

Hyperspectral Fluorescence Imaging for Molecular Biology Applications



A Major Qualifying Project

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Abstract

Fluorescence microscopy is a powerful and widely used technique in cellular and molecular biology research. One of the major drawbacks with the current technology is the inability of the UV filter cubes in microscopes to separate and discriminate between fluorophores that emit fluorescence within the same color channel. Hyperspectral imaging is a technique that can analyze spectral patterns for each pixel in an image and provide an output image that separates emission spectrum in the same color channel [1]. This major qualifying project was aimed at harnessing the power of hyperspectral imaging to analyze biological samples in applications such as fluorescence microscopy and gel imaging to achieve higher power of discrimination. In order to achieve the project objectives, we designed and constructed a printed circuit board (PCB) illumination system, developed Matlab algorithms for spectral analysis (linear unmixing and spectral angle mapper) and a LabVIEW graphical user interface (GUI). Using our system, we are able to detect fluorophores in the 350-790 nm range and distinguish emissions in the same color channel.

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Chapter 1: Introduction

Microscopes are used in a wide range of scientific and educational fields, and they can lead to many breakthroughs in technology or biotechnology. With the 3% annual growth of academic institutes, hospitals, industries, laboratories, etc.; the demand for microscopes is also increasing. Current microscopes can be purchased at a wide price range from \$30 to \$15000, making cost a critical factor in determining the proper microscope needed for each application. The applications of microscopy are constantly expanding through different fields such as chemical industry, agriculture, nanotechnology, and more [1]. Live cell imaging which is mostly done by fluorescence microscopes, also has seen a significant increase in market size in recent years. The market is dominated by North America, with Asia accounting for the fastest growth rate in the fluorescent technology market. The fluorescence resonance energy transfer (FRET) holds the largest share of the live cell imaging market. The global live cell imaging market is \$3.57 Billion in 2014 and is expected to reach \$5.45 Billion by 2019 [2].

Fluorescence microscopy is a technology that has been popular in recent years due to new technologies such as the increase in multicolor fluorescence molecules, the application of genetic encoding with the fluorescent proteins, in addition, live cell imaging with multiplexing of fluorescent labels. Advanced fluorescence microscopy techniques such as fluorescence recovery after photo-bleaching (FRAP), a technique that can determine the kinetics of diffusion through cells [3]; fluorescence correlation spectroscopy (FCS), a technique that measured the spontaneous fluorescence intensity fluctuations by a tightly focused laser beam [4], and fluorescence (or Förster) resonance energy transfer (FRET), a transfer of energy from a donor fluorophore to acceptor fluorophore [5], are examples for applying multicolor in fluorescence microscopy [6]. However, currently, many fluorescence microscopes are indistinguishable in color that have close wavelengths due to the limitations of using set fluorescent filters. This limits the number of different fluorophores or fluorescent proteins that can be used in one specimen as similar fluorophores will appear as the same wavelength in the image. To properly dye the fluorophores and genetically encoded fluorescent proteins, researchers need to consider many different constraints while using the markers such as understanding which fluorophores/proteins can pass through the microscope's filter, initial brightness, photo-stability in buffer, and excitation/emission max wavelength. The list of considerations goes on if the researchers

need to apply the fluorophores/proteins in other applications, such as flow cytometry. This selecting process is a time-consuming and difficult task prior to achieving the full sample imaging [7, 8].

This project will design an illumination system, develop a spectrum analysis software program, and test the viability of a hyperspectral fluorescence imaging platform that can distinguish proteins conjugated to different fluorescence that have close emission wavelengths after excitation. The platform should be able to capture the shape of the emission spectrum and distinguish between probes that emit in the same color channel.

Before focusing on the designing of the illumination system for the platform, gel analysis should be carried out to map out the approximate fluorescence proteins conjugated to different dyes. This means we will take multiple proteins, and individually load them under gel electrophoresis. Once this is done we will compare the emission wavelengths obtained from the gels and determine the excitation and emission wavelength for each protein. This will give us a reference excitation spectra and emission spectra of the proteins for our spectral analysis software program. The designing of the illumination system and the analysis program are classified in the following steps.

First, a light source should be designed for the different excitation wavelengths of fluorescent probes. The light source should be strong enough for the sensor/camera to detect the emission wavelengths, and the cell activity of the sample should not be influenced by way of heat or light intensity. The light source system should also be precise enough to cover the entire excitation wavelength range from approximately 350 nm to 800 nm.

By controlling the electronic components (such as changing current passing through each LED bulb by use of control components like an Arduino), emission intensity taken to the user interface of the system should be based on the same light intensity of input light with different excitatory wavelengths. Excitation wavelengths should also be able to be controlled by the user from a user interface. Depending on the different spatial arrangement of lighting components and different light sources used, a method to concentrate the light source with different materials should also be developed to focus light onto the specimen being imaged. Components such as ellipse reflectors, convex lens, and microscope condensers can be used to make the light convergent. Additional materials such as a support stand should be used to keep the scanning platform stable and withstand the weight of the LED array.

The light source should also be placed at the most suitable position for the camera to collect emission data.

After the camera records data from the specimen, the data should be organized into a 3D data cube. The analysis should not only focus on the wavelength of emission lights (as the traditional imaging techniques do), but also the light intensity. Unlike traditional imaging techniques, which divides the images into two spatial axis x and y, hyperspectral fluorescence imaging has an extra lambda axis, which takes wavelengths as measurements and records intensity as coordinates.

On the algorithm side, spectral analysis, such as spectral angle mapper (comparing the similarity between the recorded spectrum with different reference spectra by calculating the angle between the spectra) and linear unmixing (algorithm that can be used to separate mixtures of several different fluorophores at the same pixel), should be carried out to distinguish different emission outputs with different wavelengths at the same region (pixels) without taking any bleed through effects. Additional algorithms such as spectral unwrapping, spectrum analyzer, and spectroscopy should also be considered.

The viability for the hyperspectral fluorescence imaging is being determined by the marketing analysis of the hyperspectral fluorescence and the result from our spectral analysis algorithms. We will report the accuracy, precision, reproducibility of our algorithms and illumination system.

If the project is completed successfully, the system and the program should be able to distinguish any functioning fluorescence and label the region on the image with different colors. This should be done by rapidly imaging multiple pixels (array of gel blocks) at a time and then produce nearly instantaneous imaging results. The device must fit under the budget (5000 dollars) provided to us by Headwall Photonics and must be compatible with the current device (microscopes and sensors/cameras) provided by Headwall for imaging.

Chapter 2: Background review

2.1 Fluorescence imaging:

When fluorescent materials are stimulated by an external energy, such as energetic light rays, fluorescent molecules emit light by the electrons returning from an excited state to a ground state. When they return back to the ground state, they release energy, which might be detected as fluorescence signals. Not all materials could be stimulated to form fluorescence. This property has only been found when the frequencies of molecules and light are the same, and the molecules perform high fluorescence efficiency after the molecules absorb energy instead of consuming the energy during collision between molecules [9].

Compared to ordinary imaging techniques, fluorescence imaging shows many distinct advantages. It has much higher sensitivity compared to ordinary imaging techniques, such as colorimetric. The ability to image different sets of samples at a same time is another advantage of fluorescence imaging. Labeled by fluorescence with different emission wavelengths, different sample molecules can be imaged at the same time with the detection of different fluorescence signals by a sensor. Compared with radioisotopes, a higher stability has been found when using fluorescence imaging on samples containing antibodies, DNA hybridization probes and PCR primers. The fluorescence signals can exist on the samples for months. Moreover, in the majority of situations, fluorescence imaging has low toxicity to the human body. During fluorescence signaling and imaging, the experimenters only need to use gloves and ultraviolet protection safety goggles. Along with its performance and safety, fluorescence imaging also has less cost than radioisotopes [10].

The concept of the fluorescence microscopy is to use the emission light from the fluorescence molecule or chemical to look at the specimen. Fluorescence molecule's electrons will emit light when they are excited and return to their ground state. Electrons can be excited physically (absorption of light), mechanically (friction), or chemically. In fluorescence microscopy, the microscope uses a short wavelength with high energy light as a source to excite the molecule. The emission wavelength of the molecule will have a longer wavelength with lower energy. Each fluorescence molecule has its own unique excitation wavelength and emission wavelength [11].

In order to see the different emission wavelength from the different fluorescence molecules, the microscope will need to have filters to distinguish each wavelength. A fluorescent filter contains three different filters, Excitation filter, Dichromatic mirror, and the Emission filter. Excitation filters filter out the excitation wave from the light source. The dichromatic mirror will reflect the excitation wave to the specimen and pass through the emission wave. The emission filter will filter out the desired emission wave that the user wants to observe. Due to the diversity of the fluorophores and proteins, dichromatic mirrors and emission filters are not able to precisely separate close emission wavelengths that are about 10 nm away from each other. Other methods like laser scanning confocal microscopy can also distinguish different wavelengths but it also cannot distinguish close wavelengths [12].

2.2 Hyperspectral imaging:

Instead of saving images in traditional RGB data, hyperspectral imaging divides the imaging in a more detailed spectral dimension. In addition to two spatial dimensions (x and y), an extra dimension of lambda has been recorded in hyperspectral imaging, with a lot of tiny channels, each represents a narrow range of light wavelengths. When the sample is imaged by hyperspectral imaging, it will be recorded in a cube. In addition to the availability of overall spectral data for each point on the sample, the imaging data could also be shown in different spectral coverage [13].

With the increase in spectral resolution, hyperspectral imaging has a stronger ability in imaging. Compared with ordinary panchromatic and multispectral imaging, hyperspectral imaging has far wider applications. For example, it has the ability to detect and identify different geography area. With the analysis of spectral absorption features, it is possible to distinguish the specific type of vegetation coverage and material used on roads, dew and buildings. Spectral imaging is now widely used in food safety, medical diagnosis, and cosmonautic applications [14], [15], [16].

Chapter 3: Project Strategy

3.1 Initial and Revised Client Statement:

One of the problems often found in fluorescence imaging is that, when being projected on screens, many fluorescent probes are indistinguishable in color with standard sensors. By using hyperspectral fluorescence imaging techniques, the shape of the emission spectrum can be captured, and different probes can be distinguished, even if they are shown indistinguishably under traditional imaging technique. The initial client statement is present by the sponsor Headwall Photonics, Inc. directly in the quote.

“Many fluorescent probes are indistinguishable in color when imaged with standard sensors. Hyperspectral imaging can capture the shape of the emission spectrum and distinguish between probes that emit in the same channel. This project will explore how well this can be done in biological samples, such as proteins, in a research setting.”

Further discussion with the sponsor was taken to understand the goal of “exploring” the hyperspectral imaging. The deliverable for this project to our sponsor is the illumination system, the spectral analysis algorithms software, and a capability of fluorescent hyperspectral imaging.

The revised client statement for this project is “Research the usability of the hyperspectral fluorescence imaging of biological samples such as proteins and design an illumination system and spectral analysis algorithms software for common industrial/research fluorescent proteins.”

3.2 Design Requirement (Technical):

The primary technical requirements for this project can be sorted into two main categories, developing an illumination system and designing an algorithm to carry out spectral analysis. The development of an illumination system, in brief, is the designing for the light source, while the designing of an algorithm mainly focuses on distinguishing between different fluorescence and labeling them with a different color on the screen. Other requirements needed to be achieved in this project include designing a user graphic interface and so on.

3.2.1 Primary objective: Development of illumination system

1. **Providing a wide-ranged spectrum:** The development of the illumination system mainly focuses on providing a wide-ranged spectrum for triggering excitation wavelengths of fluorophores on the specimen. Different fluorophores, even if they seem to be in the same color under traditional imaging techniques, reflect with different emission wavelengths and intensities under light with certain range of wavelengths. Therefore, it becomes easier to distinguish if the light source contains a wider range of excitation wavelengths, which enlarges the difference between the responses of different fluorescence.
2. **Not affecting the subject:** Due to the fact that hyperspectral fluorescence imaging might have a wide range of applications including biological imaging, it is important that the imaging is biocompatible. Therefore, the illumination system is required to have the least influence and harm to cell activities as possible. High light intensity and heat radiation, are two main factors that could affect cells activities and even cause cell death, should be avoided.
3. **Fast scanning:** The project is required to develop a specific formation (arrangement) for the illumination system that is able to excite multiple amounts of fluorescence at once during fast scanning.
4. **Focusing source:** An illumination system should be able to converge the light source to the specimen, maintain brightness in the field, and bring uniformity in brightness.
5. **Cooling system:** A cooling system will help carry away heat produced by different lighting components, including light bulbs, wires, resistors and control components. The cooling system will protect the illumination system and increase its operation longevity.

3.2.2 Primary objective: Development of algorithm

1. **Accurately distinguish different dyes:** An algorithm is required to be developed to carry out spectral analysis on Matlab or Python. The algorithms should be able to accurately distinguish different dyes and label them with different colors on screen simultaneously. It is also required to prevent bleed through, something often found in traditional imaging techniques when multiple fluorophores appear on the same channel.

2. **User friendly:** A user graphic interface should be developed for users to carry out functions such as changing excitation wavelength and adding references for new fluorescence.

3.2.3 Design Constraints

There are a few design constraints that the team needs to consider when designing the illumination system:

1. **Stability:** Due to the platform that the sponsor wants to mount the illumination system on, the team needs to make the system stable and strong, so it can withstand the weight of the light components.
2. **Dimension:** The size of the light source should be as small as possible because it needs to focus on subjects that are in the gel analysis and fit into the box that is provided by Headwall.
3. **Budget:** The budget for this project is limited to \$5000.
4. **Time:** The project is expected to be finished within the 2017-2018 academic year.

3.2.4 Specification

In this project, several specifications are important to consider while designing the illumination system. Since the team is working with a sponsor company, the team needs to fulfill the specification that the sponsor provided.

1. **The wavelength of the provided dye:** The range of the light source should cover wavelengths from approximately 350 nm to 800 nm since the most common dyes on the market and the dyes provided are in that range.
2. **The dimension of the illumination system:** The box that contains the whole system has a dimension of 100mm x 100mm x 150mm.
3. **Spectral analysis algorithm:** The algorithm helps distinguish different emission wavelengths from dyes. The resolution of the algorithm should be 5 nm.
4. **Reaction time:** the image being posted on the monitor/screen after spectral analysis should be as fast as 0.5 s.

5. **Operation longevity:** the illumination system must have a lifespan of ~ 50000 hrs.

3.2.5 Design Functions

The illumination system should be able to cover all the excitation wavelengths for all fluorophores and fluorescent proteins that are commonly used on the market. The illumination system and spectral analysis algorithm will need to produce nearly instantaneous image results and distinguish close emission wavelengths from the fluorophores. Another important function of this device is that it will have no limitation on dye and it will effectively eliminate bleed through. Researchers can use any fluorophore and fluorescent proteins in any combination for this imaging system.

| Design Function | Possible Methods | | | |
|-----------------------------------------|--------------------------------------------------------------------|--------------------------------------------------------------|----------------------------|---------------------|
| Graphic User Interface | LabVIEW | Matlab | | |
| Light Source Setup | Repeated square LED modules with different LED | Integrate Headwall's LED with new LED | Create circular LED module | Arc discharge lamps |
| Light Source Drive | Use op amplifier current source | Common emitter BJT amplifier | Resistors | |
| Light Focusing Source | Compound parabolic concentrator | Ellipsoidal reflector | Optic fiber | Laser |
| The position of light source and camera | Light source on side, camera on top, & specimens lay on a platform | Light source on top, camera on top & specimens on a platform | | |
| Control Component | Arduino | RFduino | Hippo-ADK | |
| Coding Platform | Matlab | Python | | |
| Spectral Analysis | Spectral angle mapper | Spectral unwrapping | Linear Unmixing | Spectrum analyzer |

Table 1: Function-Means Table

In order for the system to be user-friendly, the team decided to use a graphical interface to control the illumination system. LabVIEW and Matlab are two great software for programming a graphical interface for the user. There are many ways to fit the light source into the platform that is provided by Headwall, a square LED module and a circular LED module are two methods that the team will try first, since the LED provided by Headwall is white light and will not achieve the desired

wavelengths, and the arc lamp is too big for the box. The driver for the LED can be an op-amp. Current source or a BJT amplifier. If the load of the LED is large, the team will use a BJT amplifier because the BJT will provide a current that will not be affected by the load. There are four ways to focus the light source, an ellipsoidal reflector will be the first option that the team will test. Headwall has an ellipsoidal reflector that is in a position and can be easily modified. The position of the light source and the camera sensor will be positioned at the top of the specimen. Arduino is the first choice for controlling the system since the team has prior experience using the component. The software platform that the team will use to program the spectral analysis will be Matlab and Python. Matlab for calculation and Python for software interface. Lastly, the team will use different algorithms such as Spectral angle mapper, Spectral unwrapping, and Linear Unmixing to analyze the emission spectra.

