone to capture small bubbles that can block micro and milli-sized channels; this would make liquid handling devices unusable until the bubble is removed. One way to avoid small bubbles within PDMS devices is to use PDMS degassed self-loading, which is a technique where a vacuum pump degasses the PDMS and the negative pressure inside the device is used to automatically and passively load liquid samples [14]. However, this loading technique is typically used for single-use devices. These characteristics mean that PDMS is not ideal for remote testing, since sea water contains numerous components that may adhere over time to the inner channel of the PDMS chip and make the system prone to plugging by bubbles.

There are various types of reusable biosensors, which include but are not limited to enzyme-based electrochemical sensors and nucleic acid based biosensors [16]. Some of these, especially those dependent on DNA amplification, allow for incubation of a sample, which permits the target molecule or organism to grow in number in the enclosed chamber for a determined period of time. Zamfir et al. developed a reusable carbon nanotube-based amperometric biosensor using micro-electrodes [17]. Their device is used to detect a chemical species. Its main trait is the immobilization of an enzyme onto the electrode surface of the biosensor. However, electrical based biosensors would struggle to detect bacteria in high salinity beach water samples. Also, due to the limited room in such a device, these monolayer chips may not allow the bacteria to reproduce inside the chip to optically

detectable levels for a low-cost camera, if electricity as a measurement were to be exchanged for fluorescence.

Watterson et al. developed a reusable fiber-optic nucleic acid biosensor using immobilized oligonucleotide probes and total internal refection fluorescence (TIRF). Their system utilizes probes of oligonucleotides that fix to the surface of the fused silica optical fiber and detects hybridization between target oligonucleotides and those bound to the optical fiber [18]. However, there are two disadvantages that are not suitable for our project. For this type of sensor, a single flow channel measures same sample solution that is approximately 25μL. This limited amount of analyte is proper for DNA test, but not for bacterial colony unit measurement because bacterial colonies are distributed unevenly in the water sample. In addition, their device test DNA sample which is improper for our case since DNA segmentation needs procedure of DNA extraction. This procedure will increase the time for sample preparation, which may delay the result of water quality measurement.

In our design, we choose poly (methyl methacrylate), also known as acrylic, to manufacture the chip layers. Acrylic does not adsorb small molecules, is as transparent as PDMS, is rigid, and is less hydrophobic than PDMS, which makes acrylic less prone to capturing bubbles in micro and milli sized channels. This material allows for the bacteria to be disinfected easily. The manufacturing process of laser cutting acrylic means reduced time to construct millifluidic chips. Rigid materials such as acrylic are more appropriate for building larger than micro-sized devices with high aspect ratio structures. The difficult process is to glue three layers of acrylic. To make it fully reusable and easily constructed in three dimensions, we use a medical grade adhesive and laser-cut acrylic layer to manufacture a hermetic and waterproof device for the bacteria incubation.

For the aspect of automation, we created a control and image analysis program. The program was created by Python, running on Lunix platform, which is compatible to Raspberry Pi chip. The previous types of automation often use the Arduino microcontroller. Arduino is based on the AT mega processor and C language-like programming environment [19]. Compared to C, Python language has fewer available resource referring

to real-time image analysis. That is the main reason why we use Raspberry Pi chip and Python programming language in our project.

Based on the manufacturing and the detection modalities of biosensors and the requirement of performing in-situ bacteria incubation and detection, we plan to use materials and methods compatible with milli-sized structures and volumes to build this device. Besides, we plan to integrate our millifluidic device with portable electronic components to construct a functional water quality sensor prototype capable of performing *in-situ* measurements of *enterococci* in contaminated water samples.

CHAPTER 3 – METHODOLOGY

The information of how we design and manufacture this biosensor is mentioned in work flow chart as shown in Figure 1:

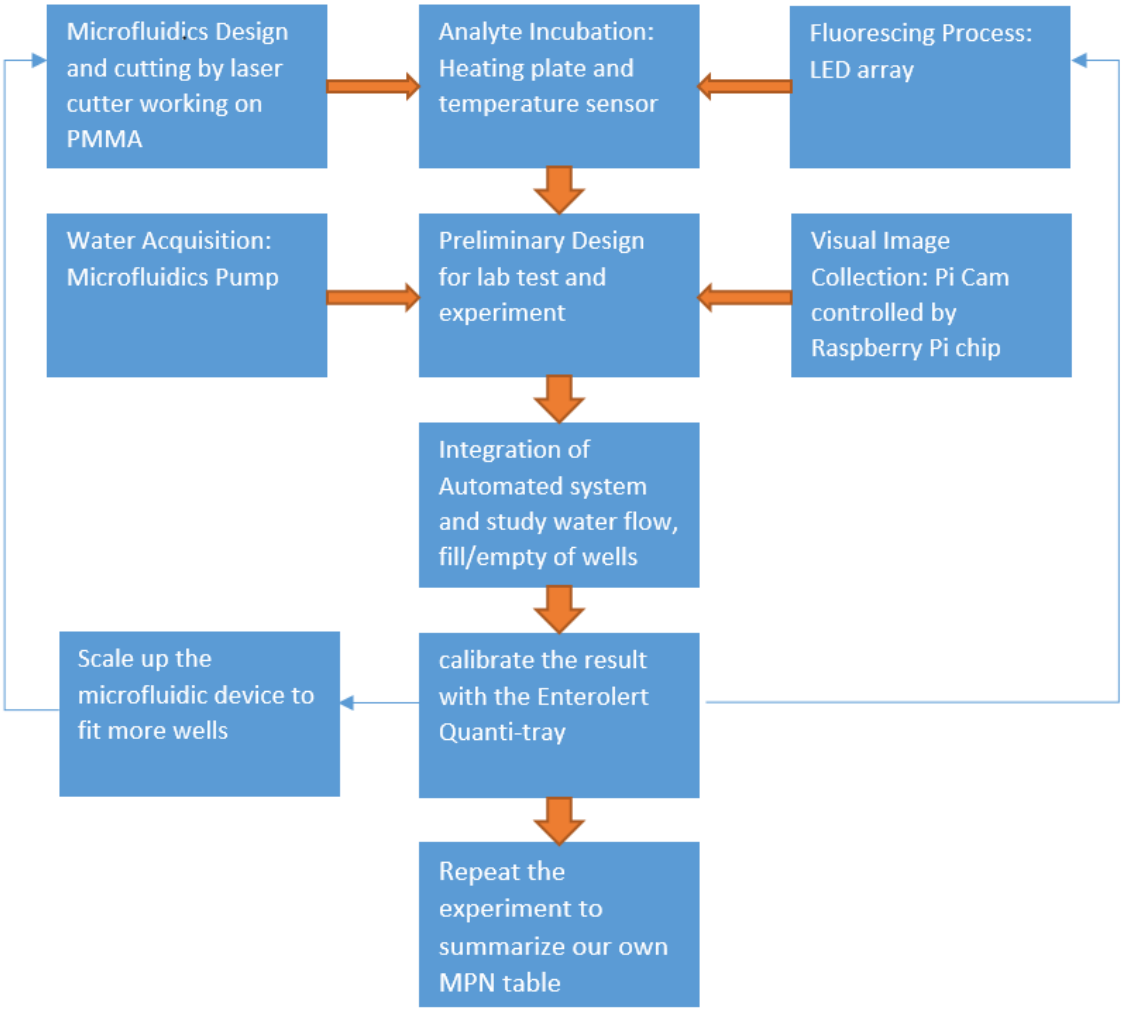

Figure 1: Flow Chart of Biosensor Project Procedure

To make sure the whole system runs well, the first step is to design the millifluidic devices. The device needs to maintain stability of physical properties under various temperature and weather conditions. Therefore, we choose acrylic as the main device material. Then the next part was to build an incubation system. For this process, a heating plate and temperature sensor are needed. Based on the standard of mEI agar test and

Enterolert method for quantifying the CFU concentration of *enterococci*, the temperature and period of incubation is 40°C+\-0.5°C degree for 24 or 28 hours. The equipment we choose needs to be temperature-consistent. A 3D printer heat bead that can be placed below the acrylic device was chosen as our incubation platform. The hotplate was attached to a power supply, electronics and controlled from a Raspberry Pi chip connected to a computer.

Next, we needed several components for performing fluorescence measurements. Those are excitation LEDs placed as an array, a 3D printing LED array holder, a CMOS camera with Red, Green and Blue pixels, and a control system for automated operation. A peristaltic pump was tested for flowing liquid samples from a reservoir to the millifluidic device. The pump was connected to a power supply, electronics and controlled by the Raspberry Pi chip. The water sample and dilution is to be pumped into millifluidic using a constant flow rate. For the purposes of this project, the peristaltic pump was only tested to show that the acrylic device can be loaded and unloaded automatically; however, complete liquid handling automation is outside of the scope of this project.

3.1 Millifluidic Device Design

For the millifluidic device, three acrylic plates were adhered using medical-grade double sided adhesive in order to manufacture a 3D biochip. The design of each 2D layer is shown in Figure 2. Figure 2 shows the configuration of each of the three layers, which are top, middle and bottom layers, respectively. The function the three layers are as follows: 1) Top layer contains laser-engraved channels connecting the inlet to three wells. 2) The middle layer has three wells (enclosed chambers) for sealing the water sample that were made by cutting the acrylic all the way through. 3) The bottom layer also has laser-engraved channels connecting the wells to the outlet. The three plates of acrylic are all in same dimension of 3.27 mm in thickness, 35.84mm in width and 104.23mm in length.

Three layers have difference design purpose. From Figure 2, there are two colors of line, which are red and black. The black line means that cutter cuts through the acrylic layer, the red line means that cutter do shallow cutting on the acrylic surface and create fluidic channel. The working process of three layers is that the water sample primarily

flows into the chip from right corner well of top layer, then flow through the channel, and reach the middle layer staying inside the three wells from the middle layer. After 24-hour incubation and data analysis, the water sample will go through the channel of the middle layer and then flow out of chip from left corner outlet.

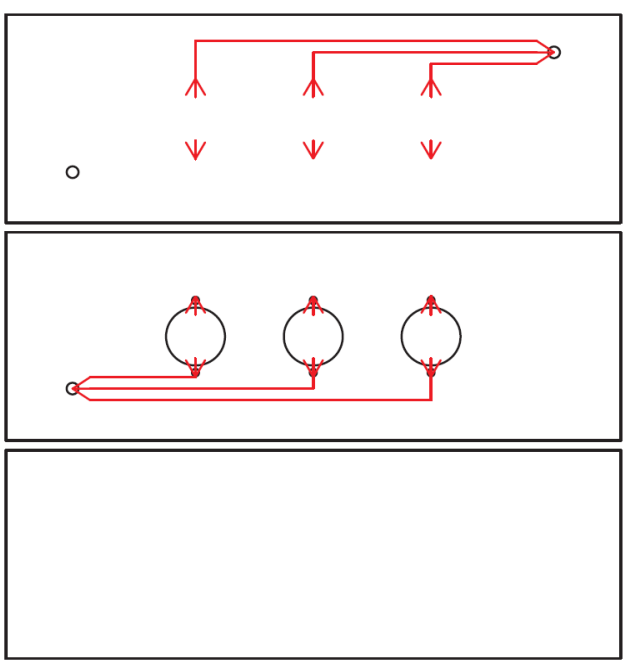

Figure 2: Chip Cutting Design

For this project, we use a biosensor chip with three wells. For the future focus, we may use chip with more wells (3×3 wells, 4×4 wells, or other configuration) for improved statistical analysis. The adhesive used for binding the chip was ARcare®90106 Clear Polyester Double-sided Adhesive tape (Adhesive Research, Inc. Pennsylvania, US). The device layer bonded with this medical-grade adhesive layer are waterproof, can be sterilized with alcohol and are reusable. The detailed information about this glue layer is shown in Figure 3.

Figure 3: Information of Double Glue Layer (image courtesy of Adhesive Research, Inc)

There are three traits of this medical glue to explain why we choose this adhesive for the biochip manufacturing. The traits of glue tape are transparent, pressure-sensitive, and chemically stable. The first trait provides waterproof and transparency for biosensor. Therefore, when we glue three layers of acrylic with two layers of glue tape (Figure 4), the chip is ready to hold and carry out the enzymatic assay for the required 24-hour period. Second, this type of glue is a pressure-sensitive adhesive. This means that the acrylic layers can fully adhere to themselves after applying manual pressure to the two contacting surfaces. Third, the chemical stability of adhesive means that it will not react with the assay reagents or the bacteria in experimental samples and that it can withstand disinfection with chemical agents. Based on the experimental experience, each glued chip can last more than 6 months under proper maintenance.

In the process of manufacturing and maintenance for the chip, there are two requirements that relate to the quality of chip. The first we need to make sure the gluing layer leaves very few bubbles on the surface of the chip. Second, we need to press on the biosensor chip using a pressing machine, such as a clamp, to make sure the glue will reshape into its original sealing at least once a month. The design of the laser-cut glue tape layer is shown as Figure 4.

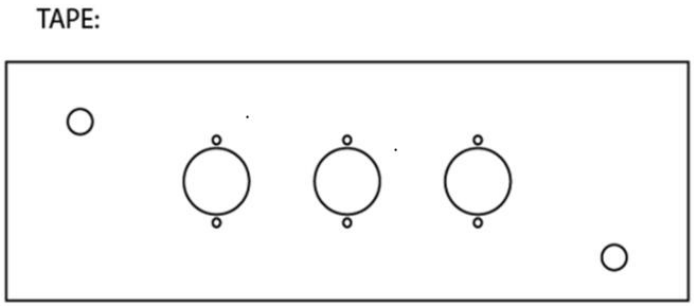

Figure 4: Laser-cut Glue layer for Chip Adhesive

3.2 Hagen–Poiseuille and Hydraulic Resistance

The hydraulic resistance is a measure of the resistance to flow generated by the physical constraints of a channel. It is an important parameter for the design of *micro* and *milli*fluidic devices especially for understanding flow rate as a function of applied inlet pressure [20]. To perform simultaneous washes of liquid samples to and from each well, the channels need to be designed to have equal hydraulic resistances from the inlet to the outlet.

The Hagen-Poiseuille law states that flow rate Q in a channel is proportional to the applied pressure drop ΔP.

$$\Delta P = R_h Q$$

The Reynold number (Re) is a measure of the interplay between viscous and inertial forces in a liquid during flow. It is a function of the fluid properties, fluid velocity and a critical length. In order to calculate Reynold number for a semicircular cross-sectional area, the critical length used is the hydraulic Diameter, D_h, where A is the area of semicircular cross-section, and P is perimeter of the cross-section of semicircular channel.

$$Re = \frac{\rho V_0 D_h}{\eta}$$

$$D_h = \frac{A}{P}$$

Based on visual observations, we estimate that the cross-sectional shape of area of the channel is semicircular and the channel diameter, D, is approximately 500μm. In order to calculate the Reynolds number, we calculate a flow rate, Q, by dividing the millifluidic chip volume of 3mL by the time it typically took to fill this channel, 30 seconds. Average flow velocity, V_0 is calculated to be is 0.25m/s.

$$V_0 = \frac{2Q}{\pi(\frac{D}{2})^2}$$

The liquid used is water where the density, ρ, is 1000kg/m^3 and the viscosity, η, mPas = 0.001Pa-s. Therefore, the Reynold number across the channels is estimated to be 155. This means that flow is mostly laminar but, with inertial considerations. It is highly likely that the flow into and out of the wells will have eddy swirls due to vorticity effects. The most important factor in the millifluidic device is whether the wells will fill and empty simultaneously or not. Achieving fully laminar flow is not critical; in fact, some convection will be useful in washing away the standing solution.

The generic equation for the hydraulic resistance under laminar flow as a function of channel shape is

$$R_{hydraulic} = 2\eta L \times \frac{P^2}{A^3}$$

Where A is the area of the cross section and P is the perimeter of the cross section [21]. The hydraulic resistance for a semicircular shape is then

$$R_{hydraulic} = \frac{8}{\pi}\eta L \times \frac{1}{(\frac{D}{2})^3}$$

Where η is the viscosity for liquid sample and L is the total length of the channel. For laminar flow if hydraulic resistance is the same for each channel, the amount of time during which the liquid sample being washed in and out of chip each will theoretically be the same. In practice, the device is always full of air before we begin pumping water sample into the chip. As we start pumping water into the device, air within the device means that the air containing part of the channel will have less hydraulic resistance than the water containing part of the channel given the difference in the viscosity of these two

media. Also in our system, a Reynolds number of 155 implies that these hydraulic resistance equations may not apply since both inertia and viscosity play a role in liquid flow. However, they are a starting point for the design of our millifluidic system.

3.3 Fluorescence Mechanism

In this biosensor project, the fluorescence method we apply is the patented Defined Substrate Technology®(DST®) of IDEXX. When *enterococci* utilize their β-glucosidase enzyme to metabolize Enterolert's nutrient-indicator, 4-methly-umbelliferyl β-D-glucoside, the sample displays fluorescent color under UV light.

For a single vessel test, one packet of reagent powder is added to the 100mL water sample in a non-fluorescing and disinfected vessel. After powder is dissolved, this vessel is incubated at 41°C for 24h. Then we obtain the results at 24 h (before 28 h) using a ultraviolet lamp for fluorescence readings of the sample. If no fluorescence is observed, the test is negative (clean water sample). If the sample fluoresces blue, it is confirmed positive (bacterial water sample).

For the florescent measurement of our acrylic device, the water sample and reagent are mixed and fully dissolved in the sterilized bottle. Then this sample is pumped into the chip using a 5ml syringe. The sample is set on the heat plate for incubation. The period of incubation is 24 hours. In each hour of incubation, the UV LEDs with wavelength of 361nm shine the wells under a dark environment and the CMOS camera capture the image data and save data in storage card.

3.4 Water Acquisition System

In the current stage of development towards this biosensor, we use a 5mL syringe and clear anti-microbial tubing to perform water acquisition. The volume of device is 3mL, and needs 30 seconds to manually fill up. Therefore, the flow rate is approximately 0.1 mL/s. Micropumps can be used to fully automate liquid handling in future experiments.

In general, pumps are classified into two groups: mechanical and non-mechanical device [22]. Mechanical pumps usually contain movable component called mechanical

actuator. The actuator uses driving force to transfer the liquid sample. The driving force can be produced by utilizing piezoelectric, electrostatic, thermo-pneumatic, pneumatic or magnetic effects. The usual types of mechanical micropumps are reciprocating pump, syringe pump and peristaltic pump. Among those types, we chose to use a peristaltic pump as the driving force for water acquisition. Peristaltic pump operates without valves and can achieve a constant flow rate [23]. The working principle of peristaltic pump is that the roller inside the pump alternates compression and relaxation of the tube to draw the liquid sample into the tube.

In the development of prototype of biosensor, we implemented an experiment to test the electric circuit for micropump and demonstrate the linear behavior of volumetric flow rate. In this experiment, peristaltic pump is control by Raspberry Pi chip programing. Considering the limit of power supply from Raspberry Pi is 5V, A MOSFET relay works as transistor to connect the pump with power supply with 9V and 12V. An experiment was implemented for testing the preliminary design. Result of the testing are shown in Figure 5 and numerical details are in Table 1. The experiment is achieved by varying the PWM at a constant supply voltage and calculated the flow rate. The detailed information of how to calculate the flow rate is mentioned below.

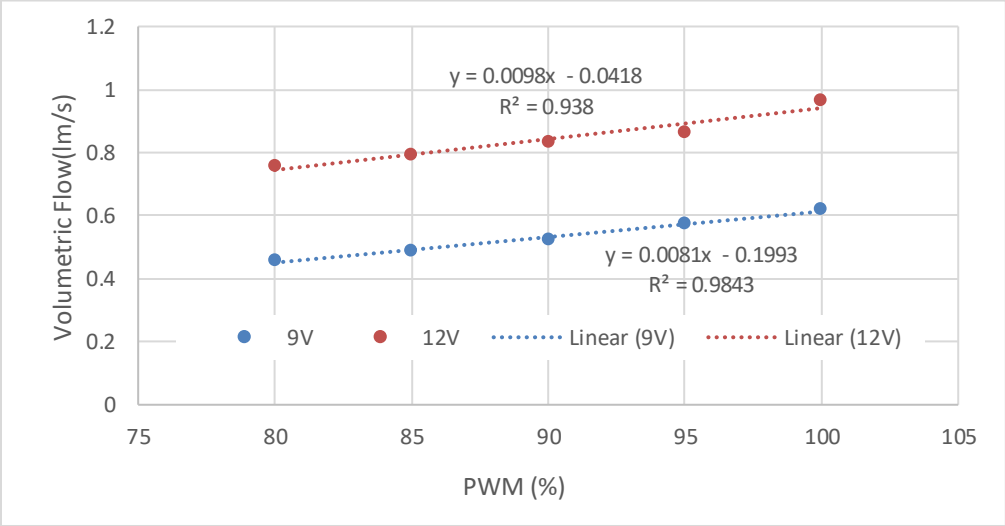

Figure 5: Pump Flow Rate Chart

Table 1: Detailed Information of Pump Flow Rate

PWM \ Voltage	9 V	12 V	PWM \ Voltage	9V	12V
100%	50.0g	70.7g	100%	0.6 ml/s	1.0 ml/s
95%	47.1g	64.6g	95%	0.6 ml/s	0.9 ml/s
90%	44.1g	62.8g	90%	0.5 ml/s	0.8 ml/s
85%	42.0g	60.4g	85%	0.5 ml/s	0.8 ml/s
80%	40.4g	58.1g	80%	0.5 ml/s	0.8 ml/s

In this experiment two parameters were tested, PWM (Pulse Width Modulation) and voltage. Volumetric flow [mL/s] Vs. PWM [%] calibration curves were created by processing the information, Figure 5 and Table 1. First the beaker mass was subtracted from the [beaker + water] mass. The beaker mass is 12.9g. This water mass was converted to Volume in cm^3 (which is equal to milliliters) by dividing the water mass by the water density at ambient temperature (0.9982 g/cm^3 or g/mL). The resulting volume was divided by the run time (60 seconds) to obtain an average volumetric flow in [mL/s]. This experiment did not take into account water evaporation. More experiments should be performed to statistically determine the reproducibility of this data once pumps are integrated into the millifluidic device for automated fluid handling.

The peristaltic pump adds the water in a flow controllable behavior. With power supply (9V or 12V), the volumetric flow change with PWM in a linear way. Understanding this linear relationship is important for being able to control the flow rate properly. For the trendline of linear regression, values of R-square are 0.98(9V), and 0.94(12V).