3.3 Design Requirement (Standards)

Although there is no international standard established for hyperspectral fluorescence imaging techniques, the product for this project should be designed according to the following standards. Due to the fact that the product of this project is expected to enter the market, quality is a very important factor that the product should achieve as high as possible. Therefore, the standardization of quality management, ISO 9000, becomes the primary standard that should be focused on during designing. Among all ISO 9000 branches, ISO 9001(2015) is the most important standard because it establishes criterion about the specific objectives the product and organization should achieve during and after designing [17]. First of all, it stipulates that the organization which is designing this product (in the following article will be simplified as “the organization”) should determine the quality management system, the order of the system, its application in the organization, the method to achieve the quality system, and carry out necessary actions to make improvements to the product. In this project, hyperspectral fluorescence imaging microscopes should provide long-term accurate, stable and instantaneous results for all sorts of scientific research and applications. The organization should also collect user experience on the product for future improvements. Moreover, it also specifies the organization’s duty to provide specific quality and instruction manuals for every user. In this project, the instruction manual about how to use the hyperspectral fluorescence imaging microscope, both on

the mechanical platform of the microscope and the algorithm platform of the graphic user interface, should be provided to users. In addition, the organization should provide the estimation of minimum operation longevity for the product. Due to the fact that the product has not been fully developed, the estimation of minimum operation longevity could not be provided at this moment but should be provided after it has been established in the future.

ISO 19056 is another ISO standard that should be accounted for. As an international standard for illumination systems that is used in optics and optical instruments, ISO 19056 (2015) standardizes the brightness that the illumination system shows on an image [18]. It also stipulates that the brightness shown on screen/seen in the field should be uniform. In this project, the illumination system should strictly follow ISO 19056 standard. Depending on the color, light intensity and half angle of light from LED bulbs, different methods should be used to focus light on the specimen and provide uniform brightness at every pixel shown on the screen.

ISO 8039 is the standard for tolerance, value, and symbol of magnification for optics and optical instruments. Depending on their different duties, different components in the product for this project are standardized with different minimum operation longevity by ISO 8039 (2014). According to ISO 8039, the organization should provide an approximate estimation of the minimum operation longevity for different components. Compared to ISO 9001, ISO 8039 is more specific to optics and optical instruments. It also stipulates the specification of extremities for different components in the product, such as operation power and current for the illumination system. In this project, depending on the specific components used in the final product, the operation longevity and tolerance should be provided after the designing. In addition, the design should also follow ISO 19012-2, which is the standard for chromatic correction. It standardizes the minimum requirement a microscope needs to achieve in a chromatic correction to make sure the user can distinguish samples from the specimen. The product in this project should follow this standard to create a clear, distinguishable field with cells and other elements on the specimen after using algorithms such as spectral angle mapper and linear unmixing to label different colors to different fluorescence.

3.4 Management Approach

In order to finish the project throughout the 2017-2018 academic year, the team created a milestone list for design and research. Figure 1 and 2, are Gantt charts that were created to show the estimated dates for the tasks to be finished throughout the year. In A term, the team ordered all the parts that were needed to design the light sources for the illumination system. The team also received training that were needed to design the light sources for the illumination system. The team also received training for using the gel analysis and the sensor and equipment provided by the sponsor. In order to design the illumination system, background and technical research were done all throughout A term. Prototypes of the illumination system were completed and worked on during B term. LabVIEW coding for the system interface was also completed simultaneously with the illumination system. The spectral analysis software was programmed in C term, and the whole system was tested in C and D term. Data analysis and conclusions were also achieved throughout D term.

To ensure the team is keeping pace and delivering the sponsor's needs, weekly meetings throughout the year were done to discuss the goal for each week and to go over the progress of the previous week with the sponsor. Furthermore, each week the team met 3-4 times to discuss problems encountered during research, design process, building or testing. A notebook was kept throughout meetings to take note of important details and to keep track of the design process. Before incorporating all of the deliverables into the final presentation and report, sponsor and advisor approval was obtained to ensure the quality and the quantity of the project met all requirements.

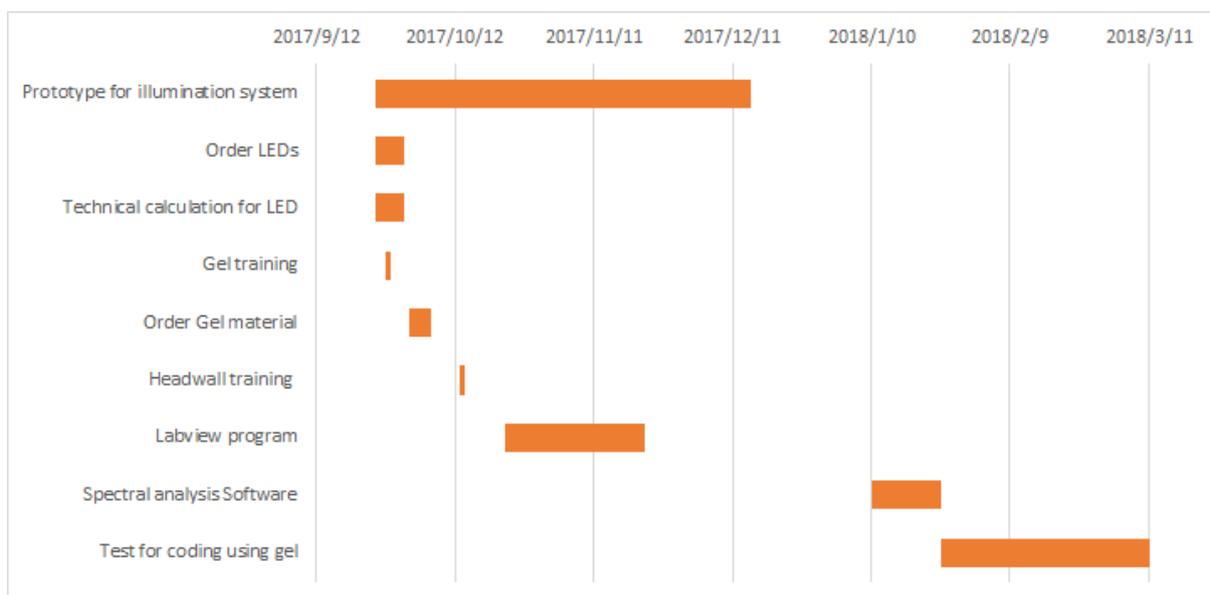


Figure 1: Gantt chart until the end of the project

3.5 Financial management

Below is a table of all the project expenses during development and testing. Headwall Photonics supplied the team with \$5000 as well as supplying the expensive fluorescent scanning platform and sensor for the project, Worcester Polytechnic Institute also supplied \$2000. After all contributions the team had a budget of \$7000 and was able to stay under budget throughout the course as the final payments for supplies came out to \$6522.

| | |
|---------------------------------------------------------|-------------------|
| LEDs (LED supply) | \$104.73 |
| Gel Electrophoresis equipment (BioRad) | \$1,005.50 |
| Alexa Fluor Dye (Fisher) | \$3,719.19 |
| LED (Allied Electronics) | \$31.21 |
| LED (Thor Labs) | \$374.97 |
| Arduino Board (Amazon) | \$49.18 |
| Fiber optic cable (Fiber Optic Products) | \$39.00 |
| Headers for PCB (Mouser) | \$13.35 |
| Epoxy (Epotek) | \$51.15 |
| Compound Parabolic Concentrator (Phoenix Electroformed) | \$406.71 |
| Acrylic Board (McMaster) | \$41.90 |
| Collimator lens (Lumatec) | \$574.05 |
| Plano Convex Lens (Edmund Optical) | \$121.80 |
| Tripod (Amazon) | \$36.99 |
| | |
| Total Cost | \$6,521.77 |
| Headwall Funding | \$5,000 |
| WPI BME Department Funding | \$2,00 |
| Remaining Budget | \$478.23 |

Table 2: Project Expenses

Chapter 4: Method and Alternative Designs

4.1 Needs Analysis

Headwall Photonics, Inc. has required the team to develop an imaging system that can process all fluorescence emissions and have no limitations with fluorophores or fluorescent proteins. Headwall Photonics, Inc. has provided an imaging platform that is currently designed for fluorescent imaging along with a fluorescent camera (pco.sCMOS) which includes two white light L1090 bulbs. In order to reach the project end goal, the team will need to develop a more advanced light source that either incorporates the current bulbs or replaces them with all new lights which effectively cover wavelengths of 350nm to 900nm uniformly. A light reflector or lens will then be constructed by the team to focus the light onto the sample in order to provide fluorescence for the camera to pick up. Another objective for the device is that it should not result in any damage to the imaged sample. A cooling system will likely be necessary in order to control the heat from the device to protect the system from being overheated and malfunction. In order to control the light source and reflector, a control panel of some sort must be designed and constructed by the team that is fully compatible with the camera and platform as well as the lighting interface. Coding must then be developed by the team to construct the fluorescence camera signal into a high-resolution image of the sample. In order to prioritize these needs and tasks, the team has developed a pairwise comparison chart seen above in table one.

In order to successfully achieve these needs, several constraints must be taken into account to meet goals and develop Headwalls desired device. One of these constraints includes the budget of five thousand dollars. As discussed in the previous section, many of the materials needed for testing and development of the device are rather costly and due to this careful consideration must be taken into account when purchasing materials in order to stay within the provided budget. Another crucial design constraint that must be accounted for is the overall size of the light source and reflector component as they must be compatible with the existing platform and camera. If the light source component is too large the integrity of the overall platform may be jeopardized and would likely not function properly.

A pairwise comparison chart shown below indicates the order of significance to our objectives. 1 indicates the objective on the left is more important than the objective on the top. 0 indicates the

objective on the left is less important than the objective on the top, and 0.5 indicates the equal importance of two objectives.

| Objectives | Software Analysis | Cover wavelengths | Effectively project light | Mix light wavelengths evenly | Cooling system | User Friendly | Control Interface | Total |
|-------------------------------------|-------------------|-------------------|---------------------------|------------------------------|----------------|---------------|-------------------|------------|
| Software Analysis | x | 0.5 | 0.5 | 1 | 1 | 1 | 1 | 5 |
| Cover wavelengths | 0.5 | x | 0.5 | 0.5 | 1 | 1 | 1 | 4.5 |
| Effectively project light | 0.5 | 0.5 | x | 0.5 | 0.5 | 1 | 1 | 4 |
| Mix light wavelengths evenly | 0 | 0.5 | 0.5 | x | 0.5 | 1 | 1 | 3.5 |
| Cooling system | 0 | 0 | 0.5 | 0.5 | x | 1 | 1 | 3 |
| User Friendly | 0 | 0 | 0 | 0 | 0 | x | 0.5 | 0.5 |
| Control Interface | 0 | 0 | 0 | 0 | 0 | 0.5 | x | 0.5 |

Table 3: Pairwise Comparison Chart

As shown in the table above, the most important objective is the software analysis. The sponsor wants us to show them which analysis method is best used for hyperspectral fluorescence imaging. The cover wavelengths for our light source is the second to most important objective, it provides the experiment to test our software analysis. The projection of light is a crucial component of connecting the sensor that the sponsor provided to us. An evenly mixed wavelength of light will enable the group to test and excite multiple fluorophores at once. The cooling system is needed for long term usage of the light sources. The control interface and user friendly are aspects that could improve the efficiency during operation.

4.2 Concept Maps/Modeling/Feasibility studies

In order to organize the design components and objectives prior to designing a prototype the following concept map was produced.

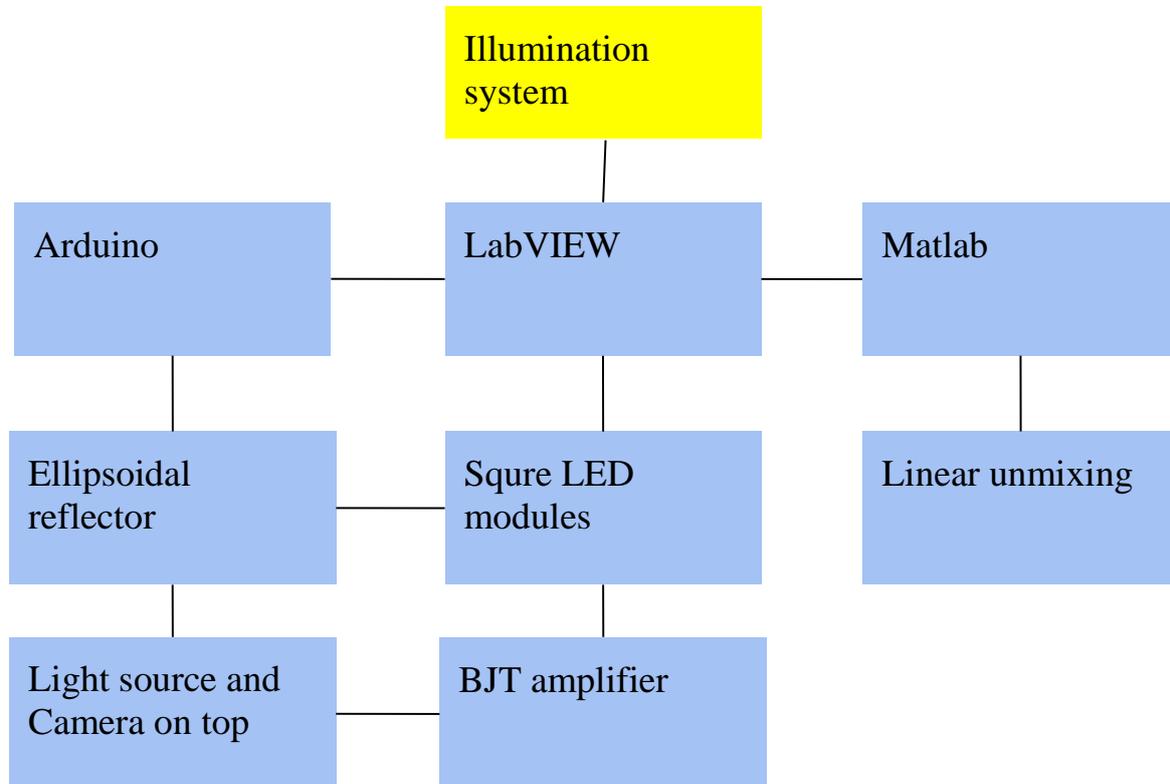


Figure 2: Concept Map

Using Table 1 and Table 2 the team was able to decide upon the best components for each part of the design and then construct them into Figure 2, the concept map. Once the concept map was finalized design prototyping and planning began. The team's first prototype will use LabVIEW to develop a graphic user interface for controlling the light source and device. The team has prior experience with LabVIEW and it provides a simple, and stable interface to use on the final device. For this project, the LabVIEW interface is the core which connects all components of the system. It must allow the users to control the current to the light source, the lights timing and power, as well as the algorithms to carry out spectral analysis. The control panel will be developed so it is easy to use and allows the user to make as many customized settings for the device as possible. On the algorithm side, Matlab will be the main platform to carry out all analysis with spectral linear unmixing.

Another portion of the designs illumination source includes the actual light setup and drive. Currently several LEDs have been selected that evenly cover wavelengths from 350nm to 900nm and an amplifier source will soon be selected. Currently a BJT amplifier is the top choice for the first prototype and an Arduino will be used as the control component. As there are currently 14 LEDs selected for the device, a circular module will be created using three of each LED arranged in an evenly distributed manner. This will provide a light source capable of emitting the entire desired light spectrum with even intensity and focus. The current focusing strategy considered mainly includes the idea of using an ellipsoidal reflector to converge light into a one-dimensional area, which is a line, with uniform light intensity and spectrum at different places on the line, while the LED matrix is arranged as a rectangle, each row of LED's emit light with the same range of wavelength. With this general idea of concept, the relative position between sensor (camera) and illumination system, was determined to be on the same side (above the specimen).

In order to determine resistance required for the many different LED's that have been selected, Figure 3 shows the calculations have been used prior to developing a full electrical schematic for the light source.

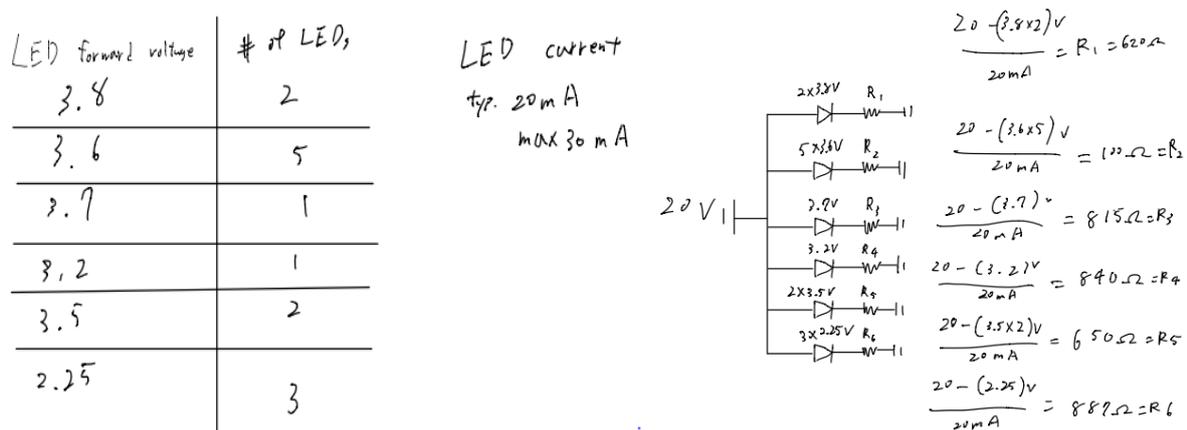


Figure 3: Calculation of resistors for corresponding LEDs

In order to direct all of the light onto the small sample area being imaged an ellipsoidal reflector will be developed to focus the light. While other reflectors may still be considered, currently the

ellipsoidal reflector seems the most viable as it reflects nearly all the light into one small region. The diagram below illustrated the usage of the ellipsoidal reflector in our prototype design.

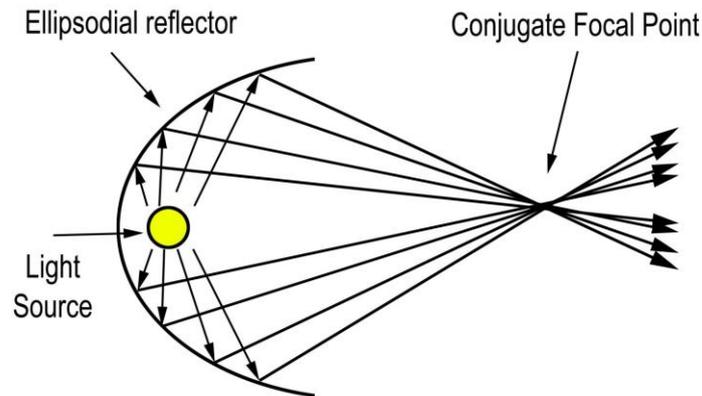


Figure 4: Schematic diagram of a reflector

The final portion of the prototype includes the data analysis and coding to take the camera's output and configure it into an image of the sample. The sponsor Headwall Photonics is requiring the group to use Matlab to code the analysis of the image as most of their computer software uses and is fully compatible with Matlab. The Matlab code will be written to use linear unmixing and unwrapping in order to create a detailed image of the sample from the camera's signal. This method analyzes the fluorescence in each pixel of the image and determines the type or shape of the fluorescence. The following equation is the basic algorithm used in linear unmixing for fluorescence [6].

$$I(\lambda) = \sum_i C_i * R_i(\lambda) \quad [4.1]$$

C_i = the concentration of dye

$R_i(\lambda)$ = the reference spectrum of a pure dye (from spectral image)

This method allows the device to examine the image pixel by pixel in order to arrange it in a distinguishable way even among fluorescence with similar wavelengths. Once the pixels are examined and compiled the device will be able to effectively distinguish all wavelengths without using common

fluorescence filters. While this coding may be the most difficult aspect of the prototype it will be most crucial as the final product relies on the spectral unmixing and analysis to achieve the project goal set by Headwall.

In order to test the feasibility of the design a testing plan has been set up by the team and sponsors. It has been decided that gel analysis will be used to create a map of the most common fluorescent dyes and allow the device to be tested and calibrated based on the known fluorescence of the dyes. Once the dyes are run through the gel they will be examined using the imaging device both known and unknown to the user. By testing unknown samples and using the image output from the device to determine the dye, the devices accuracy will be tested. Once the device proves to accurately image the gels it can be put to further testing on more complex samples such as proteins and even tissues.

4.3 Alternative Design

4.3.1 Objective 1. Cover Wavelengths

The LED light sources were chosen to cover all fluorophores from a maximum excitation wavelength of 346 nm to 784 nm. The team has found 15 different wavelength LEDs that spread from 346 nm to 784 nm. The chosen wavelengths are 375 nm, 400 nm, 420 nm, 440 nm, 470 nm, 505 nm, 525 nm, 575 nm, 590 nm, 610 nm, 630 nm, 660 nm, 680 nm, 700 nm, and 780 nm. Most of the LEDs don't have accurate specification sheets, so the team didn't know the exact wavelength of the LEDs but using HyperSpec and SpectralView we were able to see that those LEDs are ± 10 nm from what they identified on the product seller's website. Figure 5 shows that the team used a 575 nm LED to excite 10 different fluorophores, and then use SpectralView to analyze the LED wavelengths. Figure 5 shows that the sensor can accurately identify the LED that was being used and provide that the LEDs do output the correct wavelength needed to image the full desired spectrum. In order to have enough light intensity, the team chose 6 LEDs clustered together in each chosen wavelength. The team also lowered the camera to 20 cm above the scanning platform so that the camera could receive more light intensity.

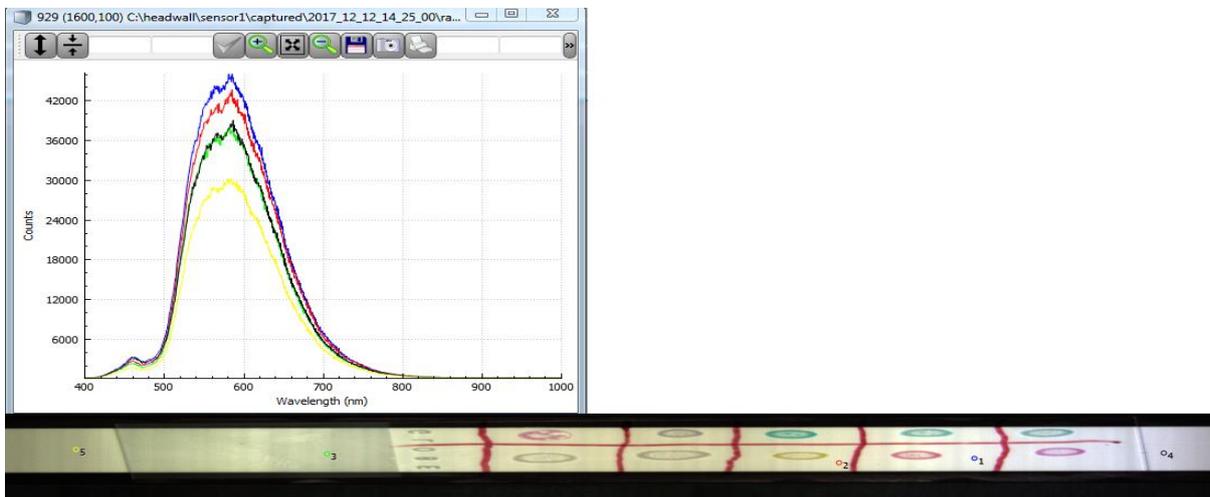


Figure 5: Wavelength of captured pixel of background with white paper

In the first design, the team chose 3 LEDs in a cluster for each wavelength, but the camera could not receive a good signal. Then the team increased 3 LEDs to 5 LEDs. Figure 5 is recorded using 5 LEDs. Figure 5, shows that one side is lighter than the other. To solve this

problem, the team had to add one more LED to each group to make light uniform and intense enough for imaging.

The LED circuit was designed using resistors and powered by an Arduino Mega board. Different wavelength LEDs have different forward voltage and forward current. LEDs are current drive sources, so resistors can produce a voltage difference and generate a current that turns on the LEDs. The anode leads of the LEDs were connected to the Arduino output pins and the cathode leads were connected to the ground. All of the resistance values are calculated using Equation 4.2. The team designed all LEDs to be powered at the typical forward current. This will provide a longer usage for LEDs.

$$R = \frac{V_{supply} - V_{forward}}{I_{forward}} \quad [4.2]$$

The LED circuit was built on a breadboard to test for orientation and the temperature of LEDs, then the team transferred the circuit on the breadboard to a PCB board. The LED schematic and the PCB board layout are shown in the appendix. Appendix A shows the alternative design schematic and PCB board layout.

4.3.2 Objective 2. Mix Light Wavelengths Evenly

After testing the LED module by shining it directly onto the ellipse reflector using the existing scanning setup, it became clear that a method must be designed to mix the different LEDs so different wavelengths don't appear in bands on the scanning platform. In order to do this the team decided it was necessary to focus all the LED light down to a smaller point. This not only allows the light to be mixed and reduce wavelength bands, it also will prevent light from escaping the ellipse reflector, allowing the device to utilize nearly one hundred percent of the LED intensity. In order to achieve this goal, a compound parabolic concentrator (CPC) has been selected to focus light into a smaller point directly from the LED module. Figure 6 illustrates how light will enter the concentrator from the wide end and then reflect off the wall of the CPC until it is focused at the smaller output end.

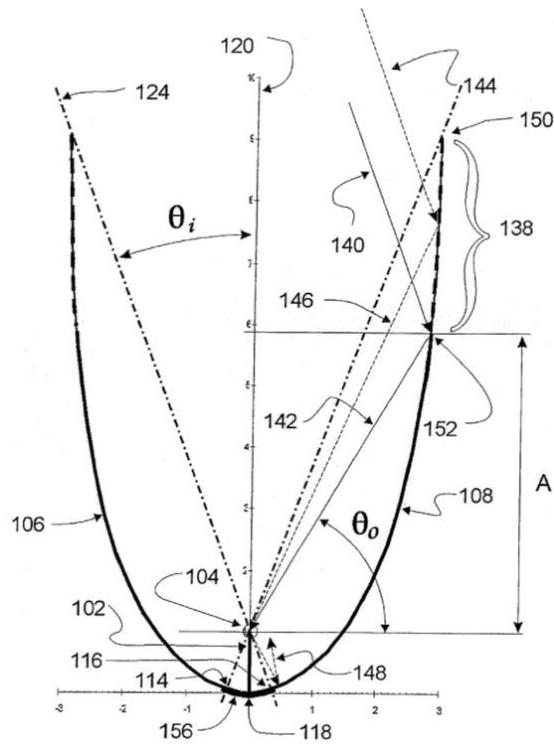


Figure 6: Schematic Diagram of Fin-type compound parabolic concentrator [22]

In order to gather all the light from the LED module a CPC has been selected that has an input hole of 3.579 inches, which should perfectly fit around the 80mm LED module. Figure 6 is the schematic for the CPC that will be ordered for this design, as stated the CPC selected is the one with a CA of 3.579 inches and a V of 0.375 inches.

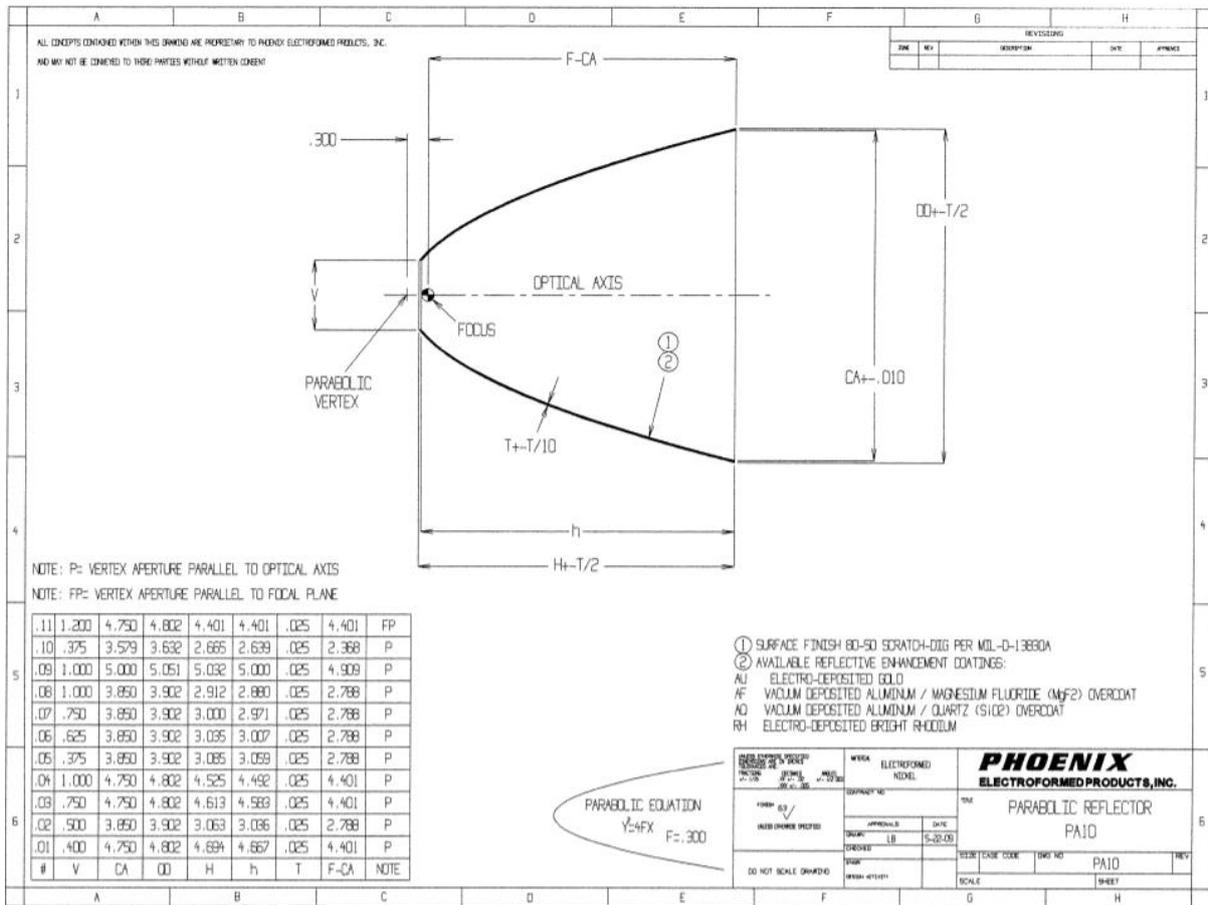


Figure 7: Compound Parabolic Concentrator [26]

4.3.3 Objective 3. Effectively Project Light

In order to thoroughly mix the different wavelengths after the light has been concentrated to a point using the CPC, fiber optic cables have been selected to further mix and carry light to the ellipse reflector. By placing end glow fiber optic cables at the small output end of the CPC, light from the LEDs will travel into fiber optic, where they will then repeatedly bounce off the optic cable walls resulting in thorough mixing of all the wavelengths used. Figure 8 below demonstrates how light waves travel through a fiber optic cable and mix together.

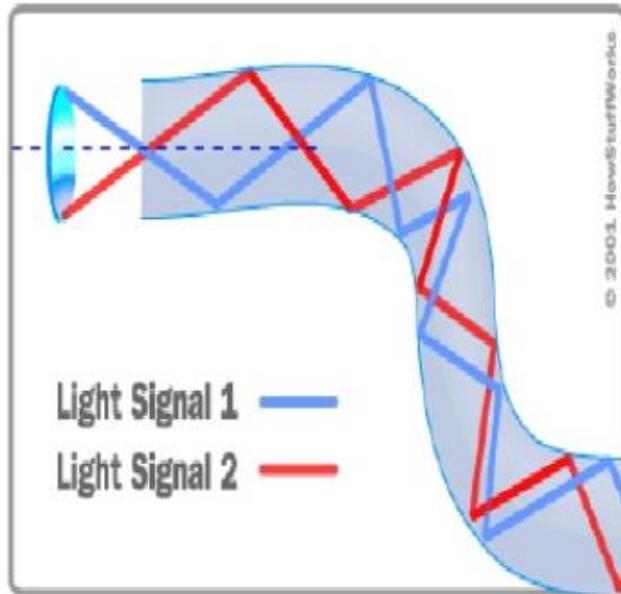


Figure 8: Fiber Optic Cable Light Movement [23]

In order to achieve the light mixing results seen in figure 8 a solid core fiber optic cable with a diameter of 10mm has been selected for testing. A backup, multi strand core fiber optic cable will also be tested to determine which type of fiber optic cable best mixes and carries light. Figure 9 is an image of the two fiber optic cables selected to complete this task.

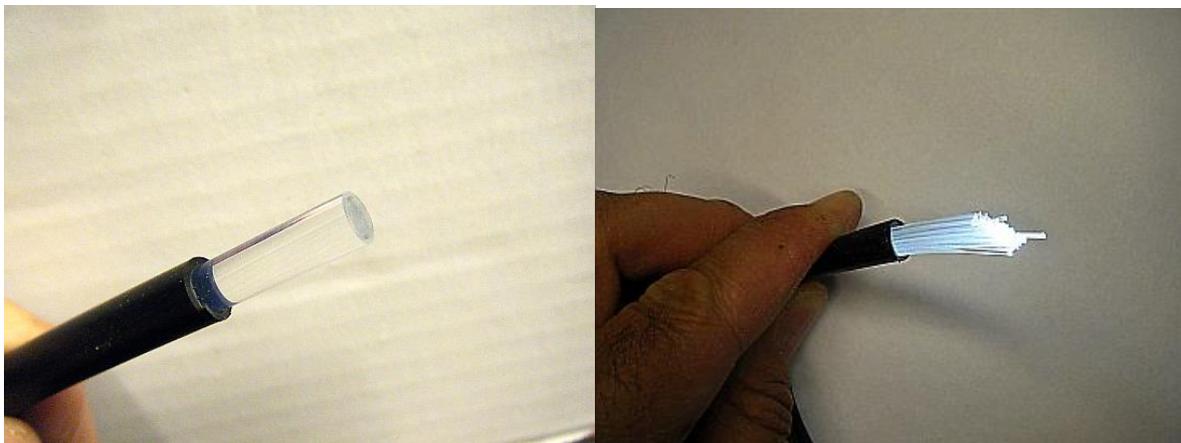


Figure 9: left solid core, right is multi strand [21]

Since the fiber optic cables generally perform better with higher wavelengths in the IR and often lack efficient transmittance as wavelengths approach UV, the team has also selected to test liquid light guides that are exceptional at UV transmittance. The light guide selected specifically transmits between 340nm and 800nm which perfectly correlates to the projects desired wavelengths.