3.5 Heat Plate for Incubation

Based on the EPA's Standard Methods for Examination of Water and Wastewater, *Enterococci* needs incubation at 41°C+\-0.5°C for 24 hours. To maintain this temperature within the determined period, we choose to use a hotplate from a 3D printer as the heat source because this type of hotplate can distribute heat evenly on its flat surface and make the device evenly warm during the incubation process. The heat plate needs 12V power supply that is not compatible to the voltage requirement of LED array and Raspberry Pi

chip. Thus, we choose an external power supply working with a MOSFET transistor and Dallas One-Wire sensor to create an on-and-off automated incubation system. To know the heat transfer behavior and temperature inside the well of middle chip layer, we devised a way to measure the temperature inside the chip based on three temperature sensors (see Figure 6 and 7).

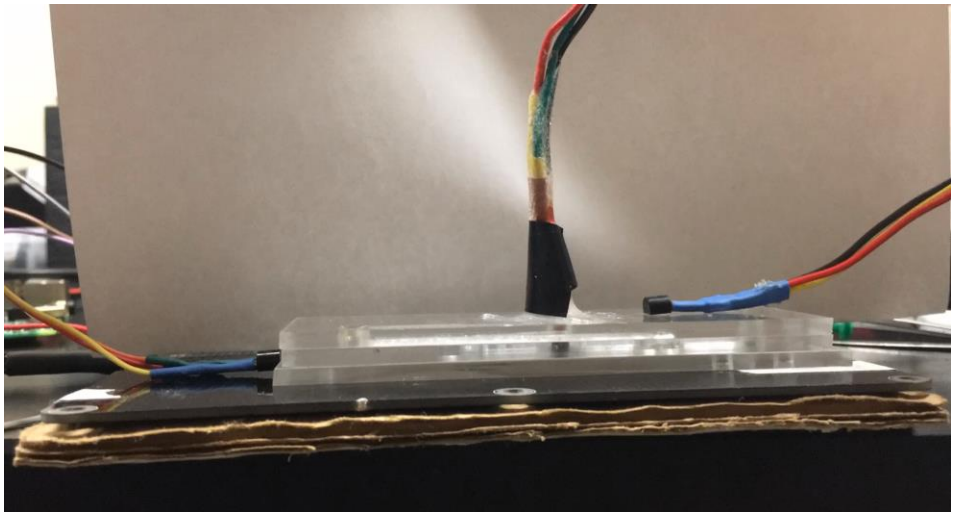

Figure 6: Side View of Temperature Sensor inside the Chip

Figure 6 displays the setting for temperature measurement experiment. There is one black colored hotplate on the bottom, one biochip in the middle, and three One-Wire temperature sensors. The one on the left is the sensor responsible for the temperature of hotplate surface. The sensor (middle) wrapped with black tape measures the temperature of the liquid inside the chip chamber. Finally, the temperature sensor on the right side is for the temperature on the surface of the chip device.

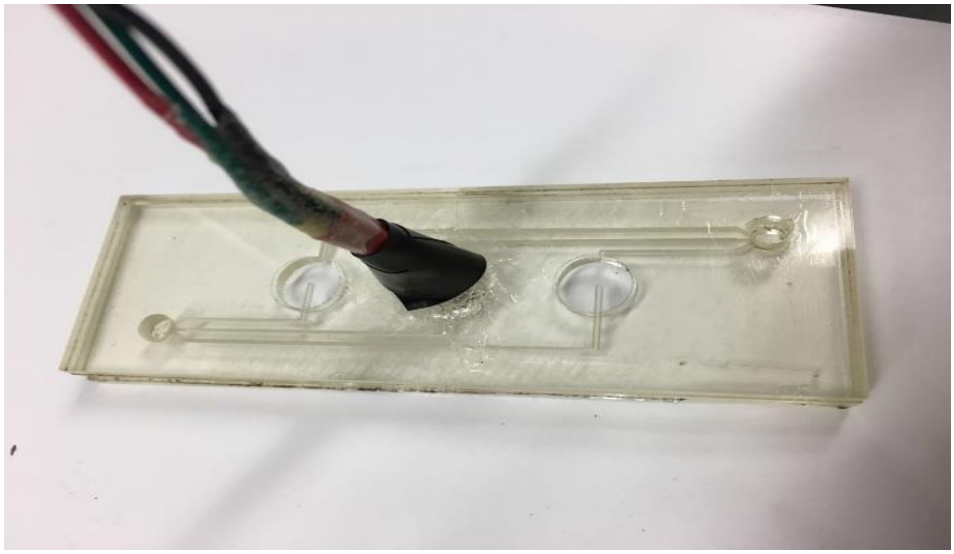

Figure 7: The Top View of Temperature Sensor inside the Chip

For the temperature measurement in the middle of the system, we drilled a hole on the top plate of the chip, then inserted a one-wire temperature sensor inside the well and sealed the chip with waterproof Silicone glue. Then we added water into the chip and started to heat up the water sample while recording the temperature data (see Figure 8).

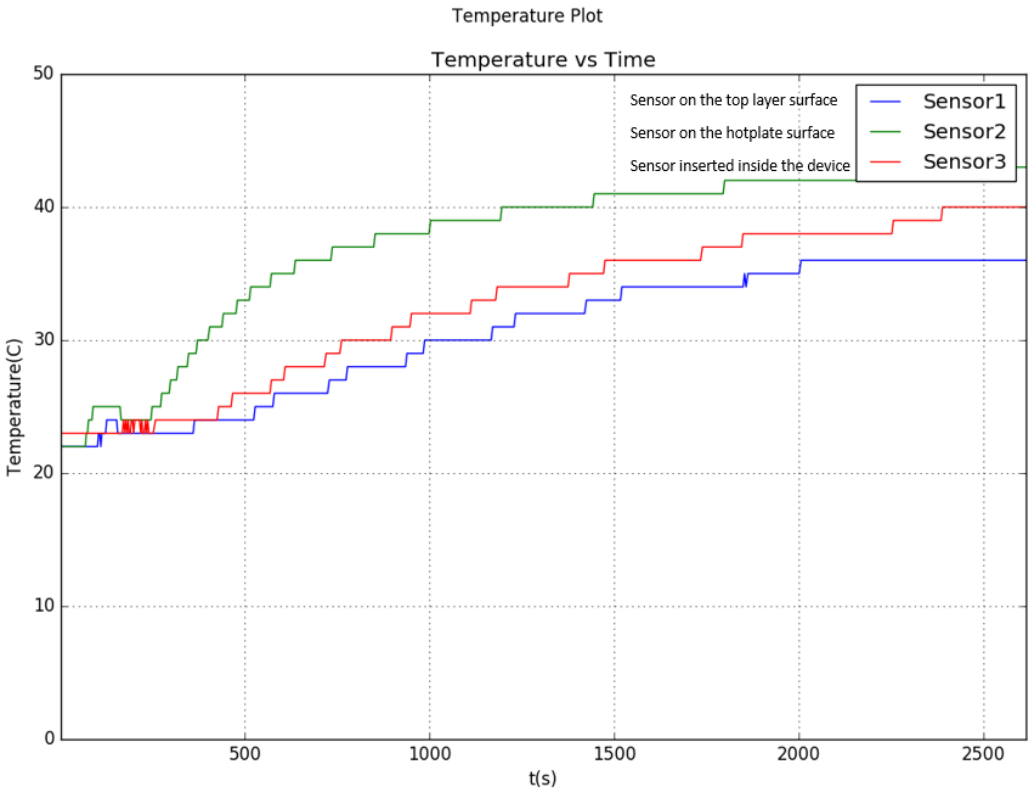

Figure 8: Temperature Measurements as a Function of Sensor Location

In the Figure 8, we see three curves increasing from 22°C to around 42°C. The green curve represents the temperature change of heat plate surface (sensor No.2). The red curve represents water in the well (sensor No.3). The blue curve represents the temperature changing on the surface of acrylic (sensor No.1). According to the temperature data from the acrylic top, the heater, and the water in the well, we can indirectly estimate the temperature inside the chip from data of sensor No.2 (hotplate surface temperature). For *enterococci* incubation, the Enterolert assay protocol requires the incubation temperature to be 41°C+\-0.5°C. Therefore, we set the incubation hot plate temperature at 41°C; however, the hotplate temperature measured was always around 40°C. This means some heat was dissipated to ambient.

The heating due to the conduction of the acrylic and the liquid inside the device from the hotplate follows a thermal dynamic model. The increases of transient temperature follow an exponential growth until reaching a steady state. The three sensors measure this

exponential increase, but each at a different growth rate. This growth rate is a function of the time constant of the system which in the thermal case is a function of the thermal capacitance. The thermal capacitance is the mass of the material multiplied by the specific heat of the material. The larger and less conductive the mass, the longer it takes for the system to reach a steady state. This experiment shows that conduction occurs across the acrylic and that the temperature of the liquid inside the device follows as exponential growth behavior until reaching a steady state. The temperature of the liquid inside the acrylic will eventually reach an equilibrium close to that of the hotplate, unless the ambient temperature is low enough to permit more heat loss than heat gain in the liquid. Some heat from the acrylic is known to be lost to ambient during 24-hour incubation cycles since the temperature of the hotplate was set at 41°C but measured at 40°C during incubation experiments. We experimentally observed that the liquid inside the millifluidic device reached 40°C in 2500 seconds, figure 8. Fluorescence indicative of bacterial growth was observed from the assay indicating that the incubation temperature was appropriate for bacterial growth. The temperature of 41°C is used by the Enterolert assay because, in contrast to other bacterial species, *Enterococcus faecalis* can grow at 41°C; therefore, this temperature acts as a selective environment for *enterococcus faecalis* growth. For the scope of this project, reaching an exact temperature of 41°C was not absolutely required since *Enterococcus* will grow at temperatures from 10°C to 45°C and selective enrichment of one bacteria species over another is not required. Future experiments can further study the behavior of temperatures in different locations of the biosensor.

3.6 Visual Image Data Collection System

The image data collection system, whose role is to detect and obtain fluorescent data from the assay, is a key component of project. The system is detailed below in three parts, which are the Raspberry Pi CMOS camera, the Raspberry Pi chip and the image data calibration.

3.6.1 Raspberry Pi CMOS Camera

In this system, we used a CMOS camera (OV5647) controlled by Raspberry Pi chip. This type of camera has several relevant advantages to our project. Primarily, this camera has 5 mega pixels (2592 x 1944 static image resolution), which can provide the clarity to resolve the expected fluorescent images. It is also compatible to Raspberry Pi, ARM, DSP platforms for research purposes. In addition, the shape of the printed circuit board is square with dimension of 36mm x 36mm (see Figure 9), which suits our need of miniaturization. Finally, CMOS lens assemblies are replaceable and are suitable for our task. In this project, we acquire fluorescent images from the biochip wells (104mm x 34mm), thus we used the lens of LS-6018 (CS mount) with technical parameters shown in Table 2. The image detection program and LED emission system are based on the physical and optical specifications. Figure 9 shows the mechanical dimension of the camera.

Table 2: The specification of CMOS Camera Lens

Model No.	LS-6018CS	**Field of View**	68°
Focal Length	6.0 mm	**Dimensions**	dia.: 28mm height: 24.2 mm
Aperture(F)	1.4	**M.O.D**	0.1 m
Mount	CS	**Weight**	29 g

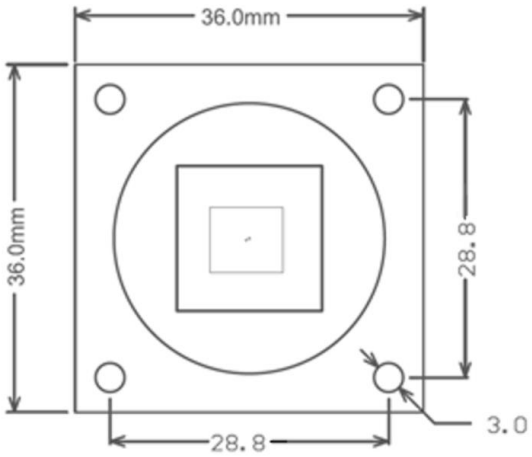

Figure 9: Cross Section of CMOS Camera

From Figure 9, we can see the printed electric board is square-size, which has the side length of 36.0mm. In each corner of the board, a small circle of 3.00mm in diameter. The distance between the two centers of the circle is 28.80mm. The diameter of the camera lens is 29.40mm. Based on these structural dimension, we design and build a light blocker to improve the result captured.

3.6.2 Raspberry Pi Microcomputer

The Raspberry Pi Microcomputer is a miniaturized computing system the size of credit card [24]. The specific model we used is Raspberry Pi 3 Model B, which was released in February 2016 and has on-board WiFi, Bluetooth and USB boot capabilities. Its default operating system is Linux, which is compatible with Python programming language. Figure 10 shows the camera size and how it connects to Raspberry Pi.

Figure 10: CMOS Camera Connected to Raspberry Pi Chip;
Raspberry Pi Chip Module Datasheet (2015), https://www.raspberrypi.org

There are several relevant advantages of this microcomputer to our project. First, this product is affordable and easy to replace. In the electrical circuit test, if this controller was damaged, we can replace it in a rapid pace and at low cost. Second, the Raspberry Pi supports the Linux platform, on which Python programming language can run. We chose Python as a compiled language for biosensor automation. Python has less-cluttered syntax and highly modular interfaces. These qualities have made it popular as a means of adding programmable interfaces to suit our needs.

Third, Raspberry Pi microcomputer has USB interfaces and a HDMI cable interface to achieve input and output functions. The camera, keyboard, mouse, and monitor are peripherals connecting to those interfaces. These USB interfaces provide a reliable and flexible way to control the chip and diagnose the program. In addition, they provide easy data storage, collection, and analysis. During the experiments, flash disk was used to transfer the data. Furthermore, the hardware is compatible to wireless network, which may achieve remote control and data cloud storage and analysis in the future. Finally, this microcomputer is powered by an outsourced 5 voltage power supply. The power source is provided by a USB interface (5V) from a desktop computer.

3.6.3 Image Data of Fluorescent Sample Test

In this experiment, the camera model we used is OV5647 CMOS Camera that is connected to the CSI port of the Raspberry Pi 3 (RPi 3). An RGB sensor (TCS34725) within the OV5647 is used to acquire fluorescent data. The RGB sensor uses the I2C protocol for digital communication with the RPi 3. We used RBG (red, green, blue) values to analyze each image data. Our chosen system uses 8-bit color graphics as a way to store and display color and light intensity information. The CMOS sensor detects photons of light and converts these photons to an electrical signal, which the software then interprets as a number between 0 and 255. Eight-bit color RGB systems allow for 256*256*256 combinations, which is equal to 16,777,216 possible colors. The RGB values measured by the sensor are a function of the intensity of each color but also of other camera variables such as brightness, contrast, white balance, shutter speed, ISO and exposure. All of these variables except the brightness are controlled automatically by the software.

The CMOS sensor does not measure and display light intensity in Lumens, it measures light intensity and color in an 8-bit value having an Arbitrary Unit (A.U.) which is a function of the camera settings and the amount of red, green and blue; where (255,255,255) corresponds to pure white, (0,0,0) corresponds to pure black, (255,0,0) corresponds to pure red, (0,255,0) corresponds to complete pure and (0,0,255) corresponds to pure blue. Each image taken by the CMOS sensor will include some amount of red, green and blue from the emission source. The RGB values for all the pixels in each image is saved as a matrix by the software. The size of the matrix is equal to the number of total pixels in each image. Each pixel is given an R, G and B value. The RGB numbers in our results represent the average value for each color present in each image.

The purpose of this experiment is to prove our automated biosensor can detect a fluorescent sample. The experiment measures the RBG value of bacterial water before and after incubation (with Enterolert reagent). In this test, we used one UV flash light (wavelength of 361nm) to excite the one well of the biochip, and then capture the image data and analyze the data by our CMOS camera GUI system (defined in Chapter 1.3). Figure 11 shows a sample of bacterial water after incubation. Figure 12 shows Figure 11

broken into Red, Green and Blue components. The accompanying histogram in Figure 12 shows the frequency at which a given value of Red, Green and Blue exists within the RGB array saved by the software.

Figure 11: Image data of Bacterial Water after Incubation

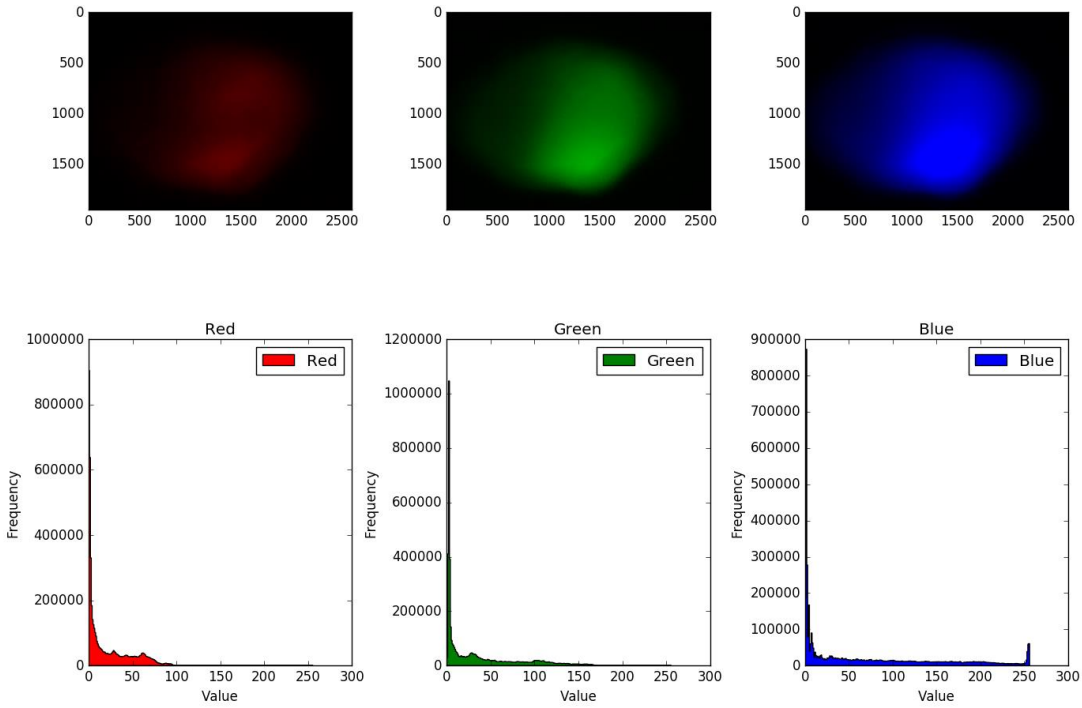

Figure 12: RBG analysis of a Bacterial Water after Incubation

Each histogram presented in Figure 12 shows the amount of times a pixel with a certain intensity from 0 to 255 appears in the image. The frequency unit represents the number of times a value appears, not Hertz or frequency of light. A histogram with high frequencies of low numbers (at or close to 0) is indicative of a dark image. A histogram with high frequencies of high numbers (at or near 255) is indicative of a bright image. Since the histograms represent colors of red, blue or green, a red histogram, for example, with high frequencies of high numbers (at or near 255) is indicative of a very red image; likewise, for the green and blue histograms. Table 3 displays the RBG data from the bacterial water sample before and after incubation. These RGB values, in Arbitrary Units, are the average value for R, G and B found in the RGB matrix.

Figure 13 is an image taken before sample incubation. Figure 14 is Figure 13 broken into Red, Green and Blue components. The accompanying histogram in Figure 14 shows high amounts of dark pixels.

Figure 13: Image Data of Bacterial Water before Incubation

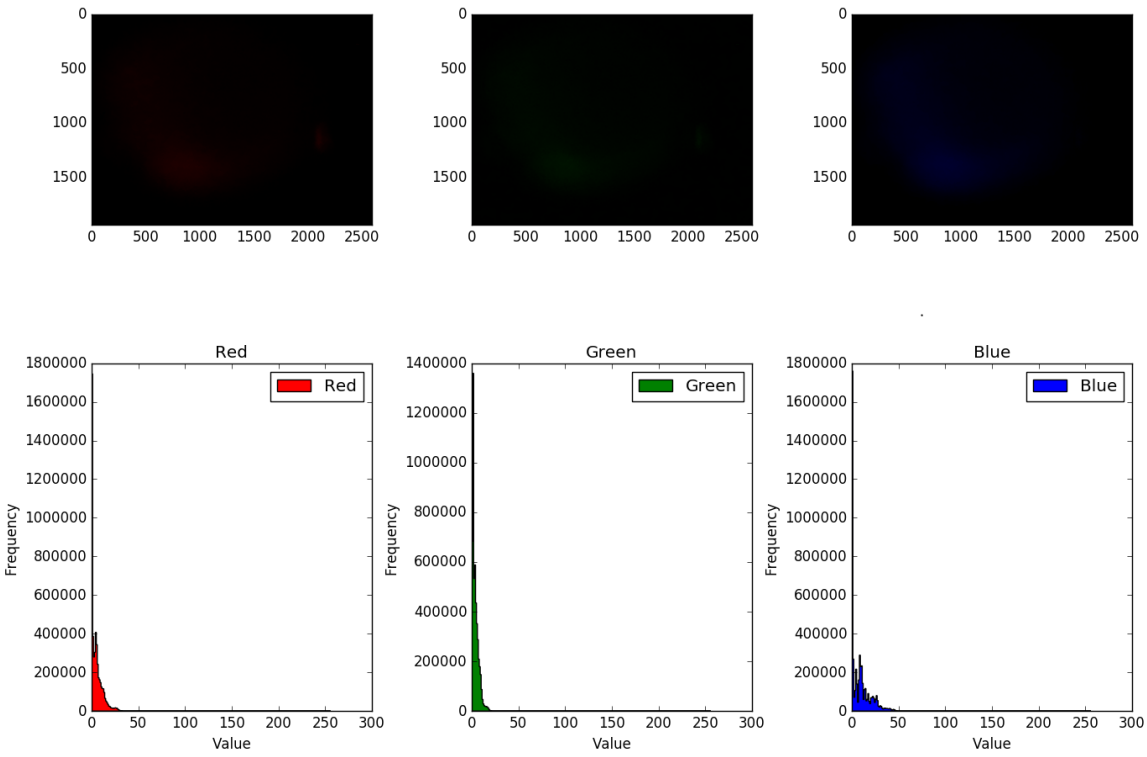

Figure 14: RGB Analysis of a bacterial water sample before incubation

Table 3: RGB Data Comparison between Fluorescence and Water Sample

	Red	**Green**	**Blue**	**Total**
Image after Incubation	22.1	34.0	71.3	71.5
Image before Incubation	4.6	3.4	8.4	8.7

Table 3 displays the RBG data from the bacterial water sample before and after incubation. The data in last column is an overall Intensity value (A.U.) generated by the Python software, which is calculated by converting the RGB values into HSV (Hue, Saturation, Value). Hue is the visual perception of color. Saturation is the amount of gray in the color, from 0 to 100 percent. Value describes the brightness or intensity of the color,

from 0-100 percent. This total intensity is calculated automatically by one of the libraries used in our Python programming (matplotlib.colors.rgb_to_hsv) and is used throughout all of our experiments. This overall Intensity correlates with the amount of red, green and blue in an image and closely tracks the highest color intensity.

Figures 11 through 14 are the proof-of-concept data, which show values of RGB before and after bacteria incubation. Figures 11 and 12 show a fluorescent image of an incubated sample contained in a single well when exposed to 361nm light. Figures 13 and 14 show a dark image of this same well excited by the same LEDs before incubation. The numerical data in Table 3 shows that the bacterial water sample (mixed with Enterolert reagent) experiences increase in RGB intensities under UV light after incubation. Red increases to 22.1 from 4.6, Green increases to 34 from 3.4, and Blue increases to 71.3 from 8.4. These results show that the color Blue has the greatest intensity after incubation. The light emission from the Enterolert assay (the fluorescence of 4-methylumbelliferone) peaks at 450nm, which correlates to blue color [25]. Therefore, this data suggests that the CMOS camera can detect fluorescence emissions as Blue using our camera GUI control system.

The percentage increase of blue intensity (749%) before and after incubation is higher than that of red (380%) but not higher than that of Green (900%). This is likely due to the Bayer filter used in CMOS sensors where 50% of one pixel detects green, 25% of one pixel detects blue and 25% of one pixel detects red; there is twice as much area for a pixel to detect green than to detect red or blue. Therefore, for a given increase in light, green color will show twice as much increase as red or blue. However, if this 900% increase is divided by two, then blue color has the highest percentage increase in the data shown in Table 3; meaning that the blue color has the highest amount of increase per unit area. The CMOS camera settings used to obtain the two sets of image data is shown in Table 4.

Table 4: CMOS Camera Setting for Image Test

Delay	50	**Resolution**	2592×1944
Brightness	50	**Contrast**	0
Shutter Speed	6000000	**Exposure**	AutoWhiteBalance
Zoom	0.34, 0.5, 0.15, 0.2	**ISO**	0

3.7 LED Emission System and Ambient Light Blocker

The purpose of building the LED emission system and ambient light blocker is to avoid the negative effect produced by ambient light and reflection of light. In the previous experiment, we used a UV flash light to excite one well and analyze the result. For the three-well measurements on the acrylic device, multiple LEDs were used to excite the sample evenly. To maintain the position and reduce the reflection of the light source, we built a LED emission holder. The role of the ambient-light blocking chamber is to fix the camera into position and remove unwanted ambient light, which affects the image data negatively.

3.7.1 Led light bulb Information and Emission System

The product ID of the LED light bulb is RLT360-BL-TO18 manufactured by Roithner Laser Technik (Wiedner, Austria). The technical specifications for these LEDs is found in Table 5.

Table 5: The Detailed Information of LED Light Bulb

Wavelength: 361nm	**Max Reverse Voltage:** 5V
Luminous Intensity: 750 μW	**Operating Temp:** -20 to +85 ℃
Max Forward Current: 30mA	**Pulse Current:** 80mA for <=10ms, duty<=1/10
Forward Voltage: 3.8V typ. @20mA	**Do not look at LED directly**

The luminous intensity of each light bulb is 750μW, which is not enough to excite an entire acrylic device. In order to optimize the fluorescent image, we used 20 LEDs to provide an adequate excitation source. 20 bulbs were connected in paralleled circuit under 5V. Based on the information on Table 5, 5V power supply was used to power the 20 LED light bulbs connected in paralleled circuit.

To fix the position of these LEDs, we built an emission system holder by 3D printing technique, Figure 15. From the figure, we can observe the beam from each light bulb goes through the side of device layers. However, when a beam of light shines through the material of acrylic, the reflection of light will be generated. To avoid the reflection being captured by CMOS camera, we designed the holder to cover the whole surface except the three-well area.

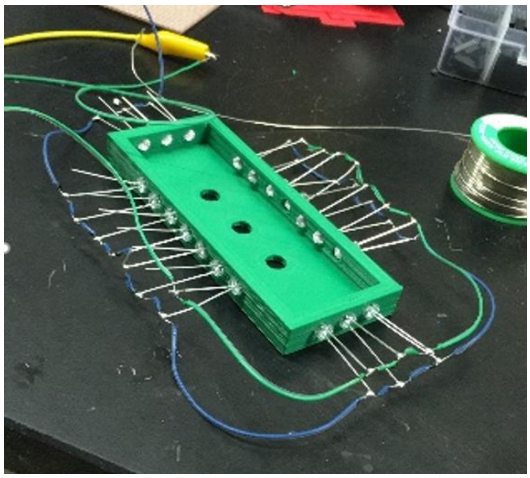

Figure 15: LED Array with Holder (Before Painting)

The parameter for the holder are the following: the height is 13.52mm, the width of cross section is 51.40mm, and length of the cross section is 120.98mm. The thickness of holder is 7.72mm, the light bulb hole diameter 5.03mm, and the diameter of hole on the top is 7.28mm.

3.7.2 Ambient Light Blocker

To fix the position of camera and preclude the factor of ambient light, we built an ambient light blocker by 3D printing technology, Figure 16. The camera lens can be inserted into light blocker and fixed on the top. The dimensions of ambient light blocker can achieve almost seamless connection with camera.

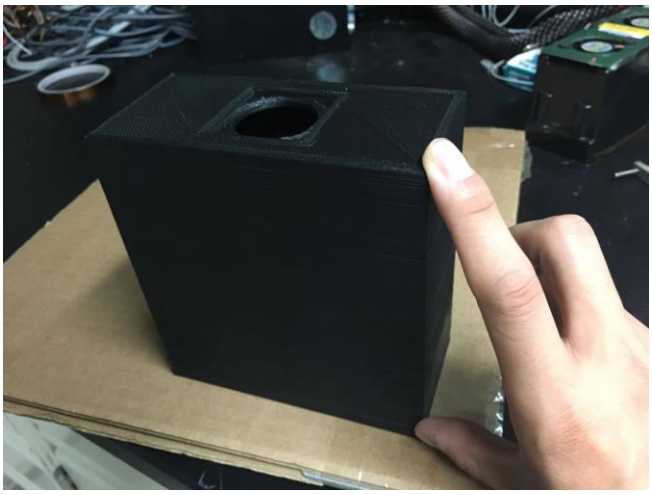

Figure 16: Ambient Light Blocker

From the figure above, the side length, width and height are 131.51mm and 61.35mm, and 136.26mm respectively. The thickness of this printed component is 5.49mm. In addition, on the top of the blocker, we can observe a rounded unprinted area for placing CMOS image sensor. The diameter of this rounded area is 32.02mm.

Finally, there would be light reflection generated by the emitted fluorescence shone on the inner surface of blocker, because 3D printing filament is Polylactic Acid (PLA) which is light reflective material. To reduce the reflection from PLA surface, we used the anti-reflection spray, Rust-Oleum Painter's Touch. This light-absorbed material will adhere to the surface of the PLA and then avoid issue of light reflection from fluorescence.

3.8 Visual Calibration System for Image Data

The purpose of this test is to enhance the image data by calibrating the parameters setting of GUI control panel. In this panel, there are four command interfaces: Camera Options, Exposure Modes, Auto White Balance, and Image Data Options (Figure 17).

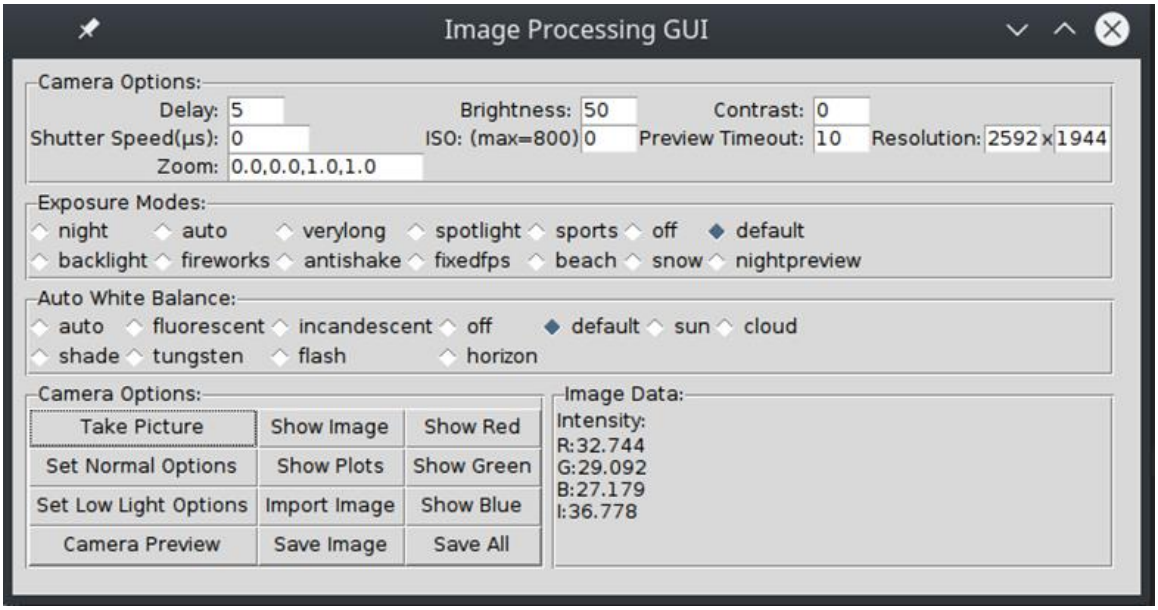

Figure 17: Control Panel for Image Processing GUI

In the interface of Camera Options, several parameters determine the quality of image data. ISO represents International Standards Organization, which is a standardized industry scale to measure how sensitive a digital image sensor is to light. Contrast is function of scale, which serves as the differentiation between the dark and bright areas of the image. The more contrast we setup, the image difference between the fluorescence and surrounding would become more evident. Brightness is the scale of visual perception in which a total amount of light come from source.

The Exposure Mode stands for the way the camera adjusts its aperture and shutter speed. Aperture is the size of how much the lens opens for the light going through. Shutter speed is the period in which the lens opens to capture light. In this experiment, we analyze an identical image data by calibrate the ISO value and two Exposure Modes--Auto Exposure Mode and Very Long Exposure (longer shutter speed) Mode.

In this experiment, we compare and analyze the image data before calibration and after calibration. The analyte is the three-well device filled with fluorescein water sample (100 micromole). For obtaining the first group of image data (see Figure 18 and 19), we set parameters: ISO 100, Brightness 50, and Contrast 15 under Auto Exposure.

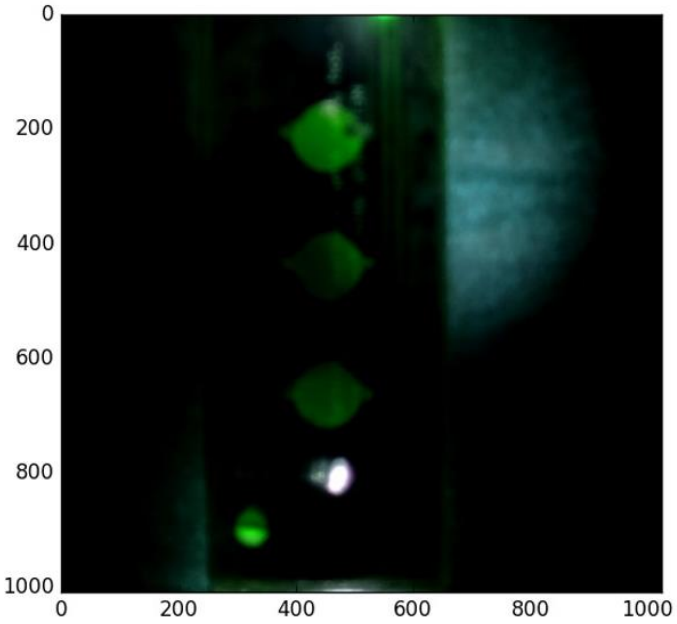

Figure 18: Image before Calibration

Figure 18 displays a dark image of three-well device data with bright reflection spot on the surface on acrylic. The light reflection issue would be solved by chip holder (see chapter 3.6.1). RGB analysis is shown in Figure 19.

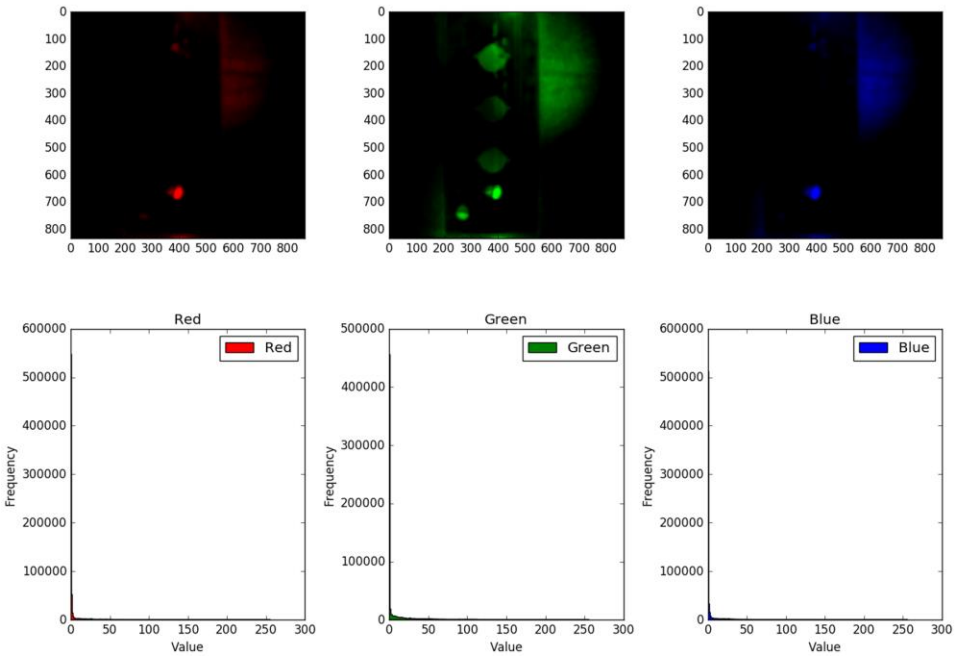

Figure 19: RBG Display before Calibration

For obtaining the second group of image data (see Figure 20 and 21), we set parameters: ISO 300, Brightness 50, and Contrast 15 under the mode of Very Long Exposure. The calibrated parameters are demonstrated in Table 6.

Table 6: Parameter Setting for Fluorescent Test

	ISO	**Brightness**	**Contrast**	**Exposure Mode**
Before Calibration	100	50	15	AutoExposureMode
After Calibration	300	50	15	VeryLongExposure

The image data we capture after calibration appears brighter (see Figure 20 and 21).

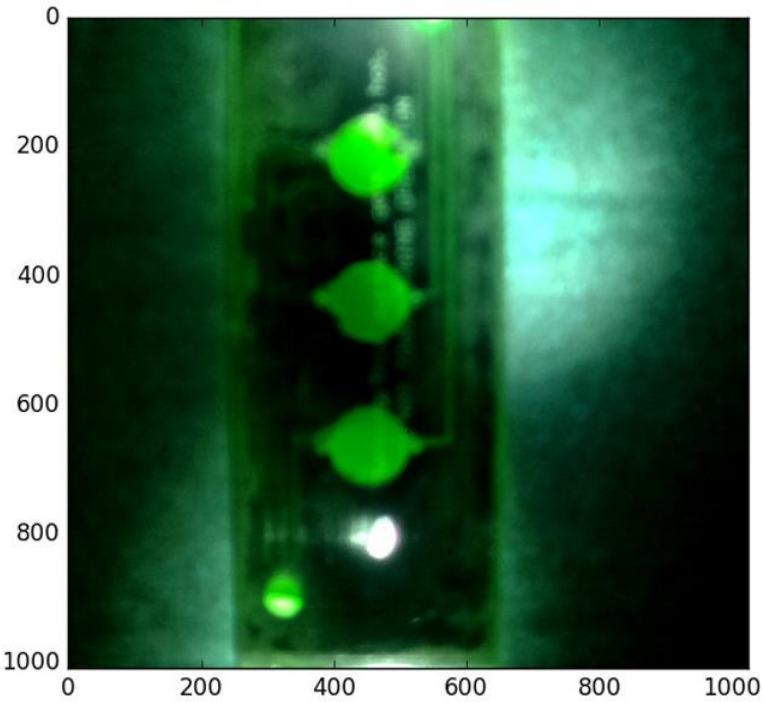

Figure 20: Image after Calibration

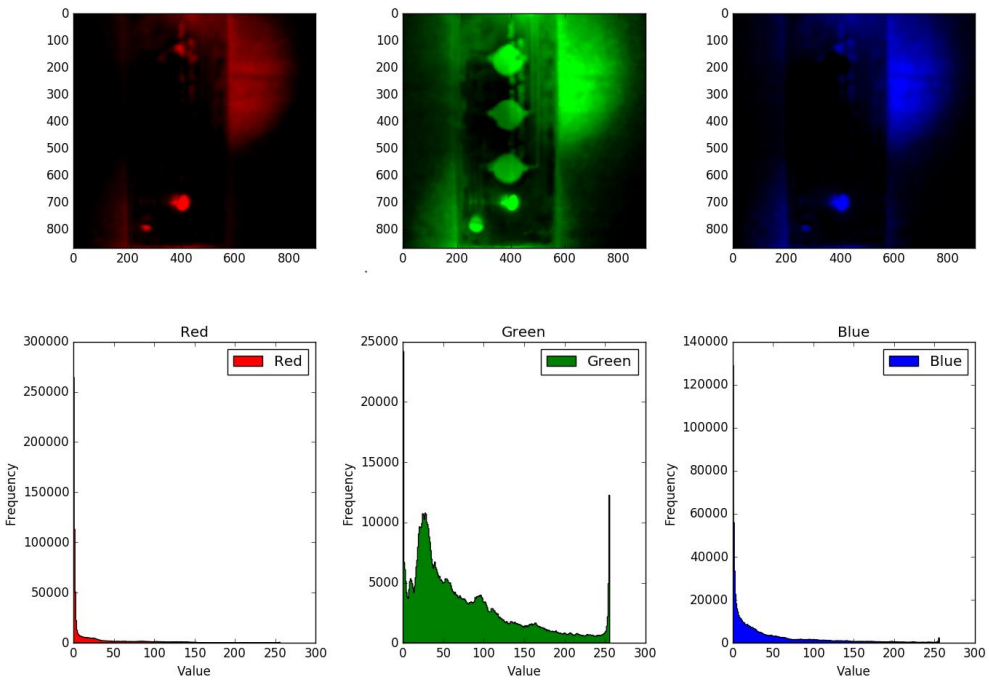

Figure 21: RBG Display after Calibration

From Figure 20 and 21, the image data appears brighter because the photon sensor in the camera becomes more sensitive to the light. In addition, Very Long Exposure mode allows the aperture to open wider and longer for fluorescence. Thus, our GUI control system of CMOS camera can adjust the technical parameters to obtain the ideal image data. Table 7 displays RGB value about the two groups of data.

Table 7: The detail RGB data before and after calibration

	Red	**Green**	**Blue**	**Total**
Before Calibration	4.3	14.7	10.4	14.9
After Calibration	22.3	75.7	40.5	75.8

3.9 Integration of Mechatronic System

3.9.1 Software Integration

The software integration is the operation to coalesce the programming of LED emission system, incubation system, CMOS Camera (RGB sensor) to form an automated program on the operation system during a 24-hour section. This integrated software is called 24-hour incubation system, which integrates the functions operation system on Linux platform, the Image Processing GUI, Coliform Control GUI, and RBG sensor GUI (see in Figures 17, 22, 23 and 24).

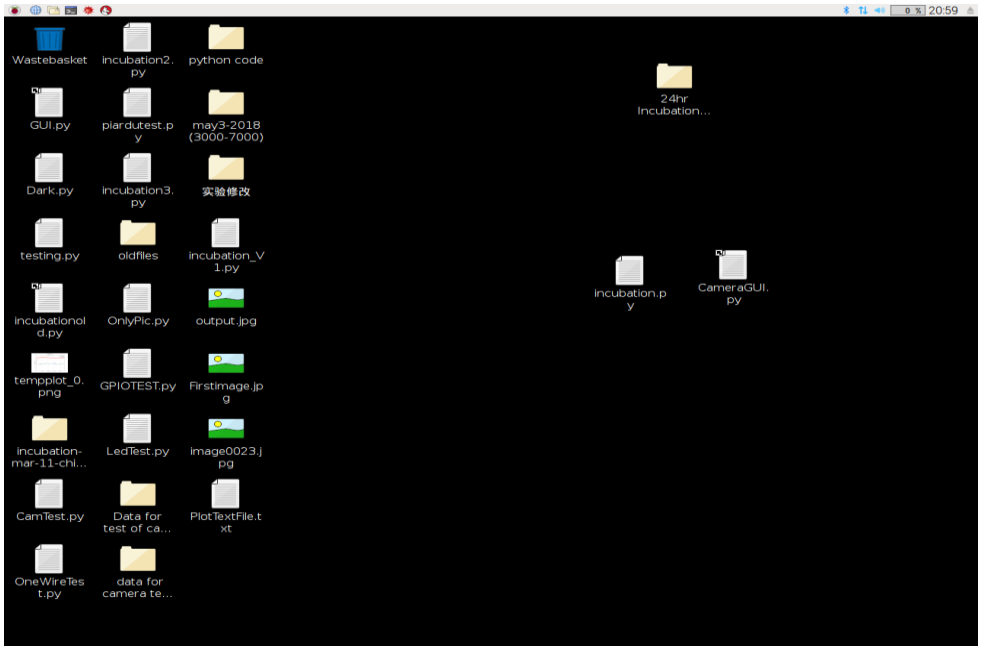

Figure 22: The Operation System Platform of Biosensor

Figure 22 is the operation system of Raspberry Pi microcomputer. This system is the platform of our biosensor on which Image processing GUI, Coliform Control GUI, and RBG sensor GUI operate. In addition, we can observe the system provide an easy access to data storage, retrieval, deletion on configuration of the desktop. The image processing GUI (mentioned in Chapter 3.7) is the data processing system that allows image data acquisition, analysis, display and calibration.

Figure 23 is the control panel for temperature sensor and peristaltic pump. In this stage, the function of temperature setting and plotting is used during 24-hour incubation, the pump control is not in use.