PRINCIPLE

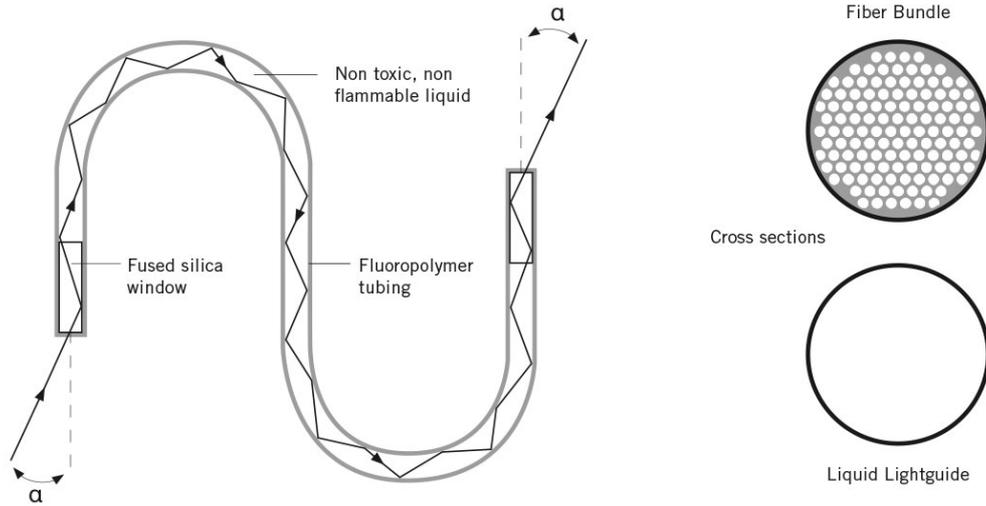


Figure 10: Liquid Light Guide [25]

After light travels through the light guide it will then be directed onto the ellipse reflector which will ultimately distribute the mixed light onto the imaging sample. Further testing is required on the fiber optic cable and light guide before it is known how well the light will enter the cable from the CPC and then be directed from the cable to the reflector. It is possible that some form of concentrating lens will be required to gather the light from the CPC and focus it at the correct acceptance angle for the light guide to capture the full light intensity.

A collimator lens will be tested for focusing light down from the CPC and funneling it into the light guide. These lenses are specifically designed to work with the liquid light guide as they can be more difficult to funnel light into as the entrance glass is smaller than the fiber optic cables being tested in this project. The figure below illustrates the usage of a collimator lens as an output for light from a light guide. This lens can also be used in reverse and allow a spread of light to be focused down to a point where the light guide is able to capture the full light intensity and transmit it to the other end of

the guide. This collimator device will be tested for use on both the input and output ends of the light guides.

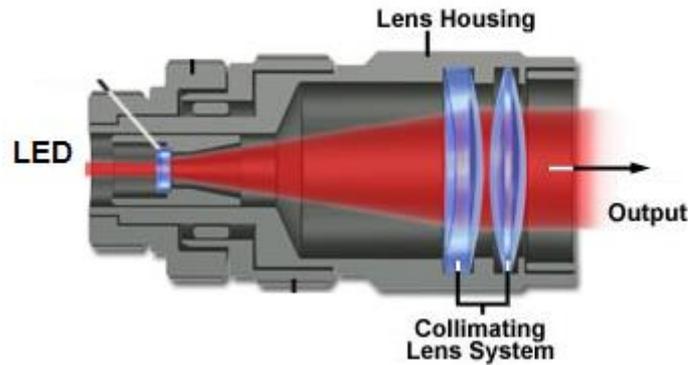


Figure 11: Collimator device [24]

Similarly, to this, another form of lens or diffuser will likely be needed to focus the light out of the CPC and in the collimator and light guide. Since the CPC has a focus point that is just inside the output hole, and the light guides are all too large to fit inside the hole of the CPC, another lens must be used to gather the focused light inside the CPC and project it out through the output hole. A plano-convex lens has been selected to achieve this goal as it works very similarly to a collimating lens but is small and easier to place inside the light guide. Below is a figure that displays the convex lens and illustrates how light waves enter the curved end of the lens and converge down into a point as it exits the concave side. If the convex lens is position properly it should effectively funnel light out of the CPC hole and into the collimator lens where it then will transmit into the lightguide, where it will mix and exit onto the imaging platform as a uniformly mixed spectrum of light.

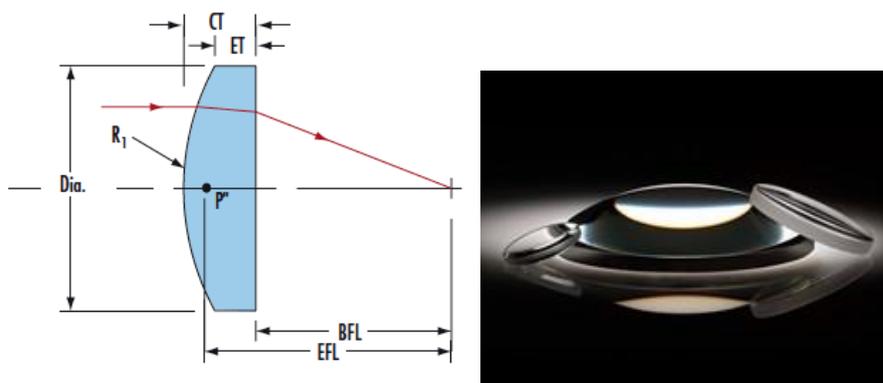


Figure 12: Collimator Lens [20]

4.3.4 Objective 4. Control Interface

A LabVIEW VI was designed as the control component for the LED light source. It has the function of assigning different light intensities to LEDs with different maximum emission wavelengths, by controlling the overall amount of LEDs turned on/off instantaneously, assigning different orders to LEDs with different maximum emission wavelengths at the same time, and showing error messages to users when one (or more) LED is not working properly.

So far, LEDs with each maximum emission wavelength have been designed with four levels of light intensity. In level 0 for LEDs with particular maximum emission wavelength, no spectra is released from LEDs. In the following levels 1, 2 and 3, each ascending level represents the powering of two more LEDs with that particular maximum emission wavelength. The changing in the light intensity of the LEDs with one particular maximum emission wavelength would not cause any change in the light intensity of LEDs with other wavelengths. As shown in the figure below, four levels are presented in sliders with different background colors. The background colors for each LED sliders depends on the color of the light emitted from corresponding LEDs.

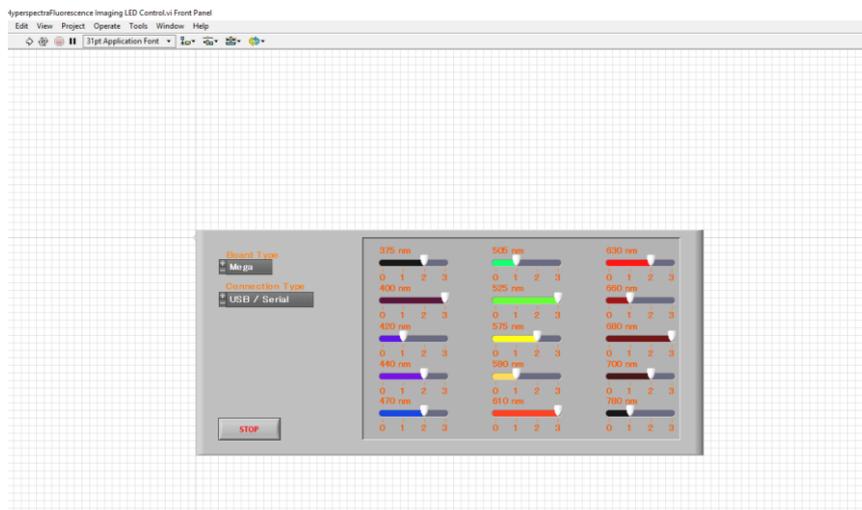


Figure 13: Front panel of LabVIEW VI for led control

In this project, Arduino Mega was selected as the control panel because of its massive number of pins. Due to the fact that changes in current passing through the LEDs would slightly change the maximum emission wavelength at the same time, the changing of light intensity could not be achieved when the LEDs with the same maximum emission wavelength are connected to the same pin. In order

to maintain the function of controlling light intensity, 45 pins were used, each of which were connected to two LEDs with the same maximum emission wavelength.

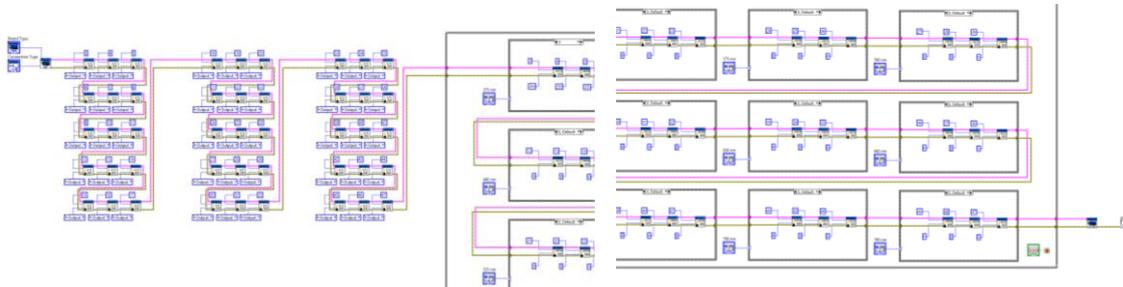


Figure 14: Board panel of LabVIEW VI for LED control (left: digital pin mode setting matrix; right: value writing matrix [partial])

4.3.5 Objective 5. Software Analysis

Matlab has been used as the software to perform spectral analysis. There are two spectral analysis method that could be performed in this project, which are spectral angle mapper and linear unmixing. The overall program could be considered as two separate parts. The first part focuses on taking reference spectrum from a data set that contains specific fluorophore(s) that the user already knows. When the script is started, a bmp image for the data is shown to the user and the user is supposed to point out the position that he/she recognizes as the fluorescence and tell the program the name of the fluorescence.

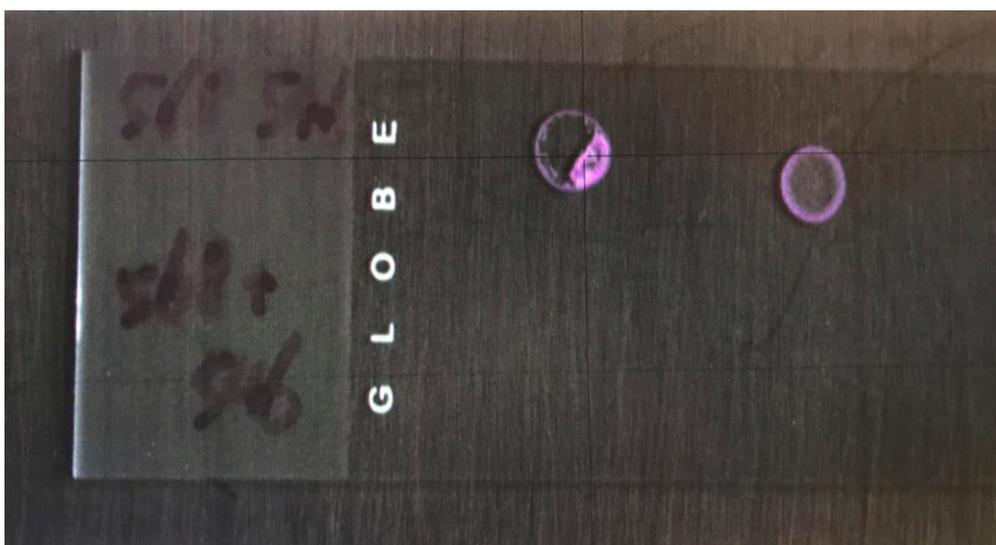


Figure 15: Matlab script for selecting reference

The second part focuses on analyzing data that contains undistinguishable fluorophore(s) which has already been recorded in previous studies. The script runs through spectral angle mapper and linear unmixing and shows the user with new image that stained pixels that contain different fluorophores with different colors.

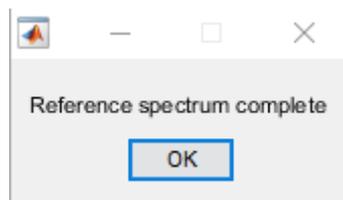


Figure 16: Success note after reference spectrum with name recorded

4.3.6 Black Box

To connect all the objectives, the team designed a black box that is able to fit all the design components. Figure 17, 18, 19, 20, and 21 show the configuration and the orientation of the components in the black box. The Black Box is made up of black Scratch-Resistant Acrylic.



Figure 17: LEDs and Arduino board

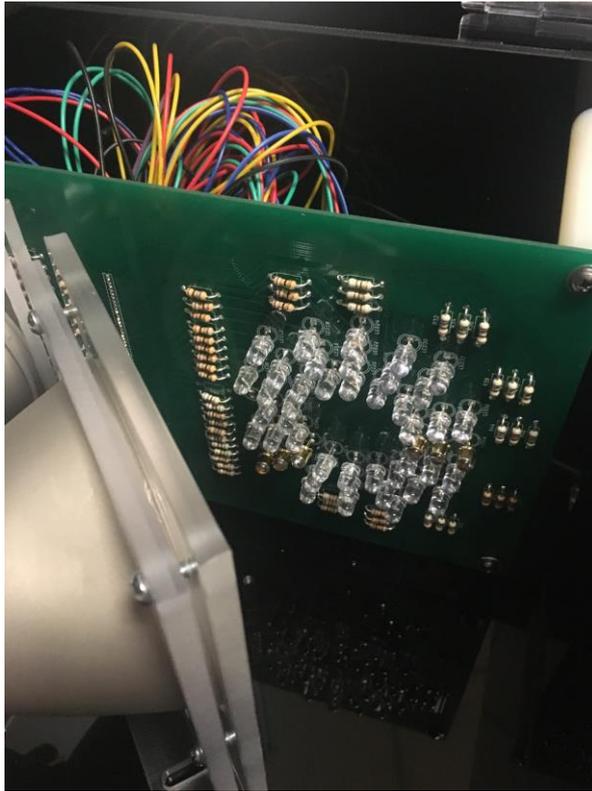


Figure 18: CPC and LEDs



Figure 19: Lens for liquid light guide

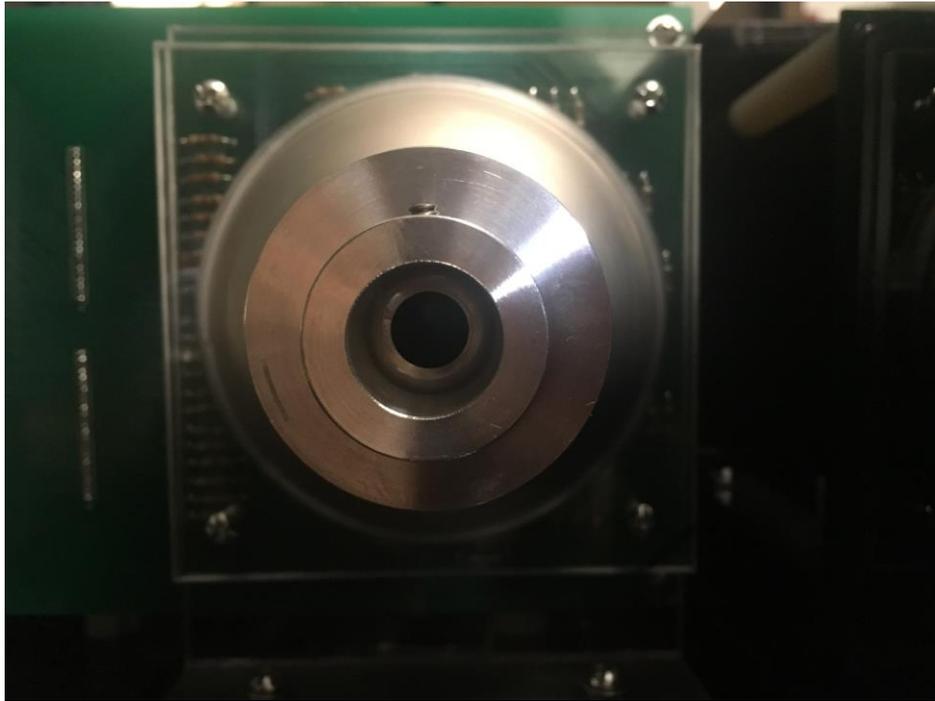


Figure 20: CPC and liquid light guide connection

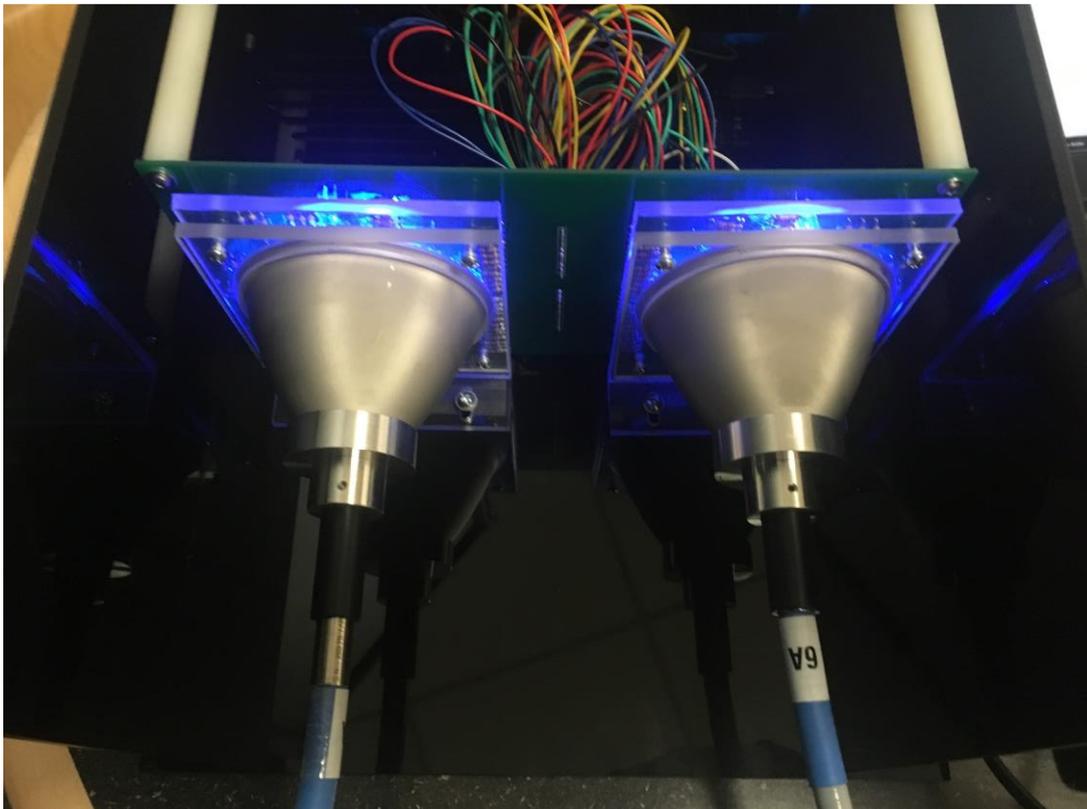


Figure 21: All components in the Black Box

4.4 Final design selection

4.4.1 Light Intensity Testing

One important objective this project needs to achieve is that the light source should be able to produce light that is strong enough for the camera to capture and accurately identify the wavelength of light reflected from the surface of the sample on the platform.

Two light intensity tests were carried out in this project. The first light intensity test was carried out on November 20th, 2017. The light source used in the first test includes one LED from each kind of emission wavelength class (15 LEDs total). As shown in the figure below, the camera did capture reflection signals from the sample. However, the light intensity was too weak and the camera could not accurately identify the wavelength of light and no fluorescence response was detected.



Figure 22: Result of first light intensity test

In order to produce light with enough light intensity, 5 more LEDs were ordered in each emission wavelength class. The second light intensity test was carried out on December 12th, 2017. Five LEDs with maximum emission wavelengths of 575 nm were used as light sources in this test. As shown in the figure below, the camera captured light with high intensity and showed a clear image on the screen. Although the right part of the image shows brighter in color, while the left part of the image shows darker in color (because the light projection on the sample was not scattered and evenly distributed), all of the readings of the spectrum on the blank region show the highest peak around 580

nm. As a result, the light intensity of five LEDs per wavelength will be enough for this project, but to make the light project uniformly on the scanning platform the team needs six LEDs per wavelength.

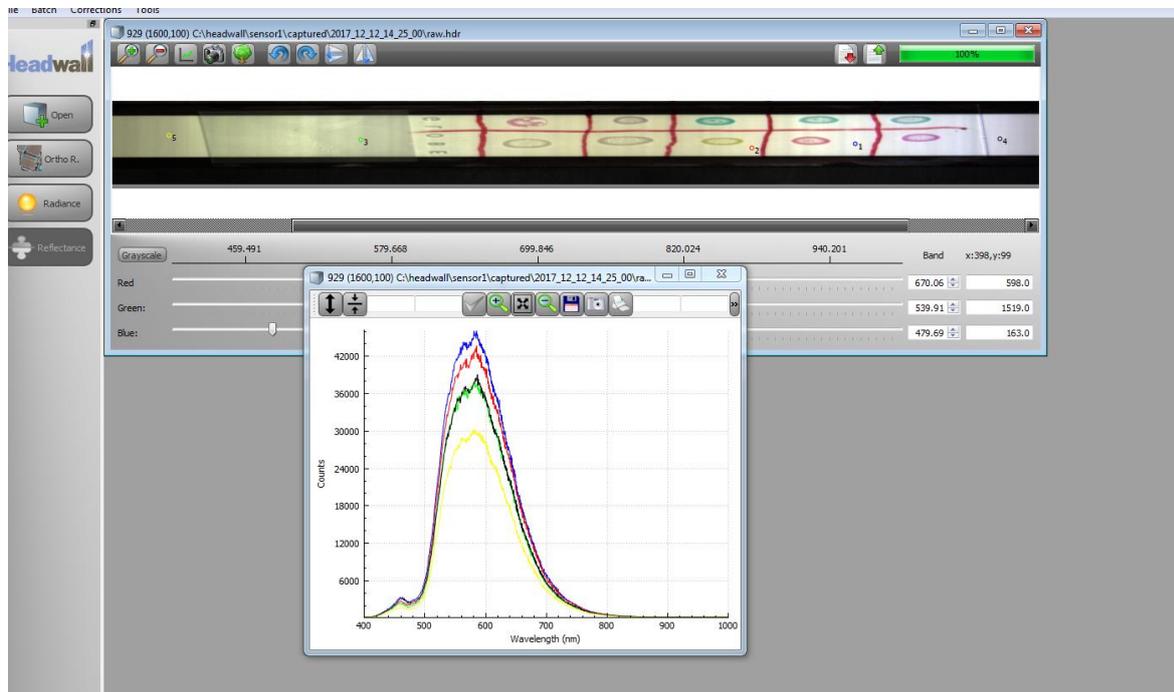


Figure 23: Result of second light intensity test

4.4.2 Objective 1: Cover Wavelengths Selection

In the final design, 555 nm LEDs were added into the design to fill the gap between 525 nm and 575 nm. The final chosen wavelengths are 375 nm, 400 nm, 420 nm, 440 nm, 470 nm, 505 nm, 525 nm, 555 nm, 575 nm, 590 nm, 610 nm, 630 nm, 660 nm, 680 nm, 700 nm, and 780 nm. From the intensity test we can see that 6 LEDs for each wavelength produce a reasonable intensity result. The configuration of the LED module is circular, as a circular design is more suitable for the focusing system. Appendix B shows the final design schematic and PCB board layout. The team separated the LEDs into two groups to get more uniform light onto the scanning platform. The hyperspectral sensor will have a better result if the light is uniform on the scanning platform.

4.4.3 Temperature Testing

Temperature testing has been done due to the design of the PCB board. The team needed to know how much heat the LEDs will generate and determined if the PCB board needs a heatsink to cool

the lighting module. For this test the team mounted all LEDs onto the breadboard and put the thermometer between the LEDs and touched the resistors. Infrared LEDs had more heat generated than the visible light LEDs due to their higher power usage.

In the Figure 23 below, the team put the thermometer close to the Infrared LEDs to measure the highest heat that the circuit will generate. The thermometer showed that room temperature before the LED were powered on was 22 degrees Celsius. The team turned on all of the LEDs and started the timer.

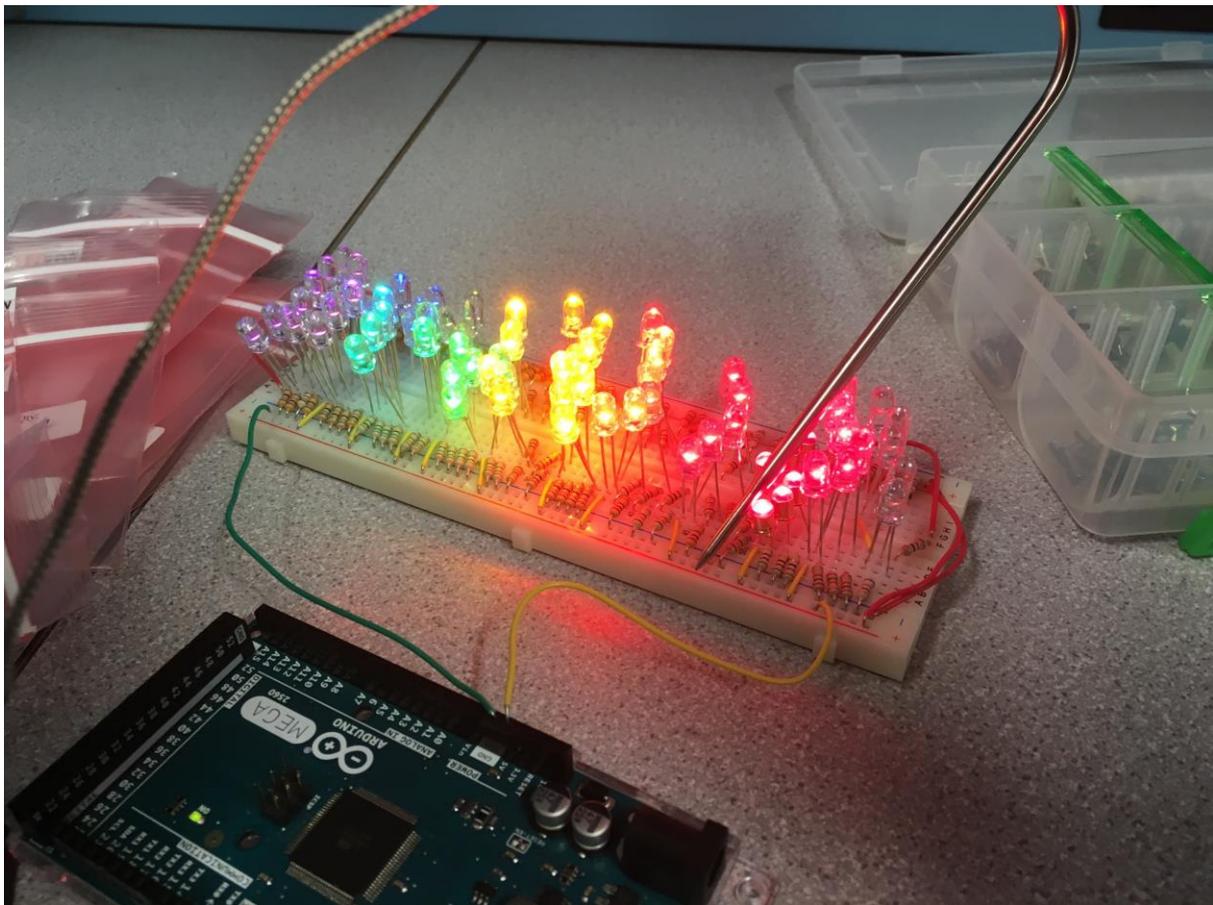


Figure 24: Temperature Test setup

Figure 25 shows that after 30 mins the thermometer measured the circuit at 24 degrees Celsius and stayed at 24-degree C for another 10 minutes. For this test, the team concluded that the PCB board does not need a heatsink to reduce the temperature created by the module. All the LEDs that the team has chosen can be operated from -30 to 80 degrees Celsius and the amount of heat released from the

LEDs to surrounding components is too minimal to cause performance change or damage and therefore can be neglected.

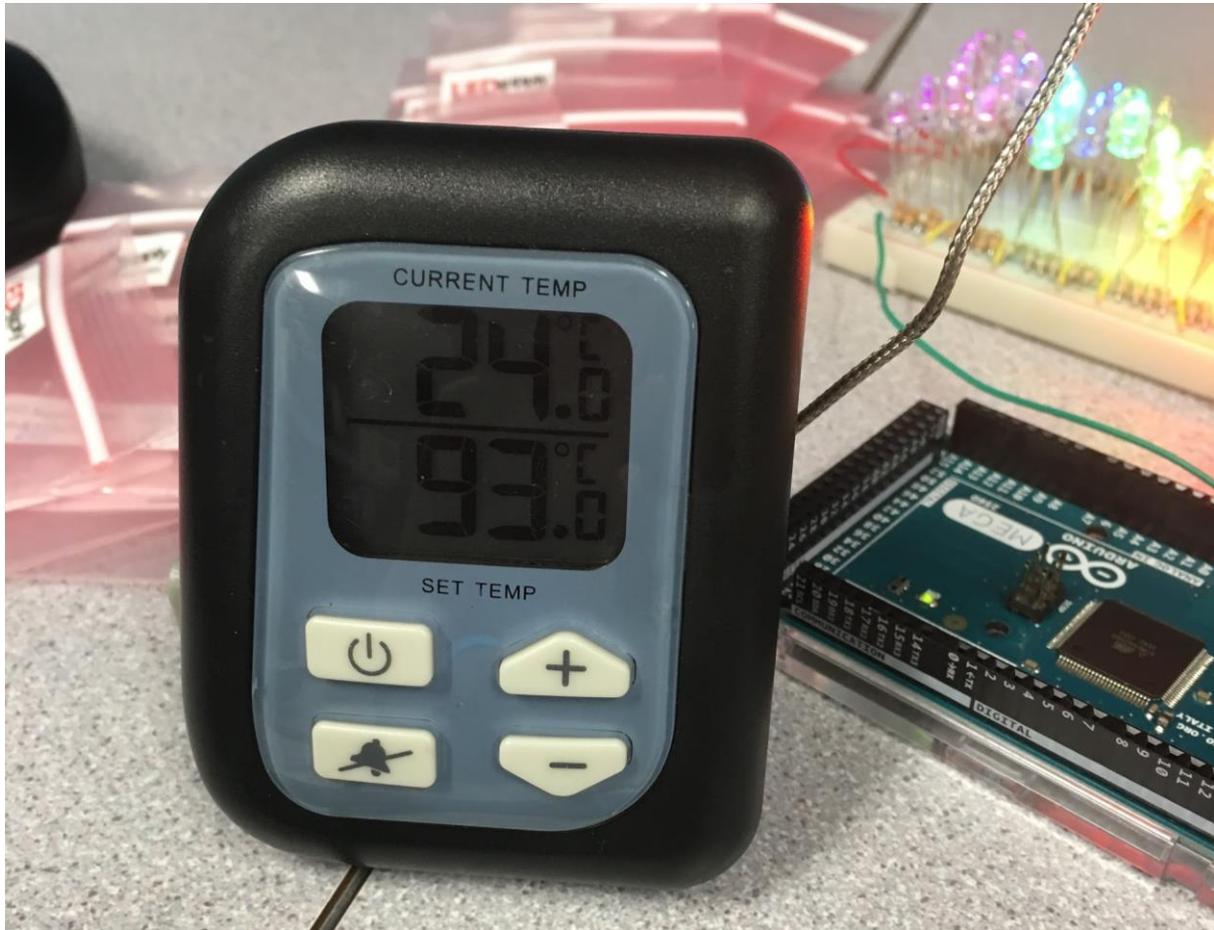


Figure 25: Result of temperature test on LEDs after 30 mins

4.4.4 Objective 2: Cooling System Selection

Based on the temperature test, the heat that is generated from the LEDs should not cause any problems, but the design should still have some air flow so that the heat will not build up in any condition. A small 12V DC 1.7 W fan can provide more than enough air flow to obtain this goal considering the generated heat from the LEDs is so low.

4.4.5 Projection Test

The concentrator and the liquid light guide were tested together with the LEDs. The team illuminated the LEDs through the CPC and used the liquid light guide to collect the mixed light. The

output of the liquid light guide was projected onto the scanning platform. The team turned on all the LEDs to excite Alexa 488 and Alexa 633. Figure 26 is the captured image from the hyperspectral sensor. Figure 27 shows the light projected onto the platform.