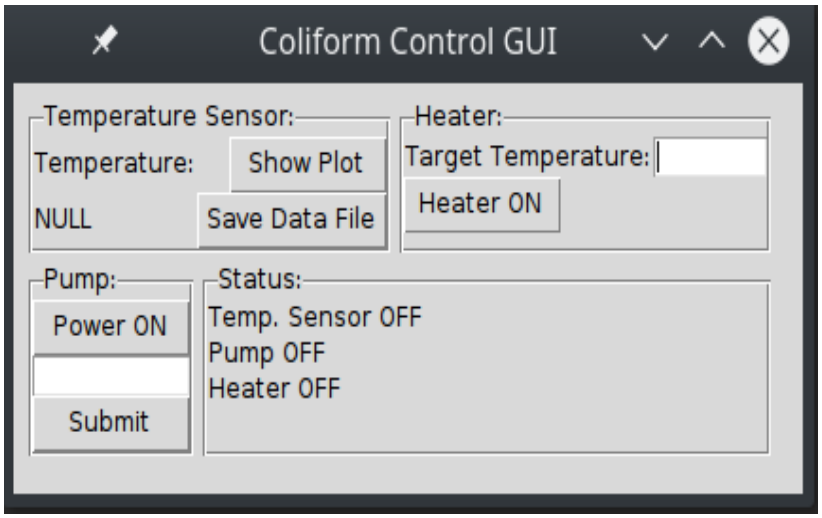

Figure 23: Control Panel of Temperature and Pump

Figure 24 is the calibration panel for RBG sensor (TCS34725). This panel can calibrate two parameters (Integration Time and Gain value) to enhance image quality without changing the exposure mode. Integration time is responsible for the sensitivity at low light levels, which can increase the sensitivity of each pixel capturing the light. Gain value represents the level of brightness without switching the exposure mode. In this project, these two parameters use default setting for RGB sensor. The image calibration does not involve varying integration time and gain value to ameliorate image quality.

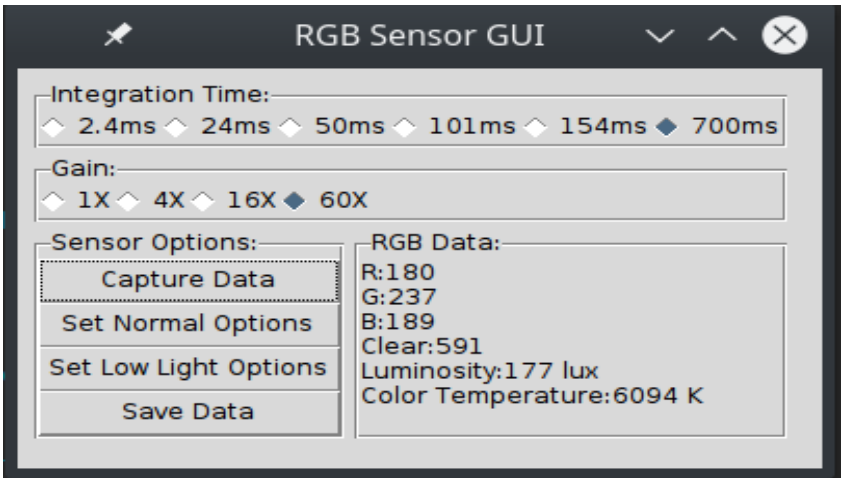

Figure 24: Control Panel for RGB Sensor

3.9.2 Hardware Integration

The different components are integrated into a miniaturized biosensor by connecting each component to an electric board (1 in^2 area). Soldering technique is applied to manufacture the electric board (see Figure 25).

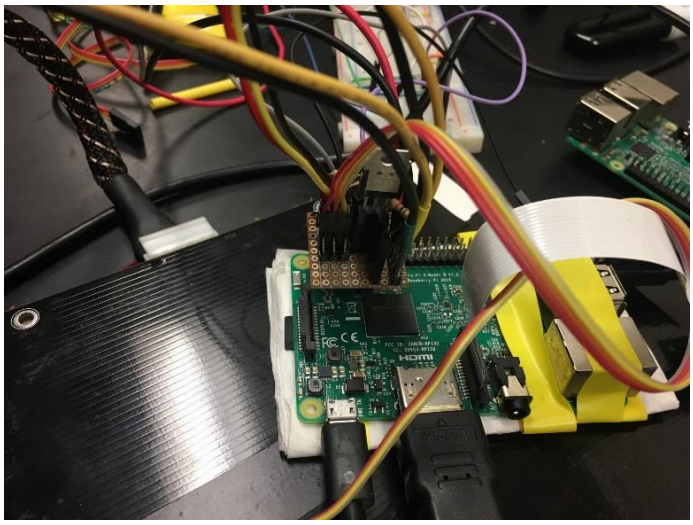

Figure 25: Integrating Chip and Raspberry Pi

In Figure 25, the piece of electrical board is on the top of Raspberry Pi (RPi) microcontroller. Its function is to integrate MOSFET for hot plate, electric wires, and pins of Raspberry Pi. The overall vision of the hardware integration is shown in Figure 26.

Figure 26 shows the overall configuration and size of this biosensor after hardware integration. The wires and MOSFET are connected to the RPi microcomputer. The CMOS image sensor beneath the microcomputer is inserted into the ambient light blocker. The blocker covers the LED holder which maintains the position of 20 LED light bulbs and three-well device. Under the device is the hot plate to keep the warmth for incubation.

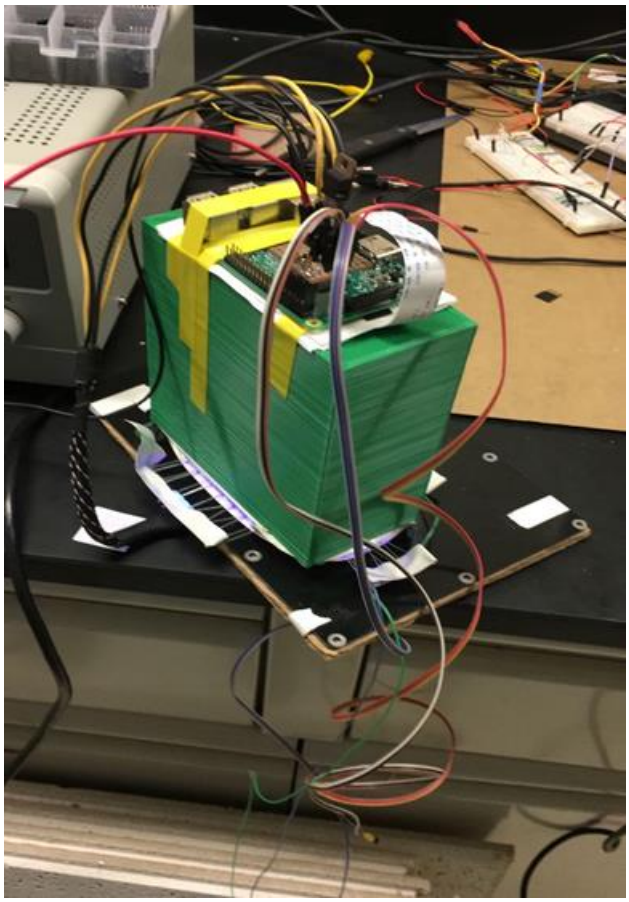

Figure 26: Integrated Water Quality Biosensor Prototype

CHAPTER 4 – DATA ACQUISITION AND MPN TABLE

For each experiment, there are two sets of data for analysis—image data obtained by biosensor and Quanti-Tray data. Biosensor data measures the presence of bacterial water and Quanti-Tray data measures the most probable concentration (100mL) of bacterial water. The statistical chart to estimate the concentration value from Quanti-Tray is Most Probable Number (MPN) Table.

4.1 Data Acquisition Procedure

4.1.1 The Test Plan for Bacterial Water Sample

For one set of experiments, we usually implement three tests for a 24-hour section. These three tests are biosensor incubation test for bacterial water sample, Quanti-Tray incubation test for bacterial water sample, and Quanti-Tray incubation test for clean bottled water mixed reagent. The measurement of clean bottled water would prove that source of water is bacteria-free and the bacterial colonies comes from the bacterial pellet (Vitroid™, Sigma Aldrich, St Louis, Missouri, United States). Vitroid discs contain viable and soluble microorganisms with the quantity (CFU) of a certified and narrow defined range, which is manufactured under reproducible conditions.

4.1.2 To obtain the Bacterial Water Sample for Test

There are two types of bacterial water samples for implementing the experiment. The first sort of liquid sample is the diluted beach water (1:10) from recreational area. The second type of liquid sample is to mix the pure water with Vitroid bacterial pellet manufactured by Sigma Aldrich, which is a corporation majoring in product of life science, technologies, and specialty chemicals. The bacterial pellet from this company specifies the mean number of colony forming units (CFU). All data analysis in Chapter 5 analyze the contaminated water samples made by this bacterial pellet (Vitroid). The procedure of making bacterial water sample from the pellet is as following: 1) we add 2ml phosphate-buffered saline (PBS) to dissolve the pellet in sterilized vessel (100mL); 2) After 3minutes,

the pellet dissolves in PBS then then we add 98 ml of clean bottled water (distilled water) into the vessel. To prepare the control group for knowing the quality of water source, 100mL clean bottled water (distilled water) is added into the vessel.

4.1.3 Mixing Fluorescent Reagent for Bacterial Water Sample and Clean (Distilled) Water

In this step, we add the enzymatic powder of Enterolert (fluorescent reagent) into the bacterial water sample and clean bottled water sample. We cover the two vessels and then shake them until the powder fully dissolve. After the dilution of powder, the two vessels yield the same color of transparent light yellow.

4.1.4 Transferring the Water Sample into Quanti-Tray and Biosensor

The volume of the three-layer device is approximately 3mL. We use syringe (5mL) to transfer the bacterial water sample from vessel to this device. Since, from our observation, 91mL liquid sample can fill all wells of one Quanti-Tray, transferring this volume from tray to biosensor would not affect the result of Quanti-Tray. After bacterial water transferring, we pour bacterial water sample and clean bottled (distilled) water sample into two sets of Quanti-Tray 2000. Then the two trays would be enclosed by the Idexx Quanti-Tray Sealer.

4.1.5 Incubating the Device and Trays for 24 Hours

Based on the standard of Enterolert, we set 24 hours as the incubation time of biosensor and Quanti-Tray. We place the two trays inside the incubator and the device on the hot plate. The temperature for incubation is 41°C±0.5°C for 24 hours. After 24-hour section, incubation process of biosensor would terminate with data storing in the hard drive and we can take the two trays out of the incubator for data analysis.

4.1.6 Acquisition and Analysis of Data from the Quanti-Tray and CMOS Sensor

To analyze the incubation results from the biosensor, we can obtain the temperature and image data from the RPi system. The detailed information about data analysis from the CMOS sensor in the biosensor is elucidated in Chapter 5. The CMOS image capture settings are shown in Table 8.

Table 8: Camera Setting for Obtaining Data from CMOS Sensor

Timelapse	One Hour	**Resolution**	2592×1944
Brightness	50	**Contrast**	0 (Auto)
Shutter Speed	0 (Auto)	**Exposure**	AutoWhiteBalance
Zoom	0.0, 0.0, 1.0, 1.0	**ISO**	0 (Auto)

To obtain each image data, we use AutoWhiteBalance with a Brightness setting of 50% to enhance the visual perception of device wells in darkness. In the exposure mode of AutoWhiteBalance, we set the Contrast, Shutter Speed, ISO equal to zero. The zero values mean these three parameters will auto adjust by the software to respond to the light conditions in the image. Usually, Contrast is approximately 50%; ISO is close to its maximum value of 800. However, Shutter Speed would vary based on the lab condition, such as ambient light, or dysfunction of LED system.

To analyze the data of two trays, we place them on a nonreflective surface and then irradiate the wells of trays under the UV light (361nm wavelength). The trays will show positive (fluorescent) and negative (non-fluorescent) wells. Based on the number of positive ones, we would refer to the Most Probable Number(MPN) Table in order to estimate the CFU concentration for bacterial water sample and to confirm whether the bottled water sample is bacteria-free.

4.2 Most Probable Number (MPN) Table

The Most Probable Number method is based on statistical theory of Poisson distribution, a method to acquire quantitative data for certain concentrations from positive/negative (incidence) data. The positive data is to indirectly estimate the original concentration via the appropriate order. The bacterial concentration of Quanti-Tray 2000

ranges from 1 to 2419 CFU. In this project, we use MPN (Most Probable Number) Table from Enterolert to analyze the bacterial water sample in Quanti-Tray. (see Figure 27).

IDEXX Quanti-Tray®/2000 MPN Table *(per 100ml)*

# Large Wells Positive	# Small Wells Positive																								
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
0	<1	1.0	2.0	3.0	4.0	5.0	6.0	7.0	8.0	9.0	10.0	11.0	12.0	13.0	14.1	15.1	16.1	17.1	18.1	19.1	20.2	21.2	22.2	23.3	24.3
1	1.0	2.0	3.0	4.0	5.0	6.0	7.1	8.1	9.1	10.1	11.1	12.1	13.2	14.2	15.2	16.2	17.3	18.3	19.3	20.4	21.4	22.4	23.5	24.5	25.6
2	2.0	3.0	4.1	5.1	6.1	7.1	8.1	9.2	10.2	11.2	12.2	13.3	14.3	15.4	16.4	17.4	18.5	19.5	20.6	21.6	22.7	23.7	24.8	25.8	26.9
3	3.1	4.1	5.1	6.1	7.2	8.2	9.2	10.3	11.3	12.4	13.4	14.5	15.5	16.5	17.6	18.6	19.7	20.8	21.8	22.9	23.9	25.0	26.1	27.1	28.2
4	4.1	5.2	6.2	7.2	8.3	9.3	10.4	11.4	12.5	13.5	14.6	15.6	16.7	17.8	18.8	19.9	21.0	22.0	23.1	24.2	25.3	26.3	27.4	28.5	29.6
5	5.2	6.3	7.3	8.4	9.4	10.5	11.5	12.6	13.7	14.7	15.8	16.9	17.9	19.0	20.1	21.2	22.2	23.3	24.4	25.5	26.6	27.7	28.8	29.9	31.0
6	6.3	7.4	8.4	9.5	10.6	11.6	12.7	13.8	14.9	16.0	17.0	18.1	19.2	20.3	21.4	22.5	23.6	24.7	25.8	26.9	28.0	29.1	30.2	31.3	32.4
7	7.5	8.5	9.6	10.7	11.8	12.8	13.9	15.0	16.1	17.2	18.3	19.4	20.5	21.6	22.7	23.8	24.9	26.0	27.1	28.3	29.4	30.5	31.6	32.8	33.9
8	8.6	9.7	10.8	11.9	13.0	14.1	15.2	16.3	17.4	18.5	19.6	20.7	21.8	22.9	24.1	25.2	26.3	27.4	28.6	29.7	30.8	32.0	33.1	34.3	35.4
9	9.8	10.9	12.0	13.1	14.2	15.3	16.4	17.6	18.7	19.8	20.9	22.0	23.2	24.3	25.4	26.6	27.7	28.9	30.0	31.2	32.3	33.5	34.6	35.8	37.0
10	11.0	12.1	13.2	14.4	15.5	16.6	17.7	18.9	20.0	21.1	22.3	23.4	24.6	25.7	26.9	28.0	29.2	30.3	31.5	32.7	33.8	35.0	36.2	37.4	38.6
11	12.2	13.4	14.5	15.6	16.8	17.9	19.1	20.2	21.4	22.5	23.7	24.8	26.0	27.2	28.3	29.5	30.7	31.9	33.0	34.2	35.4	36.6	37.8	39.0	40.2
12	13.5	14.6	15.8	16.9	18.1	19.3	20.4	21.6	22.8	23.9	25.1	26.3	27.5	28.6	29.8	31.0	32.2	33.4	34.6	35.8	37.0	38.2	39.5	40.7	41.9
13	14.8	16.0	17.1	18.3	19.5	20.6	21.8	23.0	24.2	25.4	26.6	27.8	29.0	30.2	31.4	32.6	33.8	35.0	36.2	37.5	38.7	39.9	41.2	42.4	43.6
14	16.1	17.3	18.5	19.7	20.9	22.1	23.3	24.5	25.7	26.9	28.1	29.3	30.5	31.7	33.0	34.2	35.4	36.7	37.9	39.1	40.4	41.6	42.9	44.2	45.4
15	17.5	18.7	19.9	21.1	22.3	23.5	24.7	25.9	27.2	28.4	29.6	30.9	32.1	33.3	34.6	35.8	37.1	38.4	39.6	40.9	42.2	43.4	44.7	46.0	47.3
16	18.9	20.1	21.3	22.6	23.8	25.0	26.2	27.5	28.7	30.0	31.2	32.5	33.7	35.0	36.3	37.5	38.8	40.1	41.4	42.7	44.0	45.3	46.6	47.9	49.2
17	20.3	21.6	22.8	24.1	25.3	26.6	27.8	29.1	30.3	31.6	32.9	34.1	35.4	36.7	38.0	39.3	40.6	41.9	43.2	44.5	45.9	47.2	48.5	49.8	51.2
18	21.8	23.1	24.3	25.6	26.9	28.1	29.4	30.7	32.0	33.3	34.6	35.9	37.2	38.5	39.8	41.1	42.4	43.8	45.1	46.5	47.8	49.2	50.5	51.9	53.2
19	23.3	24.6	25.9	27.2	28.5	29.8	31.1	32.4	33.7	35.0	36.3	37.6	39.0	40.3	41.6	43.0	44.3	45.7	47.1	48.4	49.8	51.2	52.6	54.0	55.4
20	24.9	26.2	27.5	28.8	30.1	31.5	32.8	34.1	35.4	36.8	38.1	39.5	40.8	42.2	43.6	44.9	46.3	47.7	49.1	50.5	51.9	53.3	54.7	56.1	57.6
21	26.5	27.9	29.2	30.5	31.8	33.2	34.5	35.9	37.3	38.6	40.0	41.4	42.8	44.1	45.5	46.9	48.4	49.8	51.2	52.6	54.1	55.5	56.9	58.4	59.9
22	28.2	29.5	30.9	32.3	33.6	35.0	36.4	37.7	39.1	40.5	41.9	43.3	44.8	46.2	47.6	49.0	50.5	51.9	53.4	54.8	56.3	57.8	59.3	60.8	62.3
23	29.9	31.3	32.7	34.1	35.5	36.8	38.3	39.7	41.1	42.5	43.9	45.4	46.8	48.3	49.7	51.2	52.7	54.2	55.6	57.1	58.6	60.2	61.7	63.2	64.7
24	31.7	33.1	34.5	35.9	37.3	38.8	40.2	41.7	43.1	44.6	46.0	47.5	49.0	50.5	52.0	53.5	55.0	56.5	58.0	59.5	61.1	62.6	64.2	65.8	67.3
25	33.6	35.0	36.4	37.9	39.3	40.8	42.2	43.7	45.2	46.7	48.2	49.7	51.2	52.7	54.3	55.8	57.3	58.9	60.5	62.0	63.6	65.2	66.8	68.4	70.0
26	35.5	36.9	38.4	39.9	41.4	42.8	44.3	45.9	47.4	48.9	50.4	52.0	53.5	55.1	56.7	58.2	59.8	61.4	63.0	64.7	66.3	67.9	69.6	71.2	72.9
27	37.4	38.9	40.4	42.0	43.5	45.0	46.5	48.1	49.6	51.2	52.8	54.4	56.0	57.6	59.2	60.8	62.4	64.1	65.7	67.4	69.1	70.8	72.5	74.2	75.9
28	39.5	41.0	42.6	44.1	45.7	47.3	48.8	50.4	52.0	53.6	55.2	56.9	58.5	60.2	61.8	63.5	65.2	66.9	68.6	70.3	72.0	73.7	75.5	77.3	79.0
29	41.7	43.2	44.8	46.4	48.0	49.6	51.2	52.8	54.5	56.1	57.8	59.5	61.2	62.9	64.6	66.3	68.0	69.8	71.5	73.3	75.1	76.9	78.7	80.5	82.4
30	43.9	45.5	47.1	48.7	50.4	52.0	53.7	55.4	57.1	58.8	60.5	62.2	64.0	65.7	67.5	69.3	71.0	72.9	74.7	76.5	78.3	80.2	82.1	84.0	85.9
31	46.2	47.9	49.5	51.2	52.9	54.6	56.3	58.1	59.8	61.6	63.3	65.1	66.9	68.7	70.5	72.4	74.2	76.1	78.0	79.9	81.8	83.7	85.7	87.6	89.6
32	48.7	50.4	52.1	53.8	55.6	57.3	59.1	60.9	62.7	64.5	66.3	68.2	70.0	71.9	73.8	75.7	77.6	79.5	81.5	83.5	85.4	87.5	89.5	91.5	93.6
33	51.2	53.0	54.8	56.5	58.3	60.2	62.0	63.8	65.7	67.6	69.5	71.4	73.3	75.2	77.2	79.2	81.2	83.2	85.2	87.3	89.3	91.4	93.6	95.7	97.8
34	53.9	55.7	57.6	59.4	61.3	63.1	65.0	67.0	68.9	70.8	72.8	74.8	76.8	78.8	80.8	82.9	85.0	87.1	89.2	91.4	93.5	95.7	97.9	100.2	102.4
35	56.8	58.6	60.5	62.4	64.4	66.3	68.3	70.3	72.3	74.3	76.3	78.4	80.5	82.6	84.7	86.9	89.1	91.3	93.5	95.7	98.0	100.3	102.6	105.0	107.3
36	59.8	61.7	63.7	65.7	67.7	69.7	71.7	73.8	75.9	78.0	80.1	82.3	84.5	86.7	88.9	91.2	93.5	95.8	98.1	100.5	102.9	105.3	107.7	110.2	112.7
37	62.9	65.0	67.0	69.1	71.2	73.3	75.4	77.6	79.8	82.0	84.2	86.5	88.8	91.1	93.4	95.8	98.2	100.6	103.1	105.6	108.1	110.7	113.3	115.9	118.6
38	66.3	68.4	70.6	72.7	74.9	77.1	79.4	81.6	83.9	86.2	88.6	91.0	93.4	95.8	98.3	100.8	103.4	105.9	108.6	111.2	113.9	116.6	119.4	122.2	125.0
39	70.0	72.2	74.4	76.7	78.9	81.3	83.6	86.0	88.4	90.9	93.4	95.9	98.4	101.0	103.6	106.3	109.0	111.8	114.6	117.4	120.3	123.2	126.1	129.2	132.2
40	73.8	76.2	78.5	80.9	83.3	85.7	88.2	90.8	93.3	95.9	98.5	101.2	103.9	106.7	109.5	112.4	115.3	118.2	121.2	124.3	127.4	130.5	133.7	137.0	140.3
41	78.0	80.5	83.0	85.5	88.0	90.6	93.3	95.9	98.7	101.4	104.3	107.1	110.0	113.0	116.0	119.1	122.2	125.4	128.7	132.0	135.4	138.8	142.3	145.9	149.5
42	82.6	85.2	87.8	90.5	93.2	96.0	98.8	101.7	104.6	107.6	110.6	113.7	116.9	120.1	123.4	126.7	130.1	133.6	137.2	140.8	144.5	148.3	152.2	156.1	160.2
43	87.6	90.4	93.2	96.0	99.0	101.9	105.0	108.1	111.2	114.5	117.8	121.1	124.6	128.1	131.7	135.4	139.1	143.0	147.0	151.0	155.2	159.4	163.8	168.2	172.8
44	93.1	96.1	99.1	102.2	105.4	108.6	111.9	115.3	118.7	122.3	125.9	129.6	133.4	137.4	141.4	145.5	149.7	154.1	158.5	163.1	167.9	172.7	177.7	182.9	188.2
45	99.3	102.5	105.8	109.2	112.6	116.2	119.8	123.6	127.4	131.4	135.4	139.6	143.9	148.3	152.9	157.6	162.4	167.4	172.6	178.0	183.5	189.2	195.1	201.2	207.5
46	106.3	109.8	113.4	117.2	121.0	125.0	129.1	133.3	137.6	142.1	146.7	151.5	156.5	161.6	167.0	172.5	178.2	184.2	190.4	196.8	203.5	210.5	217.8	225.4	233.3
47	114.3	118.3	122.4	126.6	130.9	135.4	140.1	145.0	150.0	155.3	160.7	166.4	172.3	178.5	185.0	191.8	198.9	206.4	214.2	222.4	231.0	240.0	249.5	259.5	270.0
48	123.9	128.4	133.1	137.9	143.0	148.3	153.9	159.7	165.8	172.2	178.9	186.0	193.5	201.4	209.8	218.7	228.2	238.2	248.9	260.3	272.3	285.1	298.7	313.0	328.2
49	135.5	140.8	146.4	152.3	158.5	165.0	172.0	179.3	187.2	195.6	204.6	214.3	224.7	235.9	248.1	261.3	275.5	290.9	307.6	325.5	344.8	365.4	387.3	410.6	435.2

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Figure 27: MPN table (first page only, courtesy of IDEXX Laboratories, Inc)

From Figure 27, the first page of MPN Table has 50 lines (0-49) and 25 columns (0-24). On the second page, there are 24 more columns with 50 lines. In total, there are 50 lines (0-49) and 49 (0-48) columns. Line represents the large positive wells and column stands for the small positive wells. The test range is from 1 to 2419 CFU/100mL. Knowing the positive number of large and small wells, we can estimate the concentration of bacterial water sample (see Figure 28).

Figure 28: Fluorescent Example of Quanti-Tray under UV light

Based on Figure 28, we can observe that the all large wells are positive (on the right-hand side). The small-well zone (on the left-hand side) shows 39 positive and 9 negative ones. By referring to the MPN Table (see Figure 27), the *enterococci* in the water sample is approximately 1046 CFU/100mL. With this experimental result, we can evaluate function of the automated biosensor, which is further discussed in Chapter 5.7.

CHAPTER 5 – DATA ANALYSIS

This chapter is divided into the various experiments and analysis performed with the integrated water quality biosensor. Water samples having different contamination levels were studied; 0, 23, 1046, 1500 and 6896 CFU/100mL. Each concentration of CFU was tested in the biosensor and in a Quanti-Tray 2000. For each experiment, a negative control sample was also tested in a Quanti-Tray. The data shown for each concentration include a temperature versus time scatter plot, a scatter plot having RGB (raw data) intensities versus time, a table having the percentage increase of each color over the 24-hour period compared to the first hour, and a scatter plot after discarding erroneous data including a linear regression of each color for comparing their relative increase. The raw data for each experiment is found in the Appendix.

5.1 Experiment of 0 CFU/100mL Water Sample

The experiment was implemented on May 15, 2017. Two tests were implemented simultaneously, which are the biosensor test and the Quanti-Tray test. Both biosensor and tray tests used clean bottled water (distilled water) with Enterolert reagent. They were incubated at a temperature of approximately 41°C for 24 hours. The goal of 0 CFU/100mL measurement is to observe the RGB values of a clean water sample over the 24-hour incubation period and to develop a negative control baseline for future experiments.

5.1.1 Temperature Data Chart (0 CFU/100mL)

Temperature data of ambient environment and hotplate surface of the incubated distilled water sample (0 CFU/100mL) is shown in Figure 29. There are two temperature sensors (DS18B20): TemperatureSensor1 is for the ambient temperature, and TemperatureSensor2 is responsible for the temperature of the incubator surface.

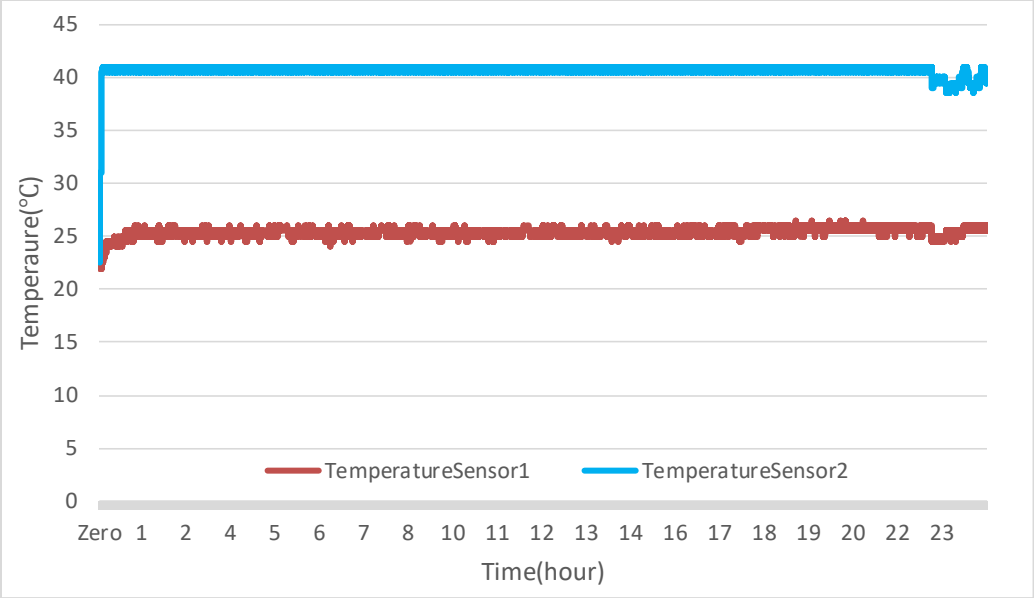


Figure 29: Incubation Temperature Data for Water Sample at 0 CFU/100mL

There are two curves colored red (Sensor 1) and blue (Sensor 2). From the behavior of two curves, the temperature of ambient environment and incubation kept consistently for 24 hours.

5.1.2 Quanti-Tray Data (0 CFU/100mL)

The Quanti-Tray test was irradiated under the UV light of 361nm wavelength for obtaining fluorescent data. The sample of clean bottled water was transferred and sealed in the tray. From Figure 30, the tray displayed all negative wells (positive results present shining blue color instead of gleaming green) before and after incubation. Referring to MPN Table, the result of all negative large and smalls wells signifies the bacterial concentration of the contaminated water sample as being 0 (<1) CFU/100mL. We regard this water sample as sterile level (0 CFU/100mL). Therefore, the bottled water can provide bacteria-free water source for biosensor test.

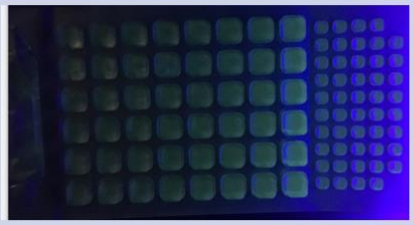
	The display of the Quanti-Tray under UV light of 361nm wavelength
1. The clean bottled water sample in Quanti-Tray (0 CFU/ 100ml) before 24-hour incubation	
2. The clean bottled water sample in Quanti-Tray (0 CFU/ 100ml) after 24-hour incubation	

Figure 30: Quanti-Tray result of Water Sample (0 CFU/100mL)

5.1.3 Biosensor Image Data and Linear Regression (0 CFU/100mL)

Two image data were taken in 1st hour and 24th hour of 24-hour period of incubation (see Figure 31). The first and last hour can show the general tendency and numerical difference of fluorescent result. We use Arbitrary Unit (A.U.) to represent the value of intensity data.

	The display of the Biosensor under UV light of 361nm wavelength
1) The clean bottled water sample in biosensor under Camera (0 CFU/ 100mL) – 1st hour Red: 5.895 Green: 7.789 Blue: 9.397 Intensity: 10.73	
2) The clean bottled water sample in biosensor under Camera (0 CFU/ 100mL) – 24th hour Red: 4.713 Green: 5.212 Blue: 9.763 Intensity: 10.42	

Figure 31: Image data of 1st and 24th hour for Water Sample at 0 CFU/100mL

In Figure 31, we obtain the numerical data of the 1st hour image: red is 5.895; Green is 7.789, Blue is 9.397 and total intensity is 10.730. The image data of 24th hour is as following: red is 4.713 (20.06% decrease); green is 5.212 (30.08% decrease); blue is 9.763 (3.89% increase) and total intensity is 10.420 (2.89% decrease). Based on discussion of Chapter 3.5.3, we use the color of blue as the main indicator to show our fluorescent variation. The other two colors serve as a negative control, which is to confirm that red and green remain constant over time while there is an increase in blue light. In this 0 CFU experiment, the A.U. value of blue color maintained stable, from 9.397 to 9.763 (3.89% increase), indicating that the biosensor detected no bacterial growth from a clean bottled water sample. This experiment serves as a negative control to show that the biosensor maintains nearly constant RGB values over a 24- hour period when the liquid sample tested in known to be clean. The detailed information is in Figure 32.

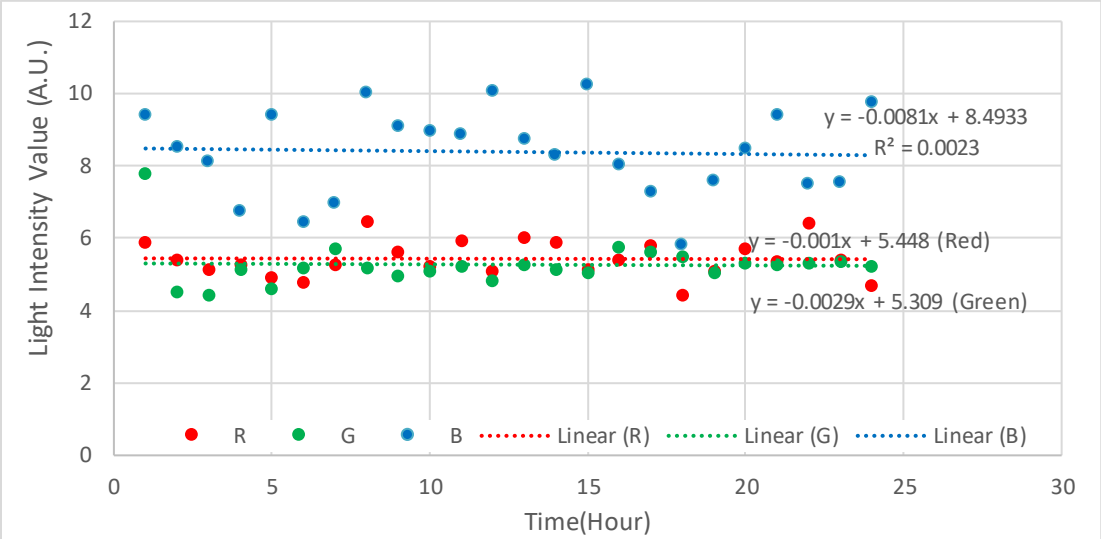


Figure 32: RGB data chart and Linear Regression for Clean Water Sample of 0 CFU/100mL

Figure 32 shows hourly RGB data from the clean water sample (distilled water sample) throughout the 24-hour incubation period. A linear regression was performed for each color and the slope was obtained. The slopes are -0.001 (red), -0.0029 (green), -0.0081 (blue). The values of the three slopes are close to zero in this negative control experiment. In the next experiments of Chapter 5 (bacterial water samples), color of blue will show greater slope value when compared to green and red. The value of R-squared value is comparatively low in this test, which is equal to 0.0023.

Table 9 shows the percent increase or decrease of RGB intensities in the 0 CFU/100mL experiment over the 24-hour period compared to the first measurement at hour one. From this table we can observe that all three colors maintained similar values throughout the 24-hour incubation period.

Table 9: Percent Change over 24-hour Period in RGB Intensity for Water Sample at 0 CFU/100mL versus First Hour

Time(hour)	Red	Green	Blue	Time(hour)	Red	Green	Blue
1	0.00	0.00	0.00	**13**	2.31	-32.59	-6.95
2	-8.06	-41.98	-9.48	**14**	0.14	-33.90	-11.58

3	-12.80	-43.27	-13.60	**15**	-13.23	-34.91	8.95
4	-10.89	-34.31	-27.99	**16**	-8.64	-26.04	-14.37
5	-16.51	-40.83	0.18	**17**	-2.03	-27.61	-22.47
6	-18.55	-33.21	-31.63	**18**	-24.53	-29.76	-37.82
7	-10.71	-26.44	-25.84	**19**	-13.40	-34.99	-19.39
8	10.00	-33.65	6.71	**20**	-3.00	-31.60	-9.87
9	-4.62	-36.07	-3.14	**21**	-9.48	-32.52	0.17
10	-11.17	-34.54	-4.48	**22**	8.73	-31.81	-20.25
11	0.87	-32.90	-5.67	**23**	-8.01	-31.25	-19.40
12	-13.74	-38.12	7.29	**24**	-20.06	-33.08	3.89

R-squared is a statistical measurement of how close the data are to the fitted regression line. The R value ranges from 0 to 1, which stands for the proportion of the variance for a dependent variable. In our case, the R squared is the difference error between the linear regression and its corresponding color intensity data. Low R squared values are due to high variability in the data. Our system suffers from low values of R-square likely due to the amount of auto adjustments performed by the python GUI software. Many of the image acquisition variables, such as White Balance, shutter speed, contrast and ISO, are automatically controlled. Other electronic issues may also add to this low R-square value, for example, changes in LED intensity, changes in ambient light, or the differences in the timing between the LED turning on and the CMOS sensor taking an image over the 24-hour period. More research needs to be performed controlling for all these variables and studying their effect on the resulting data.

5.2 Experiment of 23 CFU/100mL Water Sample

The experiment was carried out on Apr 12, 2018. Three tests were done simultaneously, which are one biosensor test and two Quanti-Tray tests. The biosensor and one Quanti-Tray measured the bacterial water sample (23 CFU/100mL). The other Quanti-Tray (experimental device No.3) measured the clean bottled water (distilled water) with Enterolert reagent. They were incubated at a temperature of around 40°C for 24 hours (see Table 10). The purpose of the biosensor test is to confirm that our device can detect a low-level contaminated water sample; 23 CFU/100mL is clean recreational water by EPA

standards. In addition, the goal of tray test filled with bacterial water sample is to confirm the CFU concentration of this sample. Finally, the Quanti-Tray filled with clean bottled water (control group) is used to ascertain that the water source is bacteria-free and that the colonies of bacteria originate from the dissolved Vitroid bacterial pellet.

Table 10: Experiment Plan for Bacterial Water Sample at 23 CFU/100mL

	Experiment Device	Solution	Incubation Device	Incubation Time
1	Biosensor Chip	Vitroid (23 CFU/mL) with Reagent	L121 Incubator @ 40 °C	24 Hours
2	Quanti-Tray	Vitroid (23 CFU/mL) with Reagent	L121 Incubator @ 41 °C	24 Hours
3	Quanti-Tray (Control Group)	Clean bottled water with Enterolert reagent	L121 Incubator @ 41 °C	24 Hours

5.2.1 Temperature Data Chart (23 CFU/100mL)

After 24-hour period, we obtained the temperature data of ambient environment and hotplate surface of the incubated bacterial water sample in ExperimentalDevice1 (see Figure 33). There are two temperature sensors (DS18B20): temperature sensor one is for the ambient temperature, and the other is responsible for the temperature of the incubator surface. The temperature reached 40℃ in around 2000 seconds and then the temperature kept warm for 24 hours.

There are two curves colored red (Sensor 1) and blue (Sensor 2). From the behavior of the red curve, the temperature of ambient environment kept consistently for 24 hours, which is close to 25℃. The blue curve represents the temperature of incubator surface. The behavior of this curve experienced variation which ranged from 35℃ to 40℃. This fluctuation of temperature may be due to the faulty of electric.

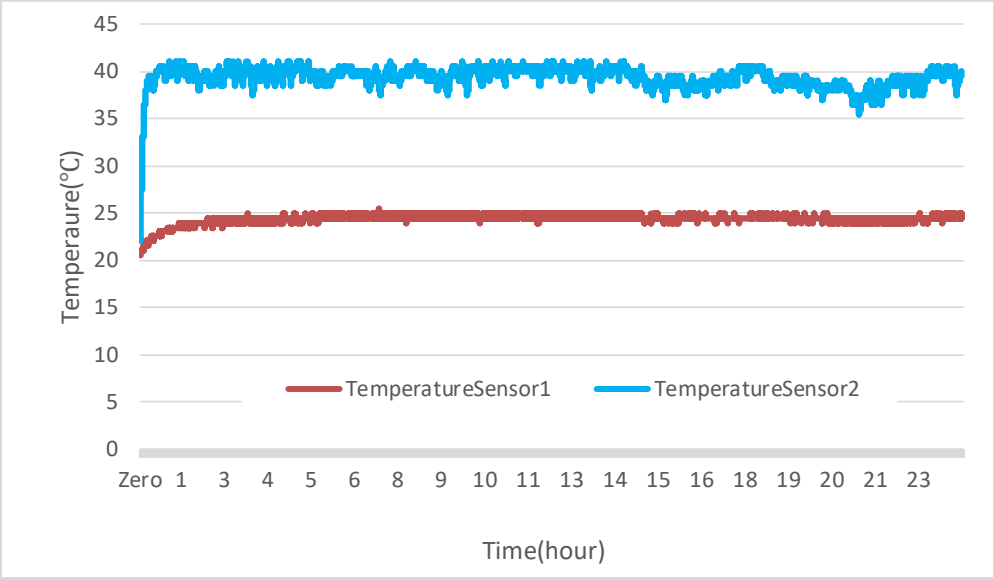


Figure 33: Incubation Temperature Data for Bacterial Water Sample at 23 CFU/100mL

5.2.2 Quanti-Tray Data (23 CFU/100mL)

For obtaining the fluorescent data, two Quanti-Tray 2000 trays were irradiated under the UV light of 361nm wavelength (see Figure 34). The two trays are experimental device No.2 and No.3. These two devices contained water samples of contaminated (bacterial) water and clean bottled water (distilled water). The sample of contaminated water exhibited fluorescent (positive) result of 16 large and 3 small wells. Referring to MPN table, the result of this fluorescence means the bacterial concentration of the contaminated water sample is approximately 23 CFU/100mL. In addition, clean bottled water sample displayed all negative wells after incubation. Based on the MPN table, the result of all negative large and smalls wells means the bacterial concentration of distilled water sample is 0 (<1) CFU/100mL. We regard this water sample as sterile level. From this point forward, we know that bottled water can provide bacteria-free water samples for the biosensor test.

	The display of the Quanti-Tray under UV light of 361nm wavelength
1. The bacterial water sample in Quanti-Tray(22.6 CFU/ 100ml)	
2. The control group with clean water	

Figure 34: Quanti-Tray result of Bacterial Water Sample at 23 CFU/100mL and Clean Water

5.2.3 Biosensor Image Data (23 CFU/100mL)

Two image data were taken in 1st hour and 24th hour of 24-hour period of incubation (see Figure 35). The first and last hour can show the general tendency of the fluorescent result. All of the measured light intensity data is presented in arbitrary units, A.U.

Figure 35 shows the numerical data of the 1st hour image: red is 6.860; green is 5.863; blue is 6.921 and total intensity is 8.172. The image data of 24th hour is as following: red is 6.589 (3.95% decrease); green is 7.739 (31.99% increase); blue is 15.503 (123.99% increase) and total intensity is 15.813 (85.14% increase, with a similar A.U. value to that of blue). The blue color has larger percentage increase, 123.99%, than the colored green and red. Therefore, the contaminated water sample experienced fluorescence indicative of bacterial growth.

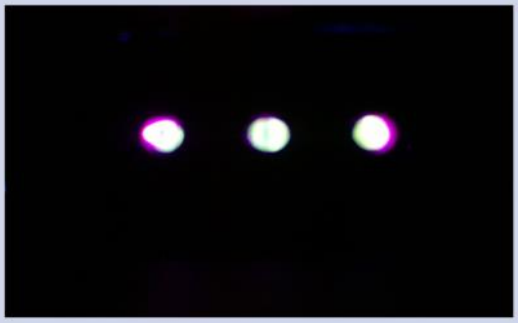
	The display of the Biosensor under UV light of 361nm wavelength
1) The bacterial water sample in biosensor under Camera (22.6 CFU/ 100ml) – 1st hour Red: 6.860 Green: 5.863 Blue: 6.921 Intensity: 8.172	
2) The bacterial water sample in biosensor under Camera (22.6 CFU/ 100ml) – 24th hour Red: 6.589 Green: 7.739 Blue: 15.503 Intensity: 15.813	

Figure 35: Image data of 1st and 24th hour for Bacterial Water Sample at 23 CFU/100mL

Figure 36 displays the raw-data scattered plot of the image data for 24-hour incubation. We can observe that value of colored blue and total intensity (color yellow) is increasing while the values of green and red stay relatively flat. In the 7th hour, the RGB data had an abnormally large value (R: 19.93; G: 21.12; B: 25.99), which is due to the ambient light entering the biosensor in the 7th hour image data. Except from the abnormal data of 7th hour, the blue value increases as the function of time.