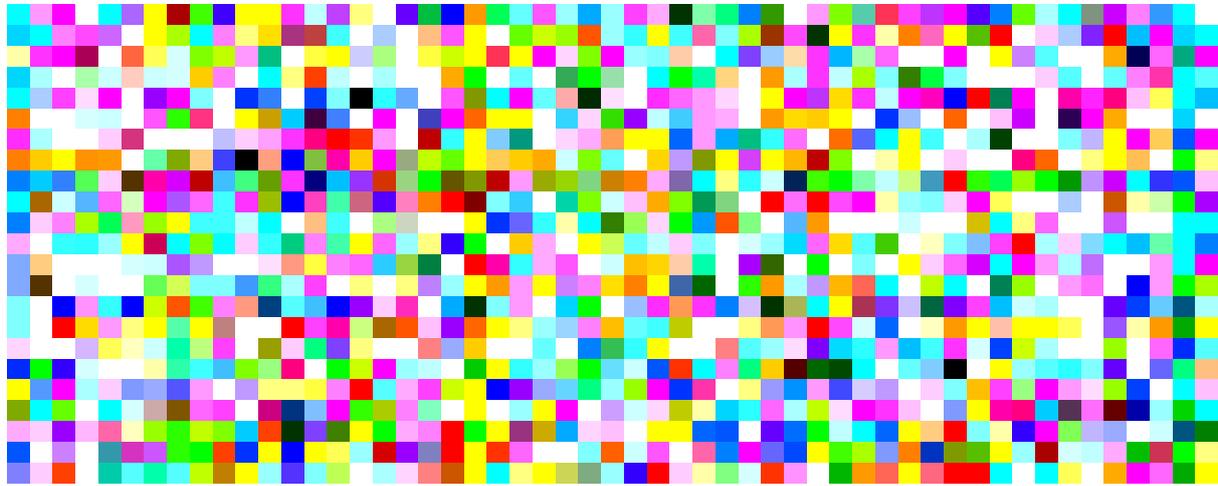


Figure 26: Output of concentrator and the liquid light guide



Figure 27: Projection of light from liquid light guide

Figure 26 didn't show anything because there was too much unwanted signals. Those signals were caused by the light not uniformly projecting onto the scanning platform. The white reference only had a certain wavelength rather than the full LED cover wavelength. This shows that the concentrator was not mixing the light as the team expected. Another reason for the large number of unwanted signals was that only a small region of light was projected onto the platform for the white reference. To solve this issue and have uniformly distributed light projected onto the platform, the team decided to remove the concentrator and the light guide and move the LED far away from the platform. Figure 28 shows the setup of the LED and projected far away from the scanning platform.

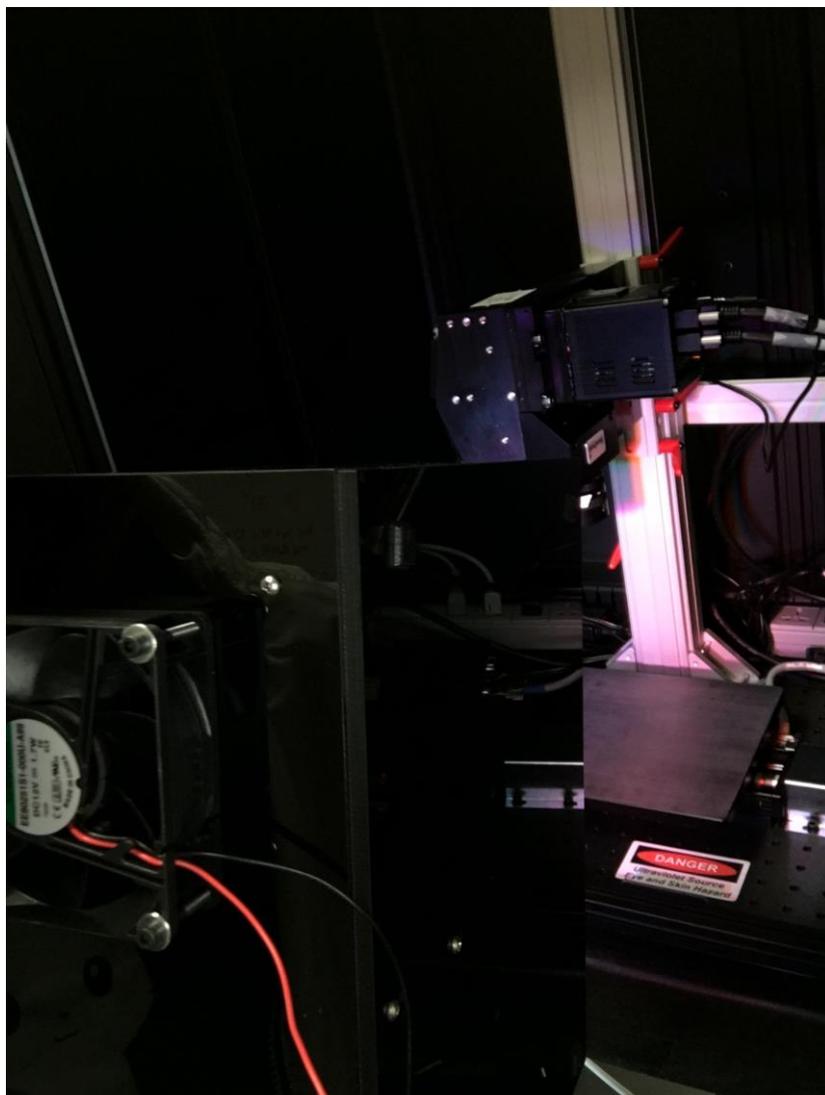


Figure 28: Projection of LEDs

Using the setup in Figure 28, the team was able to get a good signal to noise ratio, Figure 29 shows one of the samples taken from the setup. The sample used in Figure 29 and 26 are the same.



Figure 29: Projection sample

4.4.6 Illumination System Selection for Objective 3: Mix Light Wavelengths Evenly and Objective 4: Effectively Project Light

The final design for Objective 3 and 4 will be mixing the light wavelengths and projecting the light by placing the LED far enough away from the platform so that the LEDs can overlap each other and mix relatively evenly. Since the LED module was designed with multiple LEDs of each wavelength, enough light intensity is achieved by turning on all the LEDs to allow the setup in Figure 28 to produce desirable imaging results.

4.4.7 Control Interface Performance Testing

The testing of control interface includes its ability in controlling different LEDs at the same time. The LEDs were assigned with different light intensity and users tried to change the light intensity and on/off of the LEDs. The result shows that LEDs are able to show corresponding light intensity simultaneously with the control from control interface.

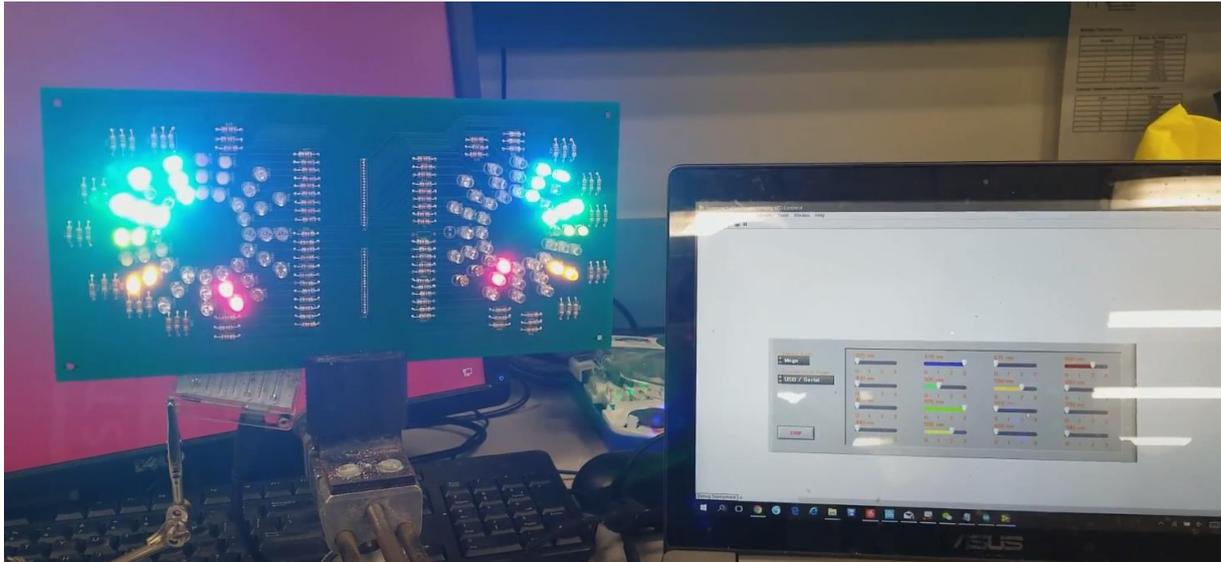


Figure 30: LED control interface testing

4.4.8 Objective 5. Control Interface Selection

The LabVIEW user interface has been used in the final design for controlling all the LEDs. As for the software analysis, spectral angle mapper has been used as the main spectral analysis tool in the final design of this project.

4.4.9 Objective 6. Software Analysis Selection

Compare to other spectral analysis methods, spectral angle mapper showed faster speed in calculation and linear unmixing showed its excellent performance. In the final test for the script, these two algorithms are selected as the algorithm for spectral analysis.

Chapter 5: Final Design Verification

To verify our spectral angle mapper, we loaded secondary antibodies conjugated with Alexa Fluor dyes onto denaturing polyacrylamide electrophoresis gels and ran them with 150 volts for 50 minutes. Each well was loaded with 30 micrograms of Alexa Fluor dye. Each dye was mixed with 15 microliters of 2x loading buffer and then micro pipetted into separate slots on the gel. The gels were taken to the scanning platform and scanned for fluorescence. Table 4 shows all the dye that were excited simultaneously with the final design.

| <u>Alexa Fluor</u> | excitation wavelength | emission wavelength |
|--------------------|-----------------------|---------------------|
| Alexa Fluor 350 | ~346 nm | 442 nm |
| Alexa Fluor 405 | 401 nm | 421 nm |
| Alexa Fluor 430 | 430 nm | 545 nm |
| Alexa Fluor 488 | 490 nm | 525 nm |
| Alexa Fluor 514 | 517 nm | 542 nm |
| Alexa Fluor 532 | 532 nm | 554 nm |
| Alexa Fluor 546 | 556 nm | 573 nm |
| Alexa Fluor 555 | 555 nm | 580 nm |
| Alexa Fluor 568 | 578 nm | 603 nm |
| Alexa Fluor 594 | 590 nm | 617 nm |
| Alexa Fluor 633 | 621 nm | 639 nm |
| Alexa Fluor 635 | 633 nm | 647 nm |
| Alexa Fluor 647 | 650 nm | 665 nm |
| Alexa Fluor 660 | 668 nm | 698 nm |
| Alexa Fluor 680 | 679 nm | 702 nm |
| Alexa Fluor 700 | 702 nm | 723 nm |
| Alexa Fluor 750 | 749 nm | 775 nm |
| Alexa Fluor 790 | 784 nm | 814 nm |

Table 4: List of dyes

5.1 Spectral Angle Mapper Testing

The performance of spectral angle mapper was tested on Alexa Fluor 488, 514 and 532 after gel electrophoresis (150 Volts for 30 minutes) as these three dyes all fall in the same color channel. To the naked eye the three dyes are indistinguishable (Figure 30). However, after applying our spectral angle mapper algorithm and false color assignment, the distinguishability of the three dyes through spectral angle mapper has been proved. The testing of spectral angle mapper script was carried out with data that contains numerous fluorophores. It confirms that spectral angle mapper can effectively separate dyes in the same color channel based on the shape of the emission spectra of individual dyes. The result showed that the script stained pixels that contains these two different dyes with different colors as shown in the figure below.

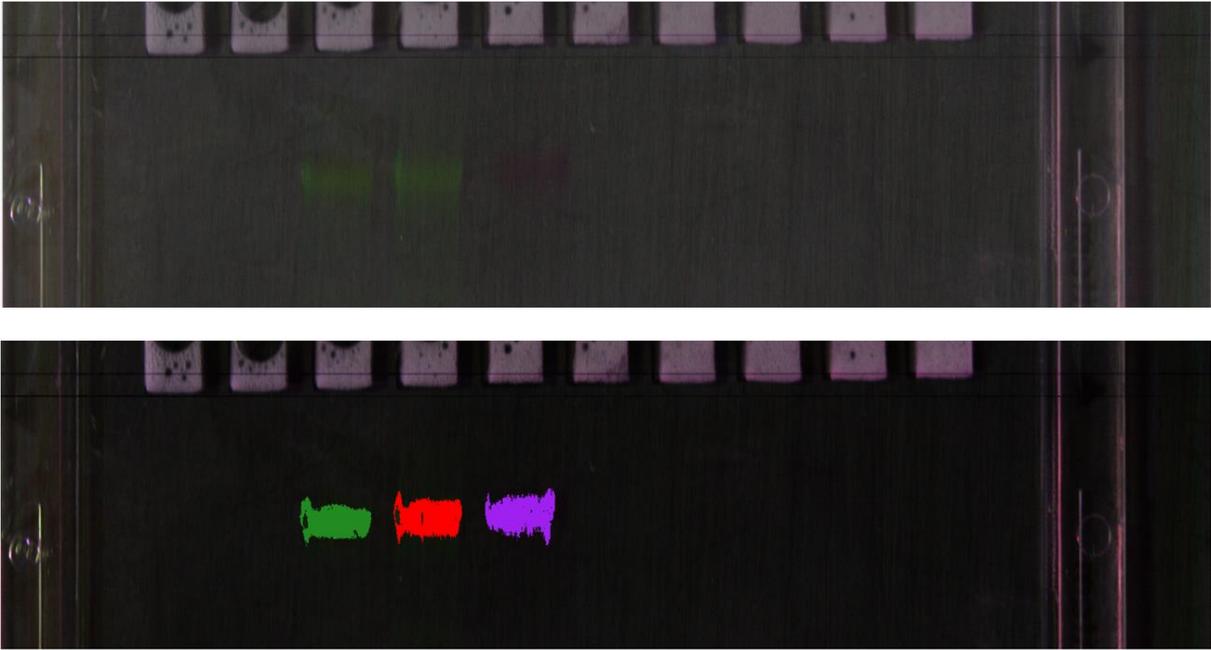


Figure 31: Alexa Fluor 488 (left in both pictures), 514 (middle in both pictures) and 532 (right in both pictures) before (picture on the top) and after (picture on the bottom) spectral angle mapper

5.2 Excitation Testing

The light source effectively excited all the dyes except Alexa 350, 750 and 790. Figure 32 shows the collected emission spectrum of Alexa 488, 514 and 532.

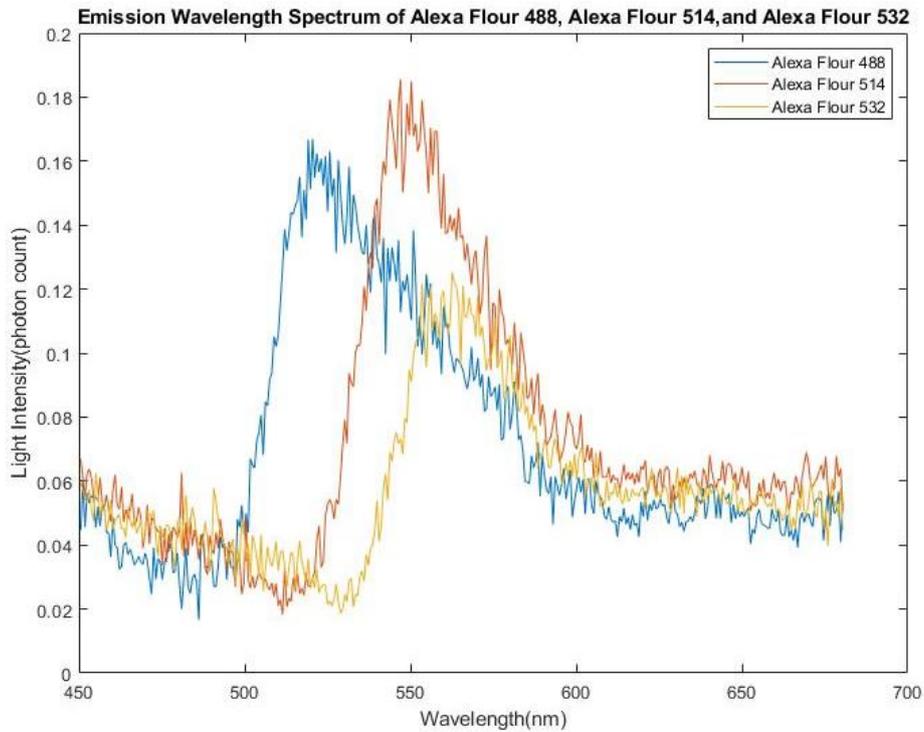


Figure 32: Spectrum curve detected by sensor (blue: Alexa Fluor 488, red: Alexa Fluor, yellow: Alexa Fluor 532)

5.3 Concentration Detection Sensitivity Testing

The detection sensitivity of the sensor versus the concentration of fluorescence was tested by applying linear unmixing on pixels that contained Alexa Fluor 488 with different loading concentrations from 20 μg down to 0.003 μg per well. All other settings for electrophoresis are identical to spectral angle mapper tests. Concentration indices, which represent the concentration of the reference spectrum in the spectrum of the pixel, were recorded by linear unmixing for samples containing different concentrations of fluorescence. The accuracy of the test could be measured by calculation of Fval in linear unmixing.