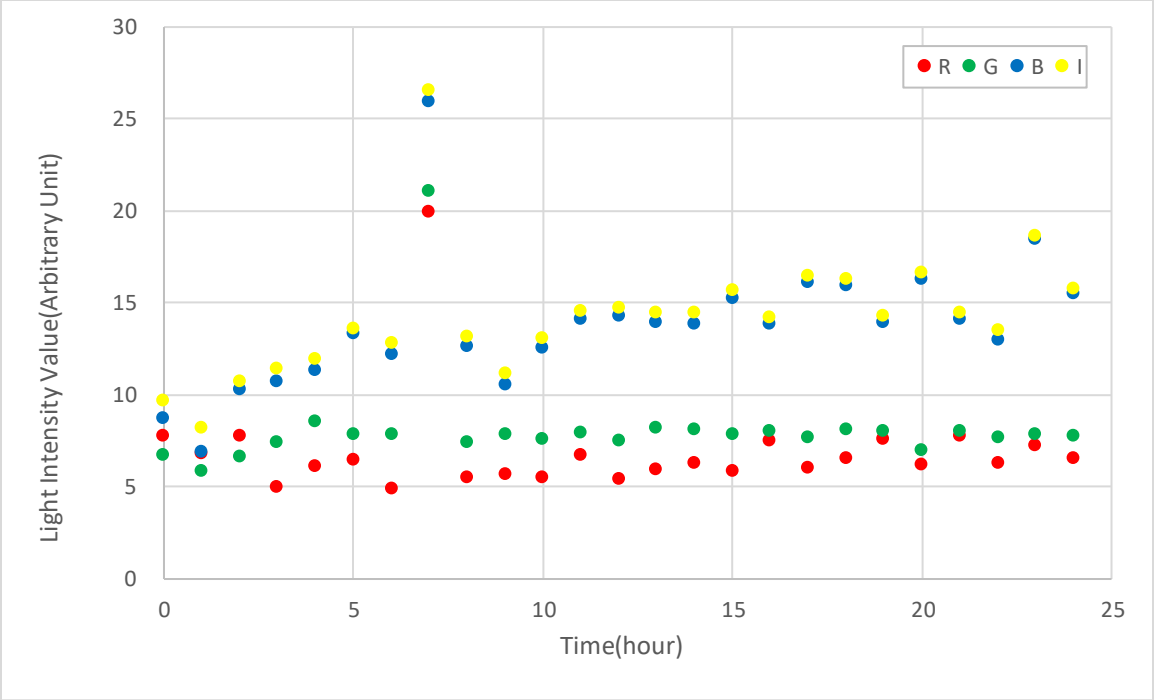


Figure 36: Raw RGB data chart for Bacterial Water Sample at 23 CFU/100mL

Table 11 shows the percent increase of RGB intensities in the 23 CFU/100mL experiment over the 24-hour period compared to the first measurement at the first hour. This table shows a greater percentage increase of blue over the 24-hour period when compared to red and green.

Table 11: Percent Change over 24-hour Period in RGB Intensity for Bacterial Water Sample at 23 CFU/100mL versus the First Hour

Time(hour)	Red	Green	Blue	Time(hour)	Red	Green	Blue
1	0	0	0	**13**	-13.53	40.50	101.93
2	12.79	13.53	48.29	**14**	-8.21	37.84	100.79
3	-27.65	26.79	55.06	**15**	-14.28	34.61	120.53
4	-11.01	45.38	63.43	**16**	9.26	37.35	99.86
5	-5.40	34.65	92.13	**17**	-12.23	30.42	132.89
6	-27.98	34.74	76.33	**18**	-4.55	37.98	129.85
7	Removed	Removed	Removed	**19**	10.36	36.55	101.03
8	-19.35	27.14	82.72	**20**	-9.44	19.67	135.30
9	-17.54	33.62	52.24	**21**	12.96	36.59	103.95
10	-19.38	29.25	81.25	**22**	-8.45	30.65	87.72
11	-1.84	35.18	103.91	**23**	5.99	33.59	166.72
12	-21.52	27.68	106.29	**24**	-3.95	31.99	123.99

5.2.4 Linear Regression Chart (23 CFU/100mL)

Bacteria follows a sigmoidal growth behavior. However, our sensor consistently measures a linear growth behavior over the 24-hour incubation period. This is highly likely due to the auto adjustments made by the CMOS camera software. We perform a linear regression as a way to quantitively measure growth of bacteria over the 24-hour incubation period and to compare the increase in blue color versus red and green. The slopes of each RGB color are measured and compared; a higher blue slope compared to red and green is indicative of bacterial growth. To plot each linear regression, we first discard any improper image data. Improper data is defined as an image where external light entered, resulting in an abnormally bright image, or the LEDs turned off, resulting in a very dark image. We observe each image to make sure it has ambient light entering from the outside before discarding it. Considering that the abnormal value at the 7^{th} hour was due to ambient light issue, we removed the 7^{th} hour data and use the rest of the hourly RGB data in the 24-hour incubation. The numerical data of R (colored red), G (colored green), B (colored blue) are plotted and we do analysis of linear regression of each color data (see Figure 37).

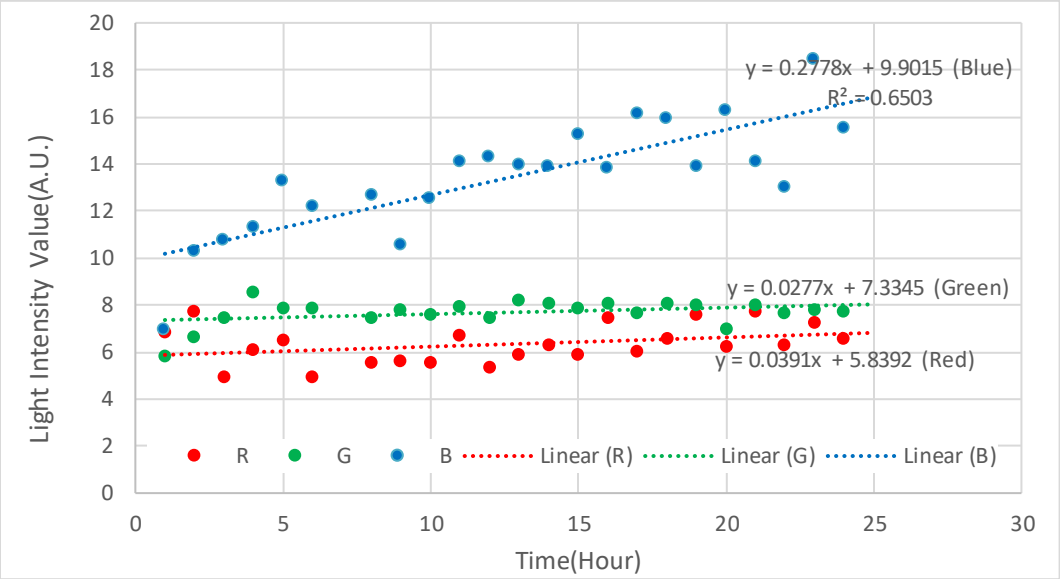


Figure 37: Linear Regression plotting for Bacterial Water Sample at 23 CFU/100mL

From Figure 37, the slope of color blue (0.277) is 902.89% greater than the slope of green (0.0277) and 610.49% that the slope of red (0.0391). Since we use the color of blue as the main indicator to show our fluorescent variation, the higher slope of blue color indicates that the biosensor has detected bacterial growth from a contaminated water sample (23 CFU/100mL). The R-squared value is 0.65, which shows high variability in the data.

The slope of the green and red are near zero throughout the 24-hour incubation period. They serve as a negative control, which is to confirm that red and green remain constant to verify the validity of an increase in blue light. Since blue increases, yet red and green remain constant, we understand that the increase in blue light is due to fluorescence and not external light sources or faulty LEDs.

5.3 Experiment of 1046 CFU/100mL Water Sample

The experiment was implemented on Mar 23, 2018. In this experiment, three tests were carried out simultaneously, which are one biosensor test and two Quanti-Tray tests. The biosensor and one Quanti-Tray (experimental device No.2) measured the bacterial

water sample (1046 CFU/100mL). The other Quanti-Tray (experimental device No.3) measured clean bottled water (distilled water) with Enterolert reagent. They were incubated at a temperature of around 41°C for 24 hours (see Table 12). The purpose of biosensor test is to confirm our device can detect the presence of a high-level contaminated water sample; 1046 CFU/100mL is considered contaminated recreational water by EPA standards. A contamination of 1046 CFU/100mL could occur in beaches around Puerto Rico. The goal of Quanti-Tray test with bacterial water sample is to confirm the CFU concentration of this sample. Finally, the Quanti-Tray filled with clean bottled water (control group) is to ascertain that the water source is bacteria-free and that the colonies of bacteria originate from the dissolved Vitroid bacterial pellet.

Table 11: Experiment Plan for Bacterial Water Sample at 1046 CFU/100mL

	Experiment Device	Solution	Incubation Device	Incubation Time
1	Biosensor Chip	Vitroid (1046 CFU/mL) with Reagent	L121 Incubator @ 41 °C	24 Hours
2	Quanti-Tray	Vitroid (1046 CFU/mL) with Reagent	L121 Incubator @ 41 °C	24 Hours
3	Quanti-Tray (Control Group)	Clean bottled water with Enterolert reagent	L121 Incubator @ 41 °C	24 Hours

5.3.1 Temperature Data Chart (1046 CFU/100mL)

Figure 38 displays the temperature data of ambient environment and hotplate surface of the incubated bacterial water sample (1046 CFU/100mL) throughout 24 hours. This temperature data was obtained via experimental device No.1. The hotplate temperature reached 41°C in around 2000 seconds and then the value maintains stable for 24 hours. There are two temperature sensors measuring data plotted in the chart. The

temperature one is for the ambient temperature, and the temperature sensor two measure the temperature on the surface of incubator.

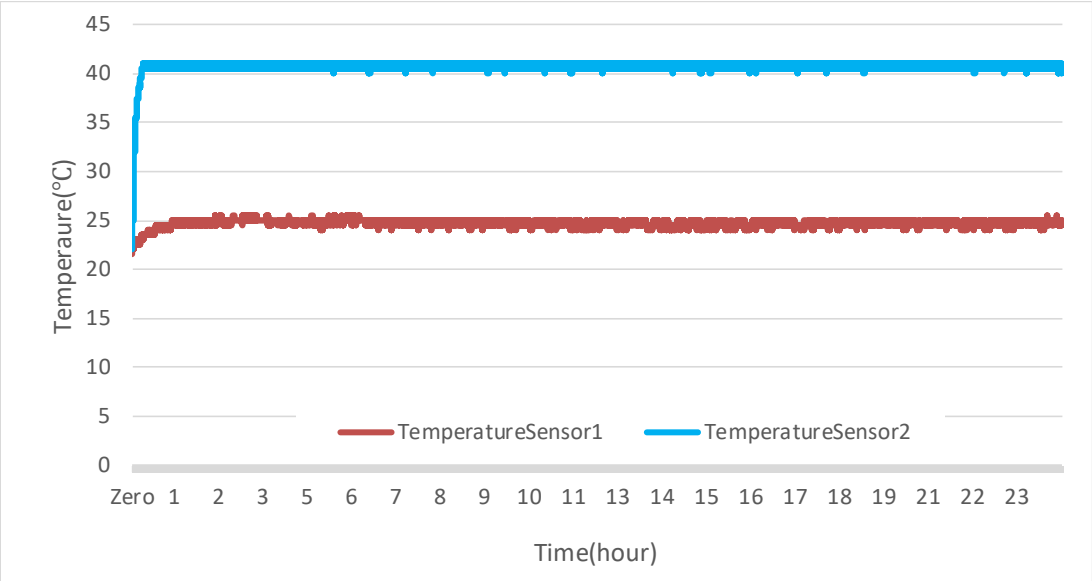


Figure 38: Temperature data for Bacterial Water Sample at 1046 CFU/100mL

5.3.2 Quanti-Tray Data (1046 CFU/100mL)

For acquiring the fluorescent data, two Quanti-Tray 2000 trays were measured under the UV light of 361nm wavelength (see Figure 39). The two trays are experimental device No.2 and No.3. These two devices contained samples of the contaminated (bacterial) water and clean bottled water, respectively. The contaminated water sample exhibited fluorescent (positive) result of 49 large and 39 small wells. Referring to MPN table, this incidence means the bacterial concentration of the inoculated contaminated water sample is 1046 CFU/100mL. In addition, the sample of clean bottled water displayed all negative wells after incubation. Based on MPN table, the result of all negative large and smalls wells means the bacterial concentration of the control group is 0 (<1) CFU/100mL. We regard this distilled water sample from control group as sterile (bacteria-free) level.

	The display of the Quanti-Tray under UV light of 361nm wavelength
1. The bacterial water sample in Quanti-Tray (1046CFU/ 100ml)	
2. The control group with clean water	

Figure 39: Quanti-Tray Data for Bacterial Water Sample (1046 CFU/100mL) and Clean Water

5.3.3 Biosensor Image Data (1046 CFU/100mL)

Two image data were taken in 1st hour and 23rd hour of 24-hour period of incubation (see Figure 40). We obtained the image data of the 1st hour: red is 10.014; green is 7.156; blue is 11.813 and total intensity is 13.096. The image data of 23rd hour is as following: red is 5.909 (41.00% decrease); green is 9.992 (39.63% increase); blue is 18.580 (57.28% increase) and total intensity is 18.961 (44.78% increase). The blue color has that highest A.U. value and the largest percentage increase, 57.28%, at the 23rd hour. Therefore, the contaminated water sample experienced a fluorescent increase after the 24-hour incubation period.

	The display of the Biosensor under UV light of 361nm wavelength
1) The bacterial water sample in biosensor under Camera (1046 CFU/ 100ml) – 1st hour Red: 10.014 Green: 7.156 Blue: 11.813 Intensity: 13.096	
2) The bacterial water sample in biosensor under Camera (1046 CFU/ 100ml) – 23rd hour Red: 5.909 Green: 9.992 Blue: 18.580 Intensity: 18.961	

Figure 40: Image data of 1st and 23rd hour for Bacterial Water Sample at 1046 CFU/100mL

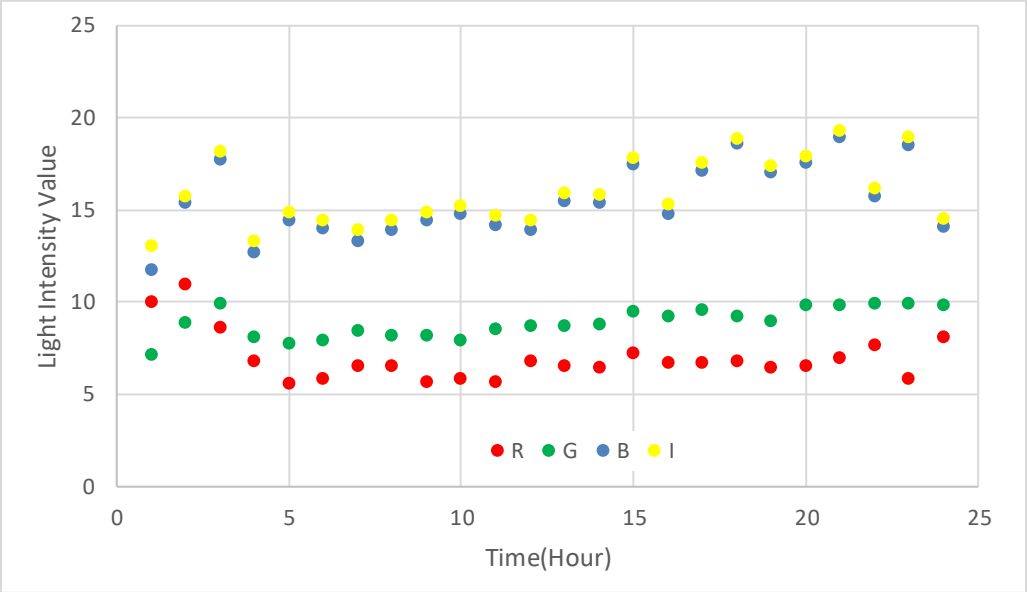


Figure 41: Raw RGB data chart for Bacterial Water Sample at 1046 CFU/100mL

Figure 41 displays the raw image data for the 24-hour incubation period. We can observe that A.U. value of blue intensity is increasing. Table 13 shows the percent increase of RGB intensities in the 1046 CFU/100mL experiment over the 24-hour period compared to the first measurement at the first hour. Notice that the increase in blue is always greater than the increase in red and greater than the increase in green in 18 out of the 24-hourly measurements.

Table 12: Percent Change over 24-hour Period in RGB Intensity for Bacterial Water Sample at 1046 CFU/100mL versus the First Hour

Time(hour)	Red	Green	Blue	Time(hour)	Red	Green	Blue
1	0.00	0.00	0.00	**13**	-34.70	21.80	31.05
2	9.44	23.94	30.40	**14**	-35.49	23.86	30.33
3	-13.26	39.07	50.66	**15**	-27.51	32.74	48.23
4	-32.12	13.69	8.07	**16**	-32.83	29.74	25.54
5	-44.32	9.22	22.24	**17**	-32.27	33.98	45.39
6	-41.64	10.68	18.53	**18**	-31.95	29.82	57.50
7	-34.68	18.89	13.18	**19**	-34.86	25.80	44.23
8	-34.53	15.06	18.15	**20**	-34.63	37.74	49.23
9	-43.27	15.00	22.27	**21**	-30.15	37.75	60.83
10	-41.74	11.36	25.71	**22**	-23.12	39.54	33.83

11	-42.97	19.55	20.26	**23**	-41.00	39.63	57.28
12	-31.76	22.40	17.89	**24**	-18.56	38.42	19.51

5.3.4 Linear Regression Chart (1046 CFU/100mL)

Figure 42 is the linear regression for RGB intensity of image data. There are no issues with external light source or non-functioning LEDs in this data set. We used the 24-hourly points of RGB data to plot the linear regression.

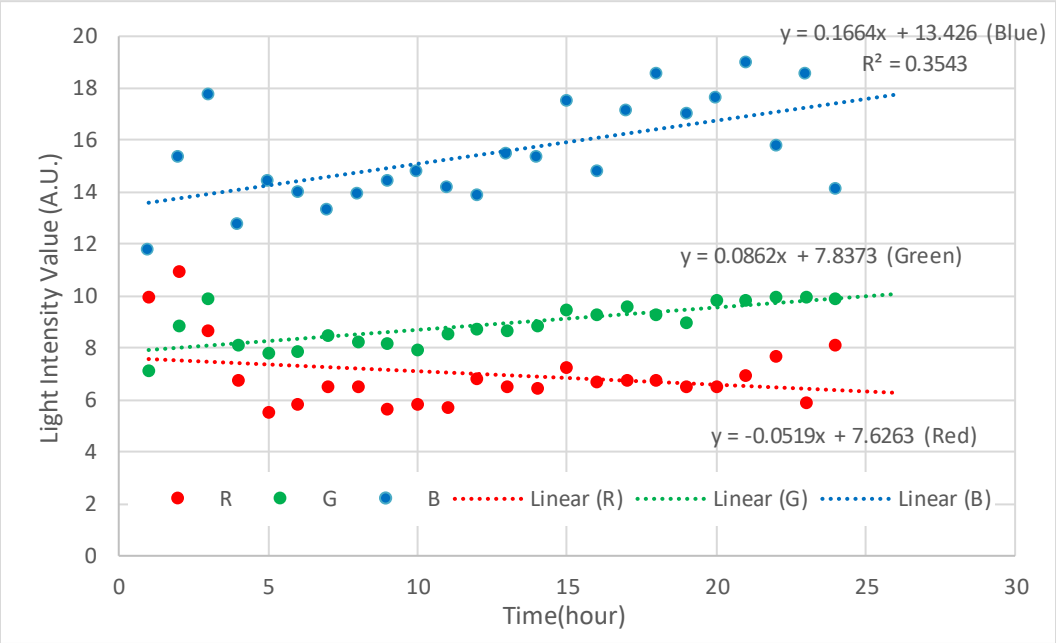


Figure 42: Linear Regression Plotting for Bacterial Water Sample at 1046 CFU/100mL

From Figure 42, the color of blue increases through 24-hour period. The color of green and red keep relatively flat. The slope of color blue (0.1664) is 93.04% greater than slopes of green (0.0862) and 420.62% that the slope of red (-0.0519). Since we use the color of blue as the main indicator to show our fluorescent variation, the higher slope of blue color indicates that the biosensor has detected bacterial growth from a contaminated water sample (1046 CFU/100mL). In addition, the R-squared value is 0.35, which shows less variability in the data than in the 23 CFU/100mL experiment. Finally, colored green

and red serve as a negative control, which is to verify the validity of an increase in blue light. Therefore, the increase in blue light is due to fluorescence and not external light sources or faulty LEDs.

5.4 Experiment of 1500 CFU/100mL Water Sample

The experiment was implemented on May 15, 2017. Three tests were done simultaneously, which are one biosensor test and two Quanti-Tray tests. The biosensor and one Quanti-Tray measured the bacterial water sample (1500 CFU/100mL). The second Quanti-Tray (experimental device No.3) measured the clean bottled water (distilled water) with Enterolert reagent. They were incubated under temperature of around 41°C for 24 hours (see Table 14). The purpose of biosensor test is to confirm our device can detect the presence of contaminated water sample at a concentration of 1500 CFU/100mL. In addition, the goal of Quanti-Tray test filled with bacterial water sample is to confirm the CFU concentration of this sample. Finally, the Quanti-Tray filled with clean bottled water (control group) is to ascertain that the water source is bacteria-free and that the colonies of bacteria originate from the dissolved Vitroid bacterial pellet.

Table 13: Experiment Plan for Bacterial Water Sample at 1500 CFU/100mL

	Experiment Device	Solution	Incubation Device	Incubation Time
1	Biosensor Chip	Vitroid (1500 CFU/mL) with Reagent	L121 Incubator @ 41 °C	24 Hours
2	Quanti-Tray	Vitroid (1500 CFU/mL) with Reagent	L121 Incubator @ 41 °C	24 Hours
3	Quanti-Tray (Control Group)	Clean bottled water with Enterolert reagent	L121 Incubator @ 41 °C	24 Hours

5.4.1 Temperature Data Chart (1500 CFU/100mL)

From Figure 43, the hotplate temperature reached 41°C in around the first hour and maintains constant for 24 hours. This temperature data was obtained via experimental device No.1. The temperature sensor No.1 is showing the ambient temperature, and the temperature sensor No.2 was placed on the surface of the incubation hot plate. The ambient temperature experienced cyclic variations, probably due to the turning on and off of an air conditioner. The blue curve represents the temperature of incubation hot plate surface, the behavior of this curve kept consistently at 41°C.

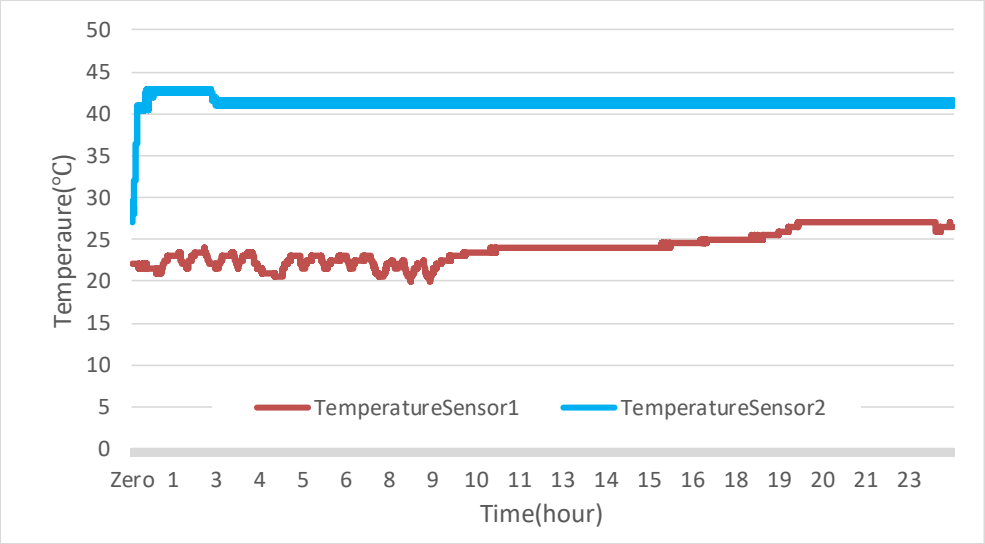


Figure 43: Temperature Data for Bacterial Water Sample at 1500 CFU/100mL

5.4.2 Quanti-Tray Data (1500 CFU/100mL)

To obtain Qaunti-Tray data, two Quanti-Tray 2000 trays were irradiated under the UV light of 361nm wavelength (see Figure 44). The two trays are experimental devices No.2 and No.3. These two devices contained water samples of contaminated (bacterial) water and clean bottled water (distilled water), respectively. The tray filled with contaminated water exhibited fluorescent (positive) result of 49 large and 45 small wells. Referring to MPN table, the result of this fluorescent incidence means the bacterial

concentration of bacterial water sample is 1500 CFU/100mL. The clean bottled (distilled) water sample displayed all negative wells after incubation. Based on MPN table, the result of all negative large and small wells means the bacterial concentration of the control group is 0 (<1) CFU/100mL. We regard this water sample from control group as sterile (bacteria-free) level.

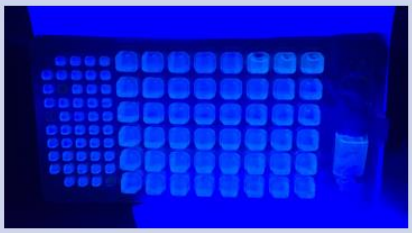
	The display of the Quanti-Tray under UV light of 361nm wavelength
1. The bacterial water sample in Quanti-Tray(1500 CFU/ 100ml)	
2. The control group with clean water	

Figure 44: Quanti-Tray Data for Bacterial Water Sample at 1500 CFU/100mL and Clean water

5.4.3 Biosensor Image Data (1500 CFU/100mL)

We obtained the image data of 4th hour and 24th hour from 24-hour period of incubation (see Figure 45). The numerical data of the 4th hour image: red is 4.327; green is 6.071; blue is 8.571 and total intensity is 9.240. The image data of 24th hour is as following: red is 15.682 (262.39% increase); green is 16.572 (172.96% increase); blue is 34.840 (306.15% increase) and total intensity is 34.874 (277.42% increase). The blue color has the largest A.U. intensity value, 34.84, and the highest percentage increase, 306.15%. We

understand then that the bacteria water sample on the biosensor (1500 CFU/100mL) has experienced a fluorescent value increase due to bacteria growth.

	The display of the Biosensor under UV light of 361nm wavelength
1) The bacterial water sample in biosensor under Camera (1500 CFU/ 100ml) – 4th hour Red: 4.3273 Green: 6.0713 Blue: 8.5781 Intensity: 9.2404	
2) The bacterial water sample in biosensor under Camera (1500 CFU/ 100ml) – 24th hour Red: 15.682 Green: 16.572 Blue: 34.840 Intensity: 34.874	

Figure 45: Image data of 4th and 24th hour for Bacterial Water Sample at 1500 CFU/100mL

Figure 46 displays the raw-data scattered plot the image data for 24-hour incubation. This data set suffers from very high variability in the data across the 24-hour period. Visual observations of each data image are necessary to understand the reason for such high data variability.

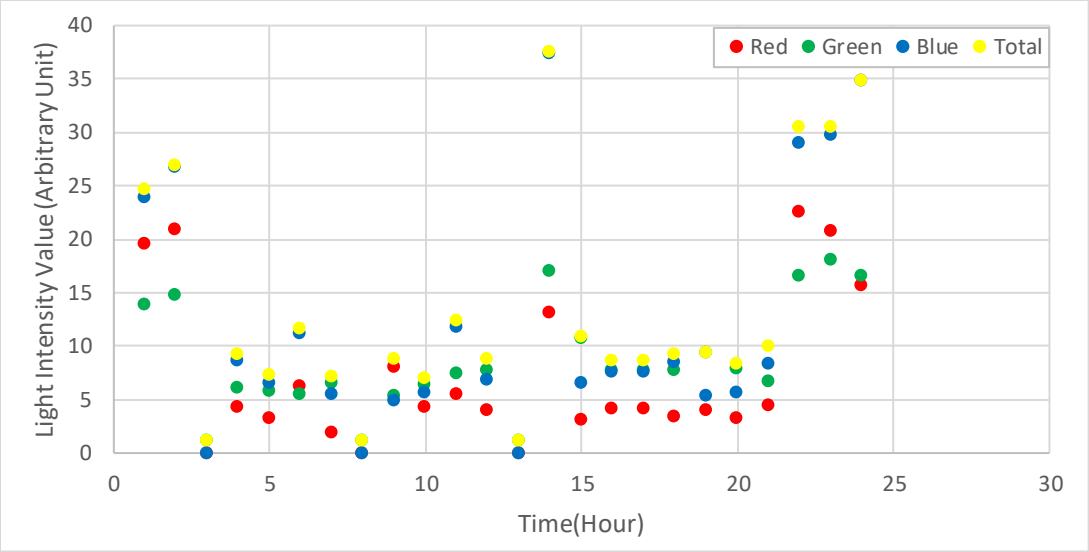


Figure 46: Raw RGBI data chart for Bacterial Water Sample at 1500 CFU/100mL

Table 15 shows the percent increase or decrease of RGB intensities in the 1500 CFU/100mL experiment over the 24-hour period compared to the first measurement at hour one, after removing the erroneous data points; the rationale for removal is explained in Section 5.4.4.

Table 14: Percent Change over 24-hour Period in RGB Intensity for Bacterial Water Sample at 1500 CFU/100mL versus the First Hour

Time(hour)	Red	Green	Blue	Time(hour)	Red	Green	Blue
1	Removed	Removed	Removed	**13**	Removed	Removed	Removed
2	Removed	Removed	Removed	**14**	204.76	180.53	336.35
3	Removed	Removed	Removed	**15**	-29.03	76.68	-24.55
4	0.00	0.00	0.00	**16**	-5.23	26.77	-10.75
5	-23.72	-5.55	-24.13	**17**	-5.23	26.77	-10.75
6	45.05	-8.73	29.93	**18**	-21.68	28.54	-0.65
7	-57.28	8.70	-36.84	**19**	-6.59	54.52	-37.33
8	Removed	Removed	Removed	**20**	-26.38	30.53	-34.85
9	86.04	-11.56	-43.79	**21**	1.08	10.60	-3.74
10	-2.25	6.02	-33.60	**22**	Removed	Removed	Removed
11	26.33	22.85	37.58	**23**	Removed	Removed	Removed
12	-7.72	26.42	-20.79	**24**	262.39	172.96	306.15

5.4.4 Linear Regression Chart (1500 CFU/100mL)

The Figure 47 below is the linear regression graph for all color image data. First, we removed the image data obtained when external light entered, which are at the 1st, 2nd, 22nd and 23rd hours. These data were removed after visually inspecting each image and observing external light entering the chamber. Then we check the groups of data with low light intensity, these are at the 3rd, 8th and 13thhour. After checking each of these images, we observed that these data were obtained under dark emission light source that is due to the LED array not turning on. After removing these six data points, we use the remaining data to make a scatter plot and do the linear regression for each of color. An interesting detail is that the majority of the removed data points all had Red values higher than Green values. Currently we don't understand the reason for this but with more study this relationship could potentially be used as a way to automatically filter for erroneous data points.

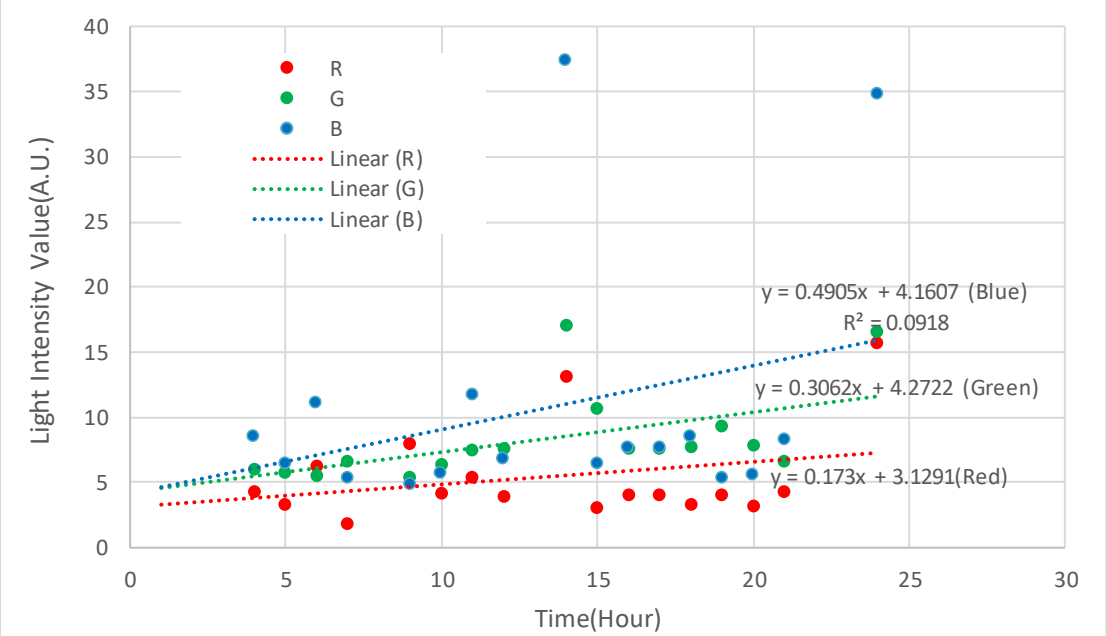


Figure 47: Linear Regression plotting for Bacterial Water Sample at 1500 CFU/100mL

In Figure 47, the color of blue increases through 24-hour period. The slope of color blue (0.4905) is 60.19% greater than the slope of green (0.3062) and 183.53% that the slope

of red (0.173). Since we use the color of blue as the main indicator to show our fluorescent variation, the higher slope of blue color indicates that the biosensor has detected bacterial growth from a contaminated water sample (1500 CFU/100mL). In addition, the R-squared value is 0.09 shows very high variability in the data. Finally, the colored green and red serve as a negative control to verify that the increase in blue light is due to fluorescence. Since by the end of the incubation period the blue increases with greater slope than red and green, we understand that the increase in blue light is due to fluorescence from bacterial growth and not external light sources or faulty LEDs.

5.5 Experiment of 6896 CFU/100mL Water Sample

The experiment was carried out on May 3, 2018. Table 16 displays the plan for this experiment. The bacterial range of Vitroid is 3000-7000 CFU, which is later proved to be 6896 CFU/100mL after incubation. This level of contamination is similar to that of raw sewage.

Table 15: Experiment Plan for Bacterial Water Sample at 3000-7000 CFU/100mL

	Experiment Device	Solution	Incubation Device	Incubation Time
1	Biosensor Chip	Vitroid (3000-7000 CFU/100mL) with Reagent	L121 Incubator @ 40 °C	24 Hours
2	Quanti-Tray	Vitroid (3000-7000 CFU/100mL) with Reagent	L121 Incubator @ 41 °C	24 Hours
3	Quanti-Tray	Vitroid ([3000-7000]/20 CFU/100mL) with Reagent	L121 Incubator @ 41 °C	24 Hours
4	Quanti-Tray (Control Group)	Clean bottled water with Enterolert reagent	L121 Incubator @ 41 °C	24 Hours

The biosensor (experimental device No.1) and one Quanti-Tray (experimental device No.2) measured the bacterial water sample (3000-7000 CFU/100mL). A second Quanti-Tray (experimental device No.3) measured a 20:1 diluted bacterial water sample ((3000-7000)/20 CFU/100mL), which was achieved by diluting 5 mL bacterial water sample from device No.2 into 95 mL of clean water with Enterolert reagent. The Quanti-Tray (experimental device No.4) measured the clean bottled water (distilled water) with Enterolert reagent. They were incubated under temperature of around 41°C for 24 hours. On the hotplate, there was heat dissipation and the surface temperature maintained around 40°C for the biosensor measurement.

The third device uses 5ml bacterial water sample (3000-7000 CFU/100mL) and 95ml clean bottled water to form a diluted bacterial water sample (dilution ratio 1:20) in order to measure the CFU with a Quanti-Tray and the MPN table. The range of MPN table is 1 - 2419 CFU/100mL. Thus, 3000-7000 water sample is untestable by MPN without dilution. Therefore, after 24-hour incubation, we can obtain the bacterial concentration from device No.3, which is the twenty times less than the concentration of device No.2.

5.5.1 Temperature Data Chart (6896 CFU/100mL)

The temperature data of ambient environment and hotplate surface of the incubated bacterial water sample for 24-hour period is shown in Figure 48. This temperature data was obtained by ExperimentalDevice1. There are two temperature sensors (DS18B20): temperature sensor one is for the ambient temperature, and the other measured the temperature of the incubator surface. The surface of incubator reached 41°C less than one hour and then the temperature of hotplate and ambient environment keep stable for 24 hours.

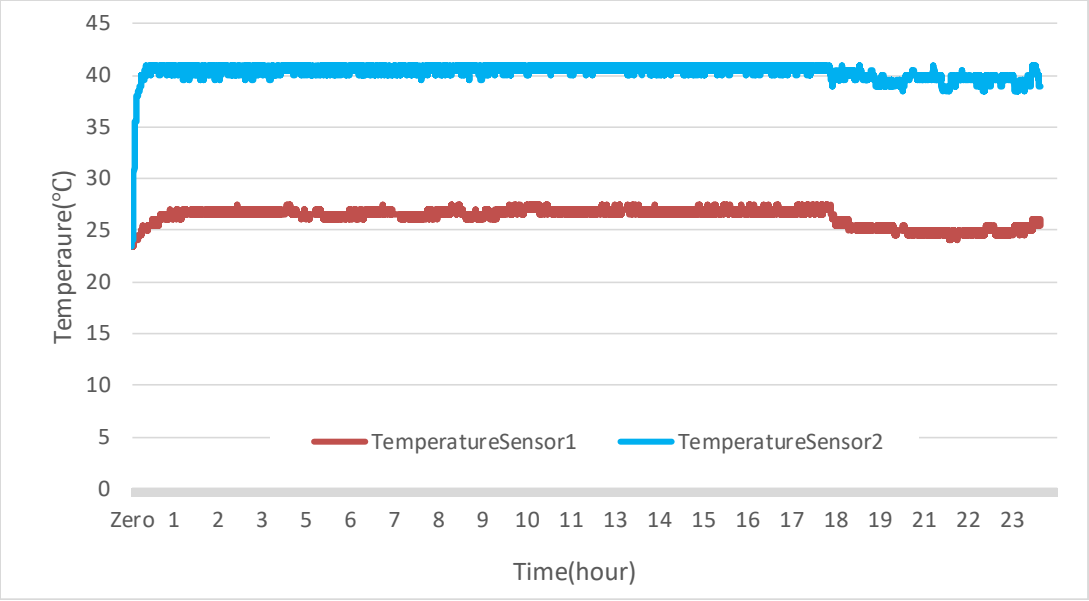


Figure 48: Temperature data for Bacterial Water Sample at 6896 CFU/100mL

5.5.2 Quanti-Tray Data (6896 CFU/100mL)

For obtaining the fluorescent data, three Quanti-Tray 2000 trays were irradiated under the UV light of 361nm wavelength (see Figure 49). The three trays are experimental device No.2 (bacterial water sample), No.3 (diluted bacterial water sample), No.4 (distilled water). The experimental device No.2 exhibited fluorescent (positive) result in every single well; 49 large and 48 small wells. Referring to MPN table, result of all positive wells means the bacterial concentration of this contaminated water sample is greater or equal to 2419 CFU/100mL. The diluted sample from device No.3 shows 49 large wells and 20 small wells, which is 344.8 CFU/100mL referring to MPN Table. Due to dilution ratio (1:20), we can obtain the bacterial concentration of this contaminated water sample in device No.2 (6896 CFU/100mL). This same high concentration (6896 CFU/100mL) is the one used in the biosensor.

Finally, the sample of distilled water (experimental device No.4) displayed all negative wells after incubation. Based on MPN table, the result of all negative large and

smalls wells means the bacterial concentration of the control group is <1 CFU/100mL. We regard distilled water sample from control group as sterile (bacteria-free) level.

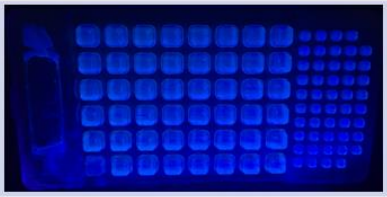
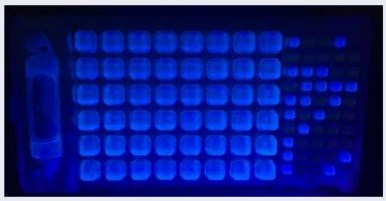
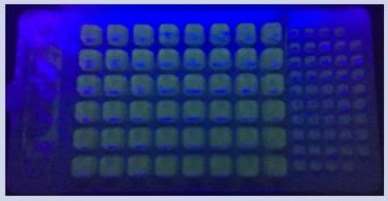
	The Display of the Biosensor under UV light of 361nm wavelength
1) The bacterial water sample in Quanti-Tray (6896 CFU/100mL)	
2) The bacterial water sample (diluted 1:20) in Quanti-Tray (6896 CFU/100mL)	
3) The control group with clean water	

Figure 49: Quanti-Tray Data for Bacterial Water Sample at 6896 CFU/100mL and Clean Water

5.5.3 Biosensor Image Data (6896 CFU/100mL)

Two image data were taken in 1st hour and 24th hour of 24-hour period of incubation (see Figure 50). We obtain the numerical data of the 1st hour image: red is 3.718; green is 5.986; blue is 6.829 and total intensity is 8.398. The image data of 24th hour is as following: red is 5.229 (28.55% decrease); green is 9.601 (60.39% increase); blue is 19.038 (178.78% increase) and total intensity is 19.382 (130.79% increase, similar to blue). The blue color has the highest intensity, 19.038 A.U., and the largest percentage increase, 178.78%, after the 24-hour incubation period. Thus, the biosensor observed an increase fluorescence after incubation in the 6896 CFU/100mL water sample.

	The display of the Biosensor under UV light of 361nm wavelength
1) The bacterial water sample in biosensor under Camera (6896 CFU/ 100mL) – 1st hour Red: 3.718 Green: 5.986 Blue: 6.829 Intensity: 8.398	
2) The bacterial water sample in biosensor under Camera (6896 CFU/100mL) – 24th hour Red: 5.229 Green: 9.601 Blue: 19.038 Intensity: 19.382	

Figure 50: Image data of 1st and 24th hour for Bacterial Water Sample at 6896 CFU/100mL

Figure 51 displays the raw-data scattered plot for image data of 24-hour incubation. We can observe that value of colored blue and total intensity (colored yellow) is increasing. The color data of green and red stay relatively flat. From the 16th hour to 21st hour of incubation, the image is almost completely dark, which is due to the LEDs not turning on. From the 22nd hour to the 24th hour, the CMOS function resumes.

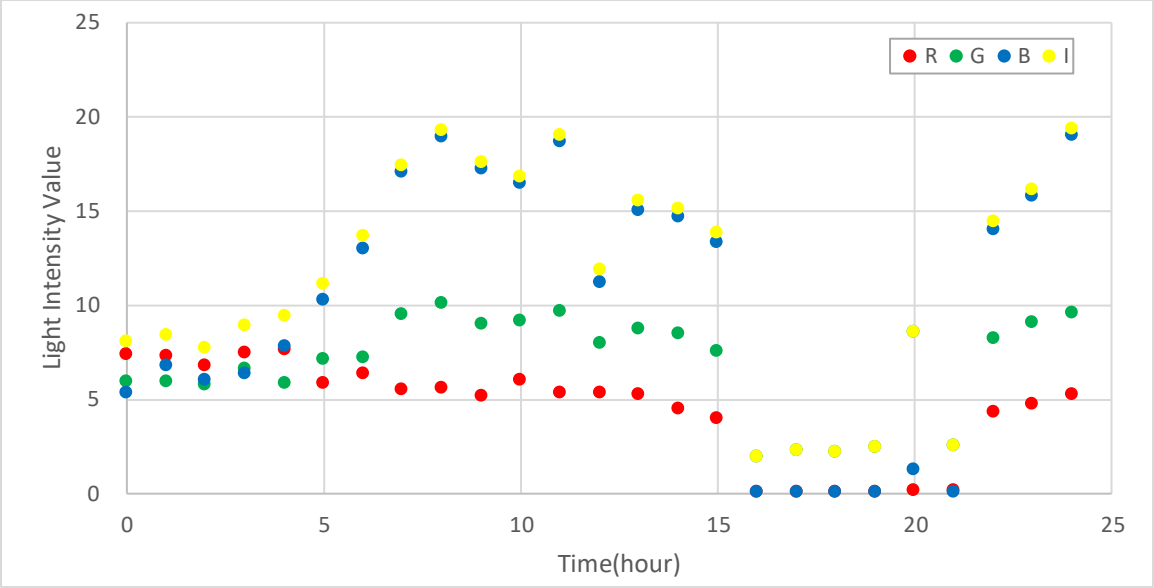


Figure 51: Raw RGBI data chart for Bacterial Water Sample at 6896 CFU/100mL

Table 17 shows the percent increase of RGB intensities in the 6898 CFU/100mL experiment over the 24-hour period compared to the first measurement at hour one. Notice the blue color experiences a larger increase in intensity versus red and green in 16 out of the 17 viable data points.