In each channel, when the spectral value of the pixel is subtracted by the sum of the product of spectral value of each reference spectrum (including all the dye being used and the background spectrum) and their corresponding concentration index (which was acquired in linear unmixing), there is a remainder value. Fval is calculated by adding up the secondary power of the remainder value from every channel. A small Fval value represents the high feasibility and accuracy of linear unmixing.

The result of the concentration detection sensitivity testing and accuracy measurement have been expressed by the consequential concentration indices and Fval indices of linear unmixing analysis as shown in Figure 33 and 34. The emission spectrum is shown in figure 35.

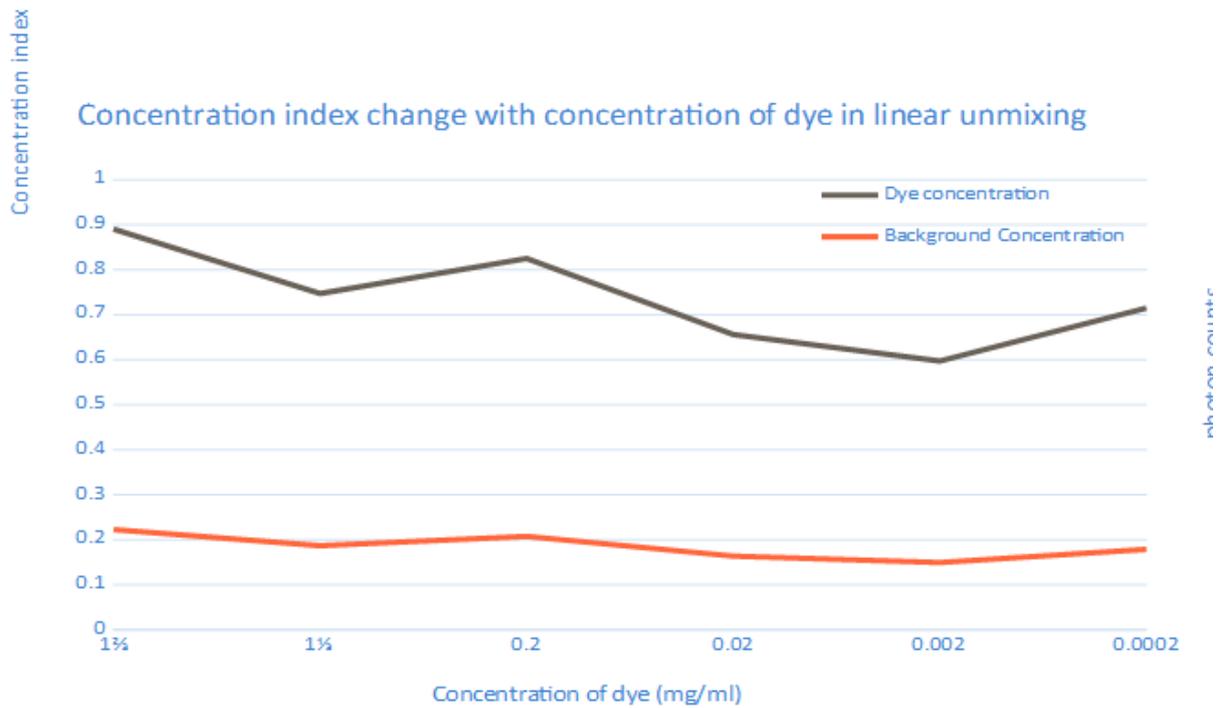


Figure 33: Graph depicting values of concentration index of Alexa Flour 488 (blue) and background noise (red) after linear unmixing

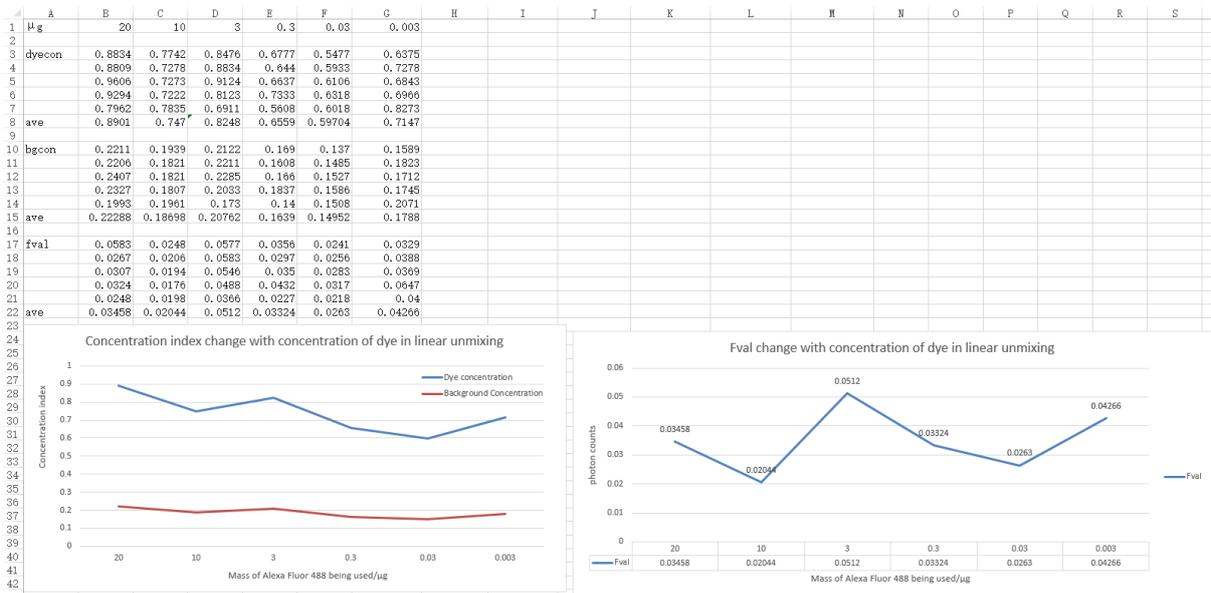


Figure 34: Raw data for Concentration Testing

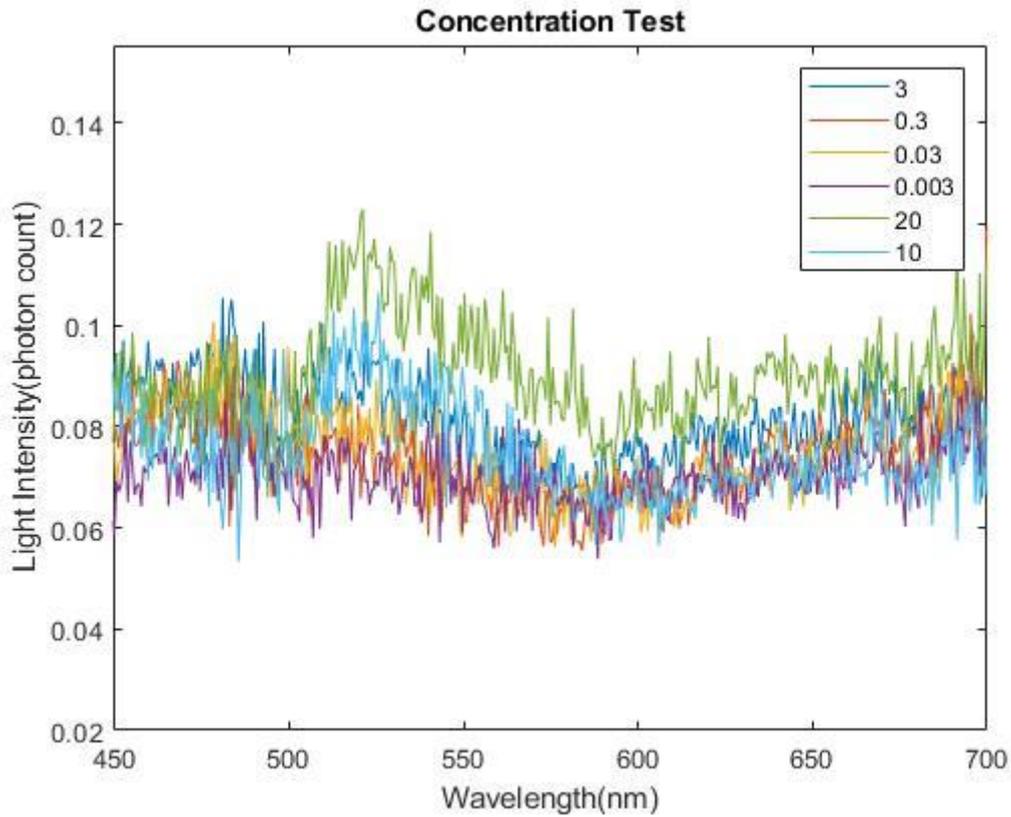


Figure 35: Emission Spectra of Different Microgram of Alexa Fluor 488

5.4 Light Intensity Detection Sensitivity Testing

The average value of maximum photon counts for the three different levels of light intensity is depicted in Figure 36.

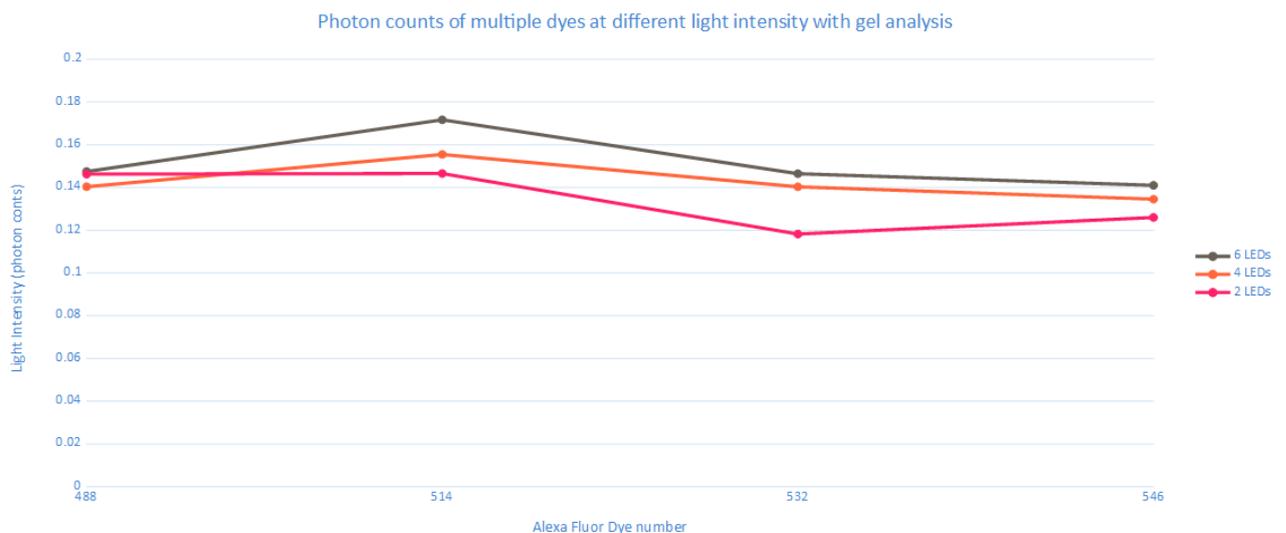


Figure 36: Graph depicting value of average maximum photon counts with 2 LEDs (green), 4 LEDs (red) and 6 LEDs (blue) on Alexa Fluor 488, 514, 532 and 546

Chapter 6: Design Validation

The primary objective for this project was to determine if an image processing algorithm could be used to measure the shape of emission signatures of dyes with similar wavelengths and distinguish the differences between them to separate them in a final output image. In order to test the newly designed device and determine if the project successfully met this objective the team set up several gels with multiple dyes of different wavelengths and scanned them with the fluorescent imaging device. The captured image data were then processed through different algorithms to identify close emission wavelength. The team used spectral angle mapper to determine dyes that are not mix together. Linear unmixing algorithm was used for identifying different concentration of spectra in a pixel. The team loaded different combination of dyes in a different well of gel electrophoresis to test the linear unmixing. Unknown combination of dyes was then created for linear unmixing to identify. The result of spectral angle mapper in Figure 31 shows that it can identify different dyes in the same emission color channel. In Figure 33, the result of linear unmixing shows that it can identify different amount of concentrations of the same dye. Unfortunately, linear unmixing did not produce a result for identifying the mixed combination of dyes. Further discussion of the failure of linear unmixing will be stated in chapter 7.

6.1 Engineering Standards

In order to be placed into production and sold to consumers the design must follow the constraints of several industry standards. The team has come up with four ISO standards that the final device will likely require in production. The first standard is ISO 9001 which covers device quality control of components, requires an instruction manual from the seller, and the device is given a minimum length of operation longevity. The second required standard is ISO 19056 which requires imaging devices to have uniform brightness and image picture output. The third ISO standard 8039 covers proper magnification symbol labeling as well as tolerance control of components. The final standard that the device should follow is ISO 19012 which covers chromatic correction and standards for imaging color in microscopes and image devices. While this device may require more manufacturing constraints and standards in the future, these four ISO standards are the ones we believe are necessary to focus on in the early stages of development of our device.

6.2 Impact of Device

6.2.1 Economics

The results of this project could one day have a positive impact on the economy as quality control of many products such as produce, or plastics is already done with fluorescence. If this design can be successfully incorporated into these fields and allow for more versatility, while eliminating the need for expensive fluorescent filters, companies could spend less money on quality control and sell products for less. Also, this product may allow a more thorough quality control resulting in a higher quality product being sold by companies which will benefit everyone. By eliminating the need for multiple filters in imaging, this projects results prove fluorescence imaging can be done with greater versatility and potentially lower cost than what is currently being used.

6.2.2 Environmental Impact

This new development in fluorescence imaging does not have any negative impacts on the environment but it may have positive impacts if used in the right field. The fluorescence device can be used for crop analysis and accurately determine what type of, and if a crop requires pesticides. This may allow farmers to use less pesticides on their crops and therefor pollute less water runoff with pesticides. Along with this application in agriculture, fluorescence can be used to scan regions for pollution in either water or soil and determine what type of pollutant exists. This may aid cleanup efforts of things such as oil spills or chemical waste spills in the environment. Through these applications this projects development in fluorescence may be useful in protecting and keeping the environment clean

6.2.3 Societal Influence

Similarly, to the economic impact of this device, it may also have an influence on society as it can aid quality control of products and accurately separate out defects in manufacturing. By doing this companies can ensure society gets the best quality products free of defects. Along with this positive impact it may negatively affect jobs as a fully automated imaging system to sort product may take away any human labor sorting that companies used to use. Aside from this potential setback, overly the

development of this fluorescence system should have an overall positive impact on society and push society forward into a new age of imaging.

6.2.4 Political Ramifications

This project may positively influence the global market as it can decrease the cost of company's product sorting and allow them to make more off products. Other uses for political reasons include military applications for reconnaissance or any other imaging necessary field. While there may not be many political issues affected by this product, it will have a trickle-down effect on the government and influence some aspects of the global market.

6.2.5 Ethical Concerns

This product will likely have positive ethical results as it may be able to help the development of drugs and disease research. Fluorescence imaging is already being used by headwall to research Alzheimer's and neurodegenerative diseases. With the results from this project, research in these fields will be able to continue to move forward and find new ways of treating and curing diseases. While these are positive ethical results, some may view military applications of the device to be negative ethical issues. The ethical results of this advancement in fluorescence imaging will ultimately be a result of how the user implements the device in their field of interest, as it has the potential for positive or negative ethical impacts.

6.2.6 Health and Safety Issues

This project could have a positive impact on the health and safety of people in several different ways. As stated in the paragraph above, fluorescent imaging is already and will continue to be used in the advancement of drugs and disease research. This project could one day assist in the development of medical breakthroughs that could ultimately save lives. Also stated above is how fluorescence can be used to separate goods in production to remove bad or spoiled goods from leaving factories. This can prevent unwanted bacteria or spoiled goods from being sold to and then consumed by users. By doing this fluorescence imaging can assist in the safety of foods and other products.

6.2.7 Manufacturability

After some further advancement of the product designed in this project by Headwall Photonics, it certainly could be manufactured relatively easy and implemented into the current device Headwall sells. The device is relatively low cost and does not contain any materials that are difficult to obtain. The algorithms and coding are also relatively simple and can for the most part be run on the current computers used by Headwall Imaging machines. These factors make this projects device capable of being manufactured easily.

6.2.8 Sustainability

In terms of renewable energy and sustainability it is possible that this fluorescence imaging device could be used in labs to help make discoveries and push research further in these fields. This device may give biologists a new look into their studies and allow them to view materials and structures in a more advanced way. Fluorescence imaging has a nearly endless number of applications in a lab setting and could easily benefit biologists looking to further research in renewable energy.