Table 16: Percent Change over 24-hour Period in RGB Intensity for Bacterial Water Sample at 6896 CFU/100mL versus the First Hour

Time(hour)	Red	Green	Blue	Time(hour)	Red	Green	Blue
1	0.00	0.00	0.00	**13**	-27.67	46.41	120.64
2	-6.93	-24.51	24.57	**14**	-38.21	41.81	114.86
3	2.66	-26.19	18.90	**15**	-45.43	25.77	95.60
4	5.01	-14.52	-0.91	**16**	Removed	Removed	Removed
5	-19.90	-23.01	37.87	**17**	Removed	Removed	Removed
6	-12.67	-13.10	-5.91	**18**	Removed	Removed	Removed
7	-24.33	-4.28	2.06	**19**	Removed	Removed	Removed
8	-23.47	-13.66	46.84	**20**	Removed	Removed	Removed
9	-29.61	-16.81	33.29	**21**	Removed	Removed	Removed
10	-18.00	-14.83	31.45	**22**	-40.57	37.23	104.72
11	-27.16	-12.69	29.82	**23**	-34.67	51.64	130.98
12	-26.93	-19.48	47.65	**24**	-28.55	60.39	178.78

5.5.4 Linear Regression Chart (6896 CFU/100mL)

Figure 52 has the RGB linear regressions for the biosensor data in the 6896 CFU/100mL experiment. Data points where the LEDs did not turn on were removed; these data points correspond from hour 16 to hour 21. The rest of the data points were used for plotting each color trendline.

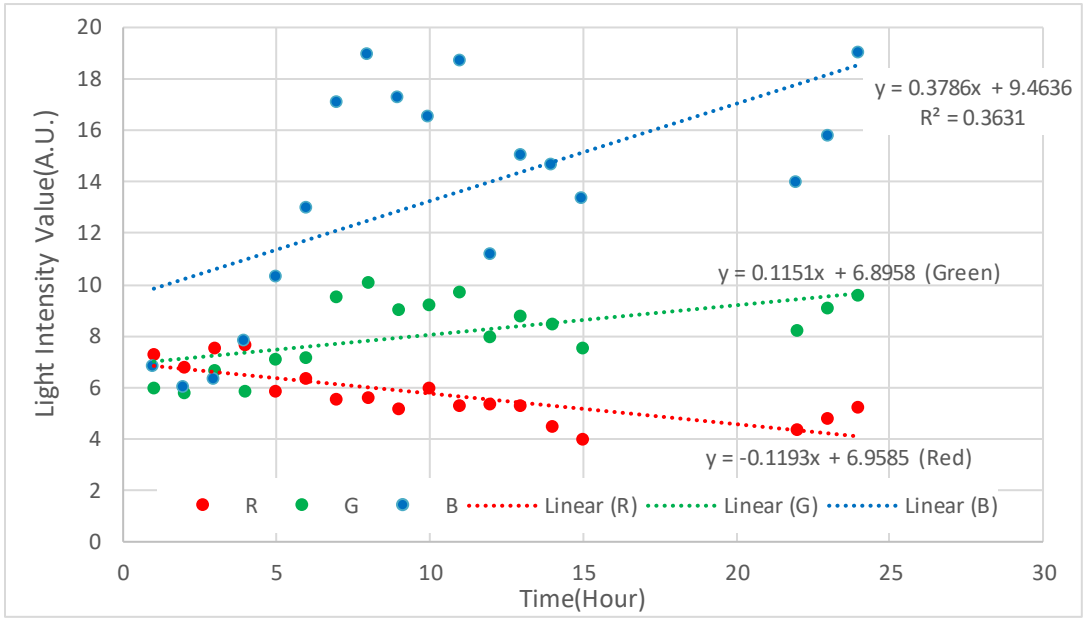


Figure 52: Linear Regression Plotting for Bacterial Water Sample at 6896 CFU/100mL

In Figure 52, the color of blue increases through 24-hour period. The color of green and red keep relatively flat. The slope of color blue (0.3786) is 228.93% greater than the slope of green (0.1151) and 417.35% that the slope of red (-0.1193). Since we use the color of blue as the main indicator to show our fluorescent variation, the higher slope of blue color indicates that the biosensor has detected bacterial growth from a contaminated water sample of 6896 CFU/100mL.

In addition, the slope of the green and red serve as a negative control to verify the validity of an increase in blue light. Since blue increases, yet red and green remain relatively constant, we understand that the increase in blue light is due to fluorescence and not external light sources.

The sigmoidal growth nature of bacteria can somewhat be observed from this data. An exponential increase can be seen from hour 1 until hour 8. It makes sense that fluorescence will grow very quickly at such a high CFU. However, intensity values decrease until increasing again at the final hour. The R-squared value of 0.36 shows high variability in the data, in part due to a linear fit over this quasi-sigmoidal behavior. As observed in previous experiments, auto adjustments of the CMOS sensor could be responsible for converting a sigmoidal growth behavior into a linear one.

5.6 Data Validation between GUI System and ImageJ

Table 18 shows a comparison between the Python GUI system and ImageJ by measuring fluorescence at the 24th hour for each bacterial concentration. The purpose of this experiment is to validate the result that we obtain from the automated biosensor Python GUI. The software ImageJ is a Java-based image processing system. It was developed by the National Institutes of Health. ImageJ is a public domain software and is widely used in the field of biological research. The purpose of this test is to certify the data we obtain can be reliable for analysis and is helpful for the data analysis in section 5.7 (light intensity versus bacterial concentration test).

In order to perform an objective comparison between the two software, we use the 24th hour data image from the 0 CFU/100mL, 23 CFU/100mL, 1046 CFU/100mL,1500 CFU/100mL and 6896 CFU/100mL experiments. An image for each well at every concentration was cut out of the original image data and analyzed individually. The RGB value of each well at each concentration was measured. Table 18 shows an image of each well at each concentration.

ImageJ RGB analysis of each individual well at the 24th hour is shown in Table 19. RGB analysis of each individual well at the 24th hour using our custom-made Python GUI system is shown in Table 20. From Table 19 and Table 20, we can observe the RBG data (the 24th hour for each bacterial concentration) obtained by the Python GUI system and ImageJ is the exact same. Therefore, we validate the data from Python GUI system used in the automated biosensor.

Table 17: Cut Image from 24th hour for Various Bacterial Concentration

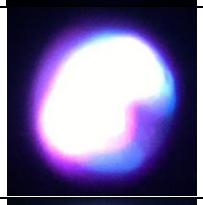
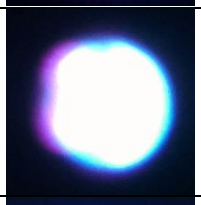
	1st Well	2nd Well	3rd Well
0 CFU/100mL			
23 CFU/100mL			
1046 CFU/100mL			
1500 CFU/100mL			
6896 CFU/100mL			

Table 18: Detailed RGB values of Cutting Wells (24th hour) obtained by ImageJ

	1st Well	2nd Well	3rd Well
0 CFU/100mL	R: 92.37 G: 79.03 B: 128.05	R: 99.25 G: 88.86 B: 140.92	R: 94.75 G: 90.5 B: 138.76
23 CFU/100mL	R: 111.2 G: 98.96 B: 149.31	R: 96.85 G: 102 B: 151.7	R: 121.25 G: 116.39 B: 162.76
1046 CFU/100mL	R: 126.4 G: 123.9 B: 152.46	R: 99.16 G: 107.89 B: 133.59	R: 104.19 G: 121.44 B: 143.38
1500 CFU/100mL	R: 103.69 G: 111.12 B: 160.48	R: 76.06 G: 92.3 B: 135.35	R: 105.73 G: 115.38 B: 162.93
6896 CFU/100mL	R: 97.21 G: 122.02 B: 160.96	R: 97.23 G: 129.92 B: 169.37	R: 77.65 G: 106.17 B: 141.16

Table 19: Detail RGB values of Cutting Wells (24th hour) obtained by Python GUI

	1st Well	2nd Well	3rd Well
0 CFU/100mL	R: 92.37 G: 79.03 B: 128.05	R: 99.25 G: 88.86 B: 140.92	R: 94.75 G: 90.5 B: 138.76
23 CFU/100mL	R: 111.2 G: 98.96 B: 149.31	R: 96.85 G: 102 B: 151.7	R: 121.25 G: 116.39 B: 162.76
1046 CFU/100mL	R: 126.4 G: 123.9 B: 152.46	R: 99.16 G: 107.89 B: 133.59	R: 104.19 G: 121.44 B: 143.38
1500 CFU/100mL	R: 103.69 G: 111.12 B: 160.48	R: 76.06 G: 92.3 B: 135.35	R: 105.73 G: 115.38 B: 162.93
6896 CFU/100mL	R: 97.21 G: 122.02 B: 160.96	R: 97.23 G: 129.92 B: 169.37	R: 77.65 G: 106.17 B: 141.16

5.7 Analysis of Light Intensity and Bacterial Concentration

The purpose of this analysis is to seek the possible relationship between florescent intensity and bacterial concentration. For doing the light intensity analysis, we choose the color of blue in 24th hour image data from 0 CFU/100mL, 23 CFU/100mL, 1046 CFU/100mL,1500 CFU/100mL 6896 CFU/100mL concentration. The blue intensity of each well for every concentration was obtained by cutting the image of each well and analyzing it individually. Then we calculated the average value of the three wells with their standard deviation. The detailed data for analysis is shown in Table 21.

Table 20: Blue Intensity Table with Average and Standard Deviation Value

CFU/100mL	Well-1-b	Well-2-b	Well-3-b	Avg (Blue)	SD Value
0	128.05	140.92	138.76	135.91	6.89
23	149.31	151.7	162.76	154.59	7.18
1046	152.46	133.59	143.38	143.14	9.44
1500	160.48	135.35	162.93	152.92	15.27
6896	160.96	169.37	141.16	156.84	14.48316

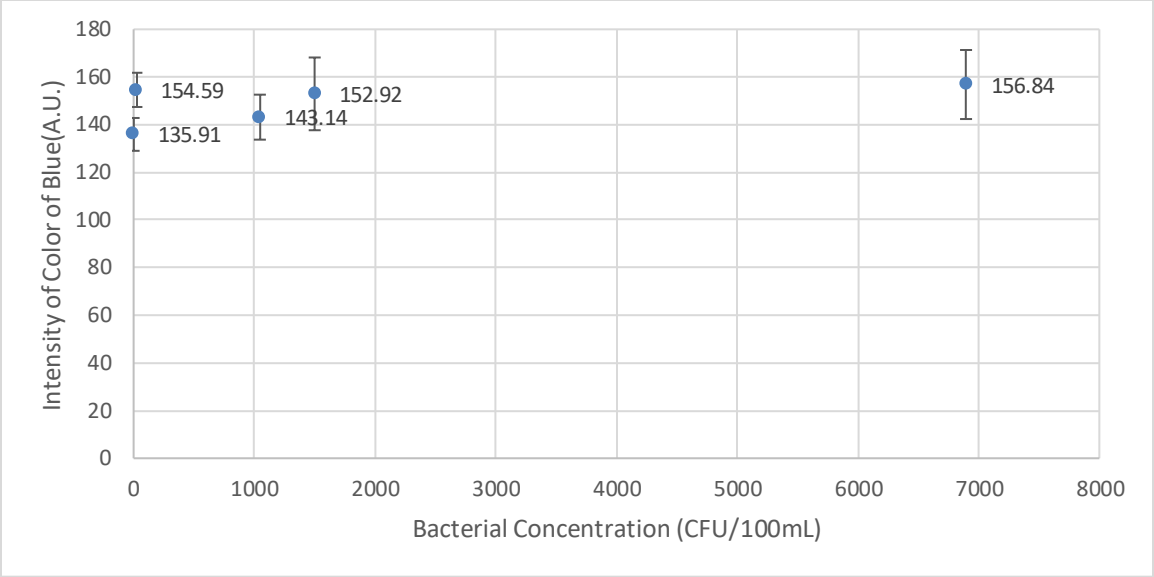


Figure 53: Average value and Standard Deviation of Blue Intensity in Four Tests

Figure 53 shows the average value of blue light intensity for each concentration; the error bars correspond to the standard deviation. The error bars in Figure 53 overlap across the five bacterial concentrations of 0 CFU/100mL, 23 CFU/100mL, 1046 CFU/100mL,1500 CFU/100mL 6896 CFU/100mL. This data suggests that there is no statistical difference in the intensity values between the different bacterial concentration and light intensity at the 24th hour. It is possible that this lack of relationship is due to the automatic adjustments performed by the CMOS sensor software in the Python GUI. Since the software automatically adjusts brightness, among other variables, we are not able to perceive a difference in light intensity as a function of bacterial concentration. Future analysis should focus on understanding and controlling how the camera translates light to electricity and controlling how the software translates these signals to Red, Green and Blue value.

CHAPTER 6 - CONCLUSION

In this project, we successfully built a prototype for a miniaturized, portable and automated water quality biosensor. We obtain several conclusions based on the process of manufacturing this biosensor and the experimental data acquired from Python GUI system.

A novel custom-made millifluidic device capable of automating the liquid handling steps of an enzyme-based water quality test was successfully manufactured from acrylic using laser cutting and bonding with medical-grade double sided adhesive. Based on our experiments, this device can be disinfected, is reusable, and is shown to be leakproof. Five sets of experiments prove the reusability the acrylic chip. In addition, this device was shown to carry out the EPA approved enzymatic water quality test.

This biosensor can perform and control the required 24-hour incubation period for the enzymatic assay. The hotplate located under the device can maintain thermal consistency over the 24-hour assay incubation period. A low-cost and portable fluorescent excitation and emission platform can be built by using 3D printing technology, an array of 361nm LEDs and a CMOS camera. A 3D printed chamber blocks external ambient light. A 3D printed LED holder allows the 20 LED lights to excite the contaminated water directly and blocks light from the LED reflecting on the acrylic wells.

Python software running on a Raspberry Pi microcomputer can be used to automate and control the system for the duration of a water quality test. This program is responsible for thermostatic temperature, fluorescence emission, data acquisition and analysis. The end result of integrating each component into a fully functioning system is the miniaturization of a water quality laboratory test into a system the size of a shoe-box.

After performing experiments with the biosensor at five different concentrations, the biosensor is proved to detect the bacterial growth by automatically collecting fluorescent image data. The concentration of these five sets of experiments are 0 (<1) CFU/100mL, 23 CFU/100mL, 1046 CFU/100mL, 1500 CFU/100mL, 6896 CFU/100mL. When the water sample known to be clean (distilled water), this biosensor can maintain

nearly constant RGB values over a 24- hour period. By analyzing the RGB data from contaminated water samples, the biosensor is capable to detect the presence of bacterial growth ranging from the very low concentration (23 CFU/100mL) to the very high concentration (6896 CFU/100mL); this concentration range is equal to clean recreational water and raw sewage spill, respectively.

Blue from the RGB data is correlated to fluorescent emissions. This makes sense since the Enterolert assay is known to emit light at 450nm, which corresponds to the colored blue. It is important to always measure Red and Green emissions. Although Red and Green colors are not directly related to fluorescent intensity, they must maintain relatively constant throughout the 24-hour incubation period to validate that the increase in blue light is due to fluorescent emissions and not external light sources. Measurements of each concentration validate the result obtained by our custom-made Python GUI compared to ImageJ software.

Our last objective was to study whether or not the biosensor is able to acquire water quality data in a quantitative and qualitative manner. Based on the measurement of clean and contaminated water samples, we show the ability of our biosensor to obtain qualitative water quality measurements. The three-well design was made to test if milliliter volumes of water could be loaded and separated using a pump; this was proven. But the three-well design was made to test if we could obtain quantitative data from our biosensor. We did not observe an obvious relationship between CFU quantity and number of wells turning on. Two reasons may explain this. Bacteria may be diffusing from one well to another over the 24-hour period. For example, in the experiment using 23CFU/100mL, this means approximately one CFU captured in the 3 milliliters tested in the acrylic device; however all three wells were seen to light up. Another reason for the lack of quantitative data could be the volumes used in each well relative to the concentration of bacteria in the 100mL sample. For example, in the 6896 CFU/100mL experiment, this means approximately 70 CFU of bacteria per milliliter-sized well; also, all three wells were seen to light up. More research is necessary to design a microfluidic system capable of obtaining quantitative data,

especially in the range of concentrations required for beach water quality 0-200 CFU/100mL.

The biosensor suffers from high variability and low R-squared linear regression values. A linear behavior can be seen in our data likely due to the auto adjustments made by the CMOS software. However, bacteria follow a sigmoidal growth behavior, which the auto adjustments in the image software could be linearizing. Future research will study how maintaining various CMOS settings constant affect the quality of the data and the observed behavior of bacteria growth. It is very important to understand how data is converted from light to electricity to 0-255 numbers. CMOS parameters such as shutter speed, brightness, contrast and ISO values have a great effect on the data and should be controlled. This analysis will help us understand why we see no sigmoidal curve growth as a function of time. Also, after controlling for CMOS variables, the relationship between color intensity over time and CFU concentration should be re-evaluated.

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APPENDIX

The content of appendix is referring to the data of red, green, blue, and overall intensity of five sets of experiment we carried out.

Table 21: RGB and Total Intensity Data for Water Sample at 0 CFU/100mL

Time (hour)	Red	Green	Blue	Total	Time (hour)	Red	Green	Blue	Total
1	5.90	7.79	9.40	10.73	13	6.03	5.25	8.74	9.38
2	5.42	4.52	8.51	9.23	14	5.90	5.15	8.31	8.83
3	5.14	4.42	8.12	8.76	15	5.12	5.07	10.24	10.8
4	5.25	5.12	6.77	7.5	16	5.39	5.76	8.05	8.89
5	4.92	4.61	9.41	9.98	17	5.78	5.64	7.29	8.04
6	4.80	5.20	6.42	7.24	18	4.45	5.47	5.84	6.72
7	5.26	5.73	6.97	7.87	19	5.11	5.06	7.58	8.11
8	6.48	5.17	10.03	10.36	20	5.72	5.33	8.47	9.12
9	5.62	4.98	9.10	9.65	21	5.34	5.26	9.41	10.09
10	5.24	5.10	8.98	9.61	22	6.41	5.31	7.49	8.11
11	5.95	5.23	8.86	9.48	23	5.42	5.36	7.57	8.26
12	5.09	4.82	10.08	10.62	24	4.71	5.21	9.76	10.42

Table 22: RGB and Total Intensity Data for Bacterial Water Sample at 22.6 CFU/100mL

Time (hour)	Red	Green	Blue	Total	Time (hour)	Red	Green	Blue	Total
1	6.86	5.86	6.92	8.17	13	5.93	8.24	13.98	14.51
2	7.74	6.66	10.26	10.75	14	6.30	8.08	13.90	14.45
3	4.96	7.43	10.73	11.41	15	5.88	7.89	15.26	15.68
4	6.10	8.52	11.31	11.96	16	7.49	8.05	13.83	14.21
5	6.49	7.90	13.30	13.62	17	6.02	7.65	16.12	16.45
6	4.94	7.90	12.20	12.84	18	6.55	8.09	15.91	16.26
7	19.93	21.12	25.99	26.57	19	7.57	8.01	13.91	14.31
8	5.53	7.45	12.65	13.16	20	6.21	7.02	16.29	16.61
9	5.66	7.83	10.54	11.20	21	7.75	8.01	14.12	14.47
10	5.53	7.58	12.54	13.08	22	6.28	7.66	12.99	13.53
11	6.73	7.93	14.11	14.57	23	7.27	7.83	18.46	18.68
12	5.38	7.49	14.28	14.72	24	6.59	7.74	15.50	15.81

Table 23: RGB and Total Intensity Data for Bacterial Water Sample at 1046 CFU/100mL

Time (hour)	Red	Green	Blue	Total	Time (hour)	Red	Green	Blue	Total
1	10.01	7.16	11.81	13.10	13	6.54	8.72	15.48	15.91
2	10.96	8.87	15.40	15.75	14	6.46	8.86	15.40	15.82
3	8.69	9.95	17.80	18.19	15	7.26	9.50	17.51	17.84
4	6.80	8.14	12.77	13.35	16	6.73	9.28	14.83	15.33
5	5.58	7.82	14.44	14.91	17	6.78	9.59	17.18	17.55
6	5.84	7.92	14.00	14.49	18	6.81	9.29	18.61	18.87
7	6.54	8.51	13.37	13.97	19	6.52	9.00	17.04	17.39
8	6.56	8.23	13.96	14.44	20	6.55	9.86	17.63	17.96
9	5.68	8.23	14.44	14.91	21	6.99	9.86	19.00	19.32
10	5.83	7.97	14.85	15.27	22	7.70	9.99	15.81	16.23
11	5.71	8.55	14.21	14.74	23	5.91	9.99	18.58	18.96
12	6.83	8.76	13.93	14.45	24	8.16	9.91	14.12	14.54

Table 24: RGB and Total Intensity Data for Bacterial Water Sample at 1500 CFU/100mL

Time (hour)	Red	Green	Blue	Total	Time (hour)	Red	Green	Blue	Total
1	19.59	13.89	23.96	24.60	13	0.01	1.14	0.00	1.14
2	20.85	14.78	26.74	26.90	14	13.19	17.03	37.43	37.46
3	0.01	1.15	0.00	1.15	15	3.07	10.73	6.47	10.85
4	4.33	6.07	8.58	9.24	16	4.10	7.70	7.66	8.67
5	3.30	5.73	6.51	7.32	17	4.10	7.70	7.66	8.67
6	6.28	5.54	11.15	11.65	18	3.39	7.80	8.52	9.25
7	1.85	6.60	5.42	7.11	19	4.04	9.38	5.38	9.38
8	0.01	1.09	0.00	1.10	20	3.19	7.93	5.59	8.35
9	8.05	5.37	4.82	8.83	21	4.37	6.72	8.26	9.94
10	4.23	6.44	5.70	6.95	22	22.61	16.50	28.98	30.49
11	5.47	7.46	11.80	12.30	23	20.82	18.08	29.69	30.45
12	3.99	7.68	6.79	8.71	24	15.68	16.57	34.84	34.87

Table 25: RGB and Total Intensity Data for Bacterial Water Sample at 6896 CFU/100mL

Time (hour)	Red	Green	Blue	Total	Time (hour)	Red	Green	Blue	Total
1	7.32	5.99	6.83	8.40	13	5.29	8.76	15.07	15.53
2	6.81	5.79	6.00	7.75	14	4.52	8.49	14.67	15.16
3	7.51	6.67	6.35	8.91	15	3.99	7.53	13.36	13.81
4	7.68	5.88	7.81	9.45	16	0.11	2.00	0.07	2.00
5	5.86	7.14	10.28	11.17	17	0.12	2.31	0.08	2.31
6	6.39	7.19	12.97	13.64	18	0.12	2.19	0.08	2.19
7	5.54	9.53	17.09	17.45	19	0.13	2.46	0.09	2.46
8	5.60	10.09	18.97	19.26	20	0.18	8.57	1.24	8.57
9	5.15	9.02	17.25	17.57	21	0.14	2.58	0.09	2.58
10	6.00	9.19	16.52	16.81	22	4.35	8.21	13.98	14.42
11	5.33	9.71	18.72	19.02	23	4.78	9.08	15.77	16.16
12	5.35	8.00	11.21	11.86	24	5.23	9.60	19.04	19.38