Chapter 7: Discussion

Figure 31 proves that spectral angle mapper is capable of separating dyes with extremely close emission spectrum that all fall in the same color channel. As stated previously, Alexa Fluor 488, 514 and 532 were put into gel electrophoresis and imaged with our device, resulting in Figure 31. As seen in Figure 31, the three dyes are indistinguishable by eye with normal fluorescence as they all appear green or are too dim to see at all. It also shows the false classified colors for each dye after the data ran through the spectral angle mapper algorithm. Alexa Fluor 488 has been classified as green, 514 as red and 532 has been given a purple classification color. Figure 32 is a graph which shows the wavelength for each dye that the spectral angle mapper has deciphered and split. According to Thermo Fisher Alexa Fluor 488, 514 and 532 should have emission wavelengths of 519, 540, and 553 respectively. Figure 32 demonstrates that the hyperspectral device was able to accurately capture these emissions and spectral angle mapper effectively separated each individual dye and outputted wavelengths for all three that correlate closely to the expected data from Thermo Fisher [19].

Current fluorescent imaging devices require filters to separate out emission wavelengths and image dyes. Due to these filters dyes with similar emission wavelengths that fall in the same color channel cannot be imaged and separated in the same scan. With spectral angle mapper, our device is capable of exciting fluorescent dyes in the same color channel and accurately predicts their emission wavelengths. The device can then separate the dyes by applying false colors, so the dyes appear different to the human eye. The current device is capable of doing this for every dye listed in the Table 3 above.

The only difficulty for the light source is the emission intensity of dyes at the far end of UV and IR ranges having very low signal to noise ratio as shown in Figure 37. Figure 37 shows the raw spectrum curve of Alexa Fluor 488 captured by the scanning platform. Noise was produced by not enough white reference for the hyperspectral sensor. The hyperspectral sensor uses the white reference to detect the emission wavelength the brighter the white reference the better the capture emission will be.

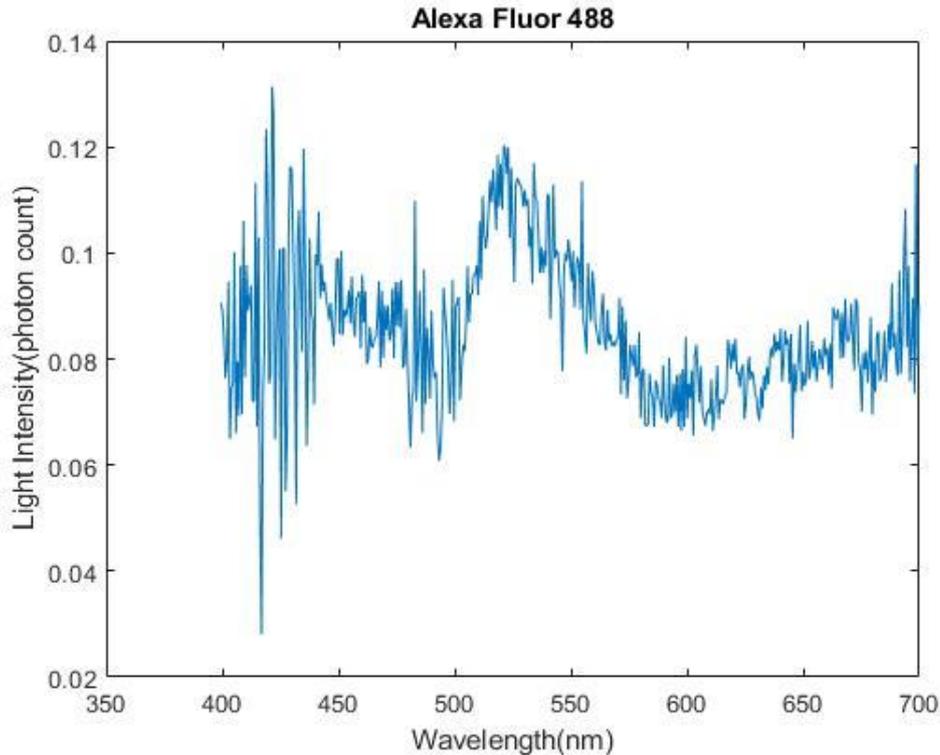


Figure 37: Raw spectrum curve

The concentration testing and light intensity testing indicates high levels of sensitivity for the sensor. A downward trend in concentration index was shown after applying linear un-mixing on the spectrums and proved that the sensor is able to detect pixels containing fluorescence with loading concentration of 0.0002 mg/ml at the beginning of gel electrophoresis. In light intensity testing, a reduction in the level of maximum photon counts had been recorded with varying LED intensities (6, 4 and 2 LEDs). However, both these reductions were small and not directly proportional to the corresponding input. The reason that caused this phenomenon was light leakage from the outside environment and low light intensity provided from the LEDs. But due to the high sensitivity of the Pco.edge sensor, the tiny reduction had been recorded.

While our device proved spectral angle mapper works perfectly and can separate dyes, linear unmixing tests were not as successful. Tests showed that with linear unmixing, the device was capable of determining the concentration of dye in the image sample but was unable to classify the dyes and separate out dye mixtures. Figure 33 shows how linear unmixing was able to detect concentrations of dye even with decreasing amounts of dye being used per sample. Figure 35, however illustrates the

large amount of noise in the linear unmixing data, which caused the detection and separation of specific dyes to be inaccurate. Due to the complexity of the linear unmixing algorithm, combined with the large amount of background noise in the data gathered, the current devices computer processor did not have the power required to run the unmixing. It is possible that with a much more powerful computer the data gathered could be unmixed using the linear unmixing algorithm and dyes may be able to be distinguished accurately but with the current device this is unknown.

View basic information about your computer 

Windows edition

Windows 10 Pro
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System

| | |
|-------------------------|----------------------------------------------------|
| Processor: | Intel(R) Core(TM) i7-4700HQ CPU @ 2.40GHz 2.40 GHz |
| Installed memory (RAM): | 8.00 GB (7.89 GB usable) |
| System type: | 64-bit Operating System, x64-based processor |
| Pen and Touch: | Touch Support with 10 Touch Points |

Computer name, domain, and workgroup settings

| | | |
|-----------------------|-------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------|
| Computer name: |  |  Change settings |
| Full computer name: |  | |
| Computer description: | | |
| Workgroup: | WORKGROUP | |

Windows activation

Windows is activated [Read the Microsoft Software License Terms](#)

Product ID: 00331-20020-00000-AA544  [Change product key](#)

Figure 38: The configuration of the computer that had failed to run through entire image with linear unmixing

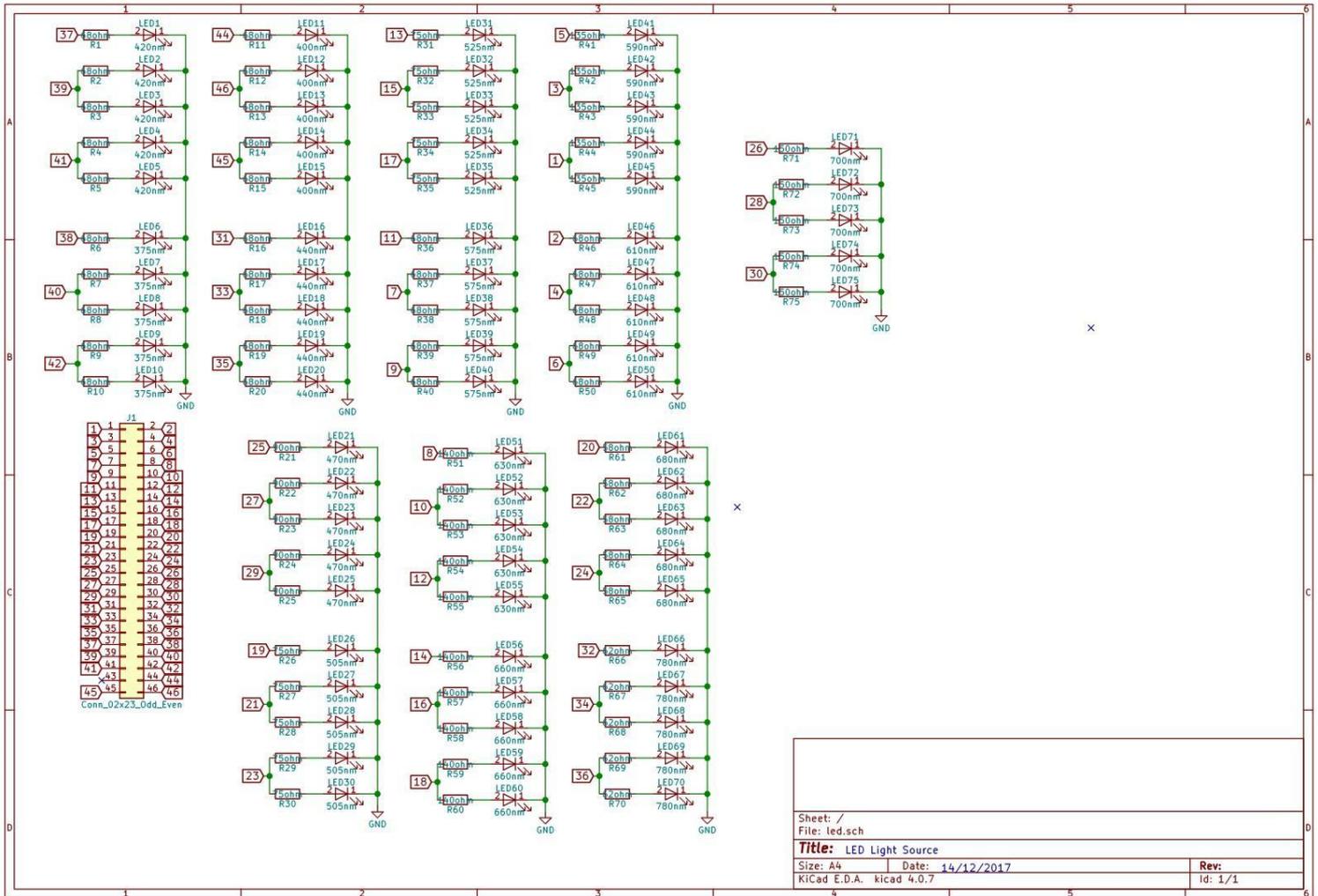
Chapter 8: Conclusions and Recommendations

The primary goal for this project was to design an illumination system, develop a spectrum analysis software program, and test the viability of a hyperspectral fluorescence imaging platform that can distinguish proteins conjugated to different fluorescence that have close emission wavelengths after excitation. The platform needed to be able to capture the shape of the emission spectrum and distinguish between probes that emit in the same color channel. After analysis of our final design we can conclude that the project successfully met this goal, specifically with the use of the spectral angle mapper algorithm. An illumination system was successfully built using 16 different wavelength LEDs with 6 LEDs per wavelength, clustered together into a circular module system. The wavelengths ranged from ultraviolet to infrared, and successfully excited dyes within this range. The LED light module combined with our spectral angle mapper algorithm allowed our device to image dyes in the same color channel and successfully determine the dye wavelengths and separate them accurately into false classified colors. Linear unmixing was not as successful, but it is possible that with an improved computer system and light source the algorithm could be able to distinguish mixed dyes successfully. We did prove that the linear unmixing algorithm is able to quantify the concentration of dye in samples, so with some improved specifications the algorithm should be able to work as fully intended.

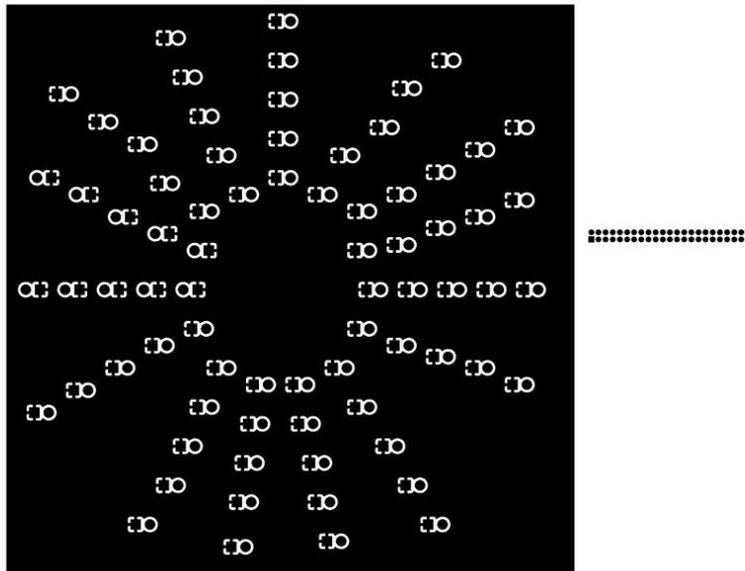
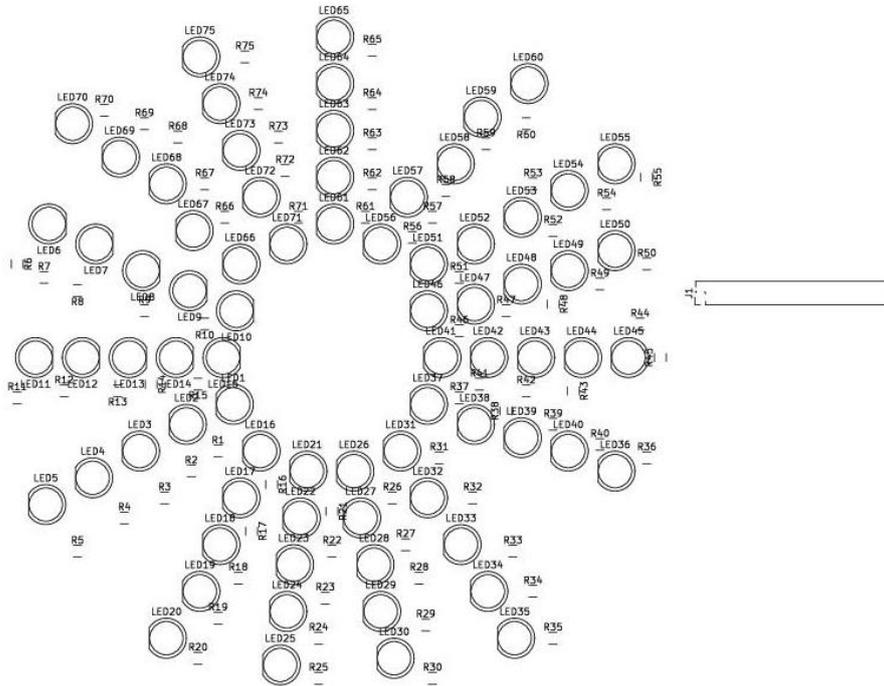
With several improvements to the current system it is possible that this device could be used in many biological imaging applications, and many other industry applications in the future. The primary improvement needed for this device is to add a more powerful light source and integrate in a better light focusing system. Unfortunately, after testing many alternative designs the team could not construct a suitable focus system for the current light source. This led to increased background noise in the scanning data specifically in the ultraviolet and infrared ranges causing some difficulty when running the algorithms. It is also possible that eliminating this background noise with a stronger light source could aid the linear unmixing algorithm and make it more practical for use in this system. In order to further improve linear unmixing results it is important to further revise the algorithm code to make it as efficient as possible. Also adding a more powerful computer system may allow linear unmixing to successfully identify overlapped dyes and give accurate concentrations for the dyes scanned. Ultimately with these changes this device could one day be integrated into microscopes to allow imaging of cells

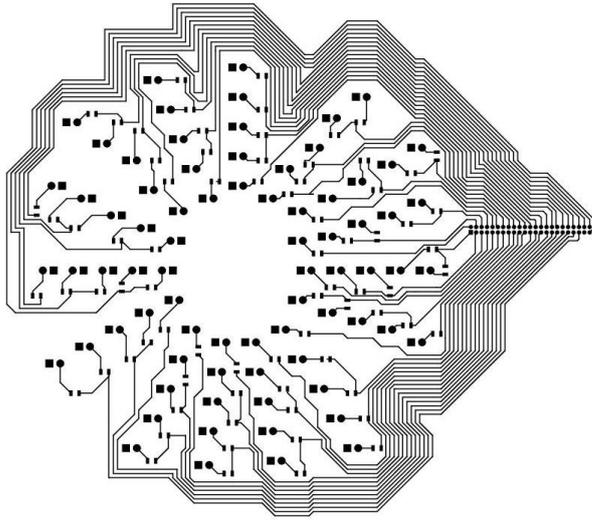
and organelles, using many dyes at once to allow quantification or analysis of a wide range of molecules and structures in one image. It is possible that this could proceed into successful whole animal imaging capable of showing nearly any desired structures in the body in one scan. As this current design successfully proved the concept of spectral angle mapper and illustrates the potential for linear unmixing it is likely that future developments will result in a huge market for this product with many different possible applications.

APPENDIX A: First Design Schematic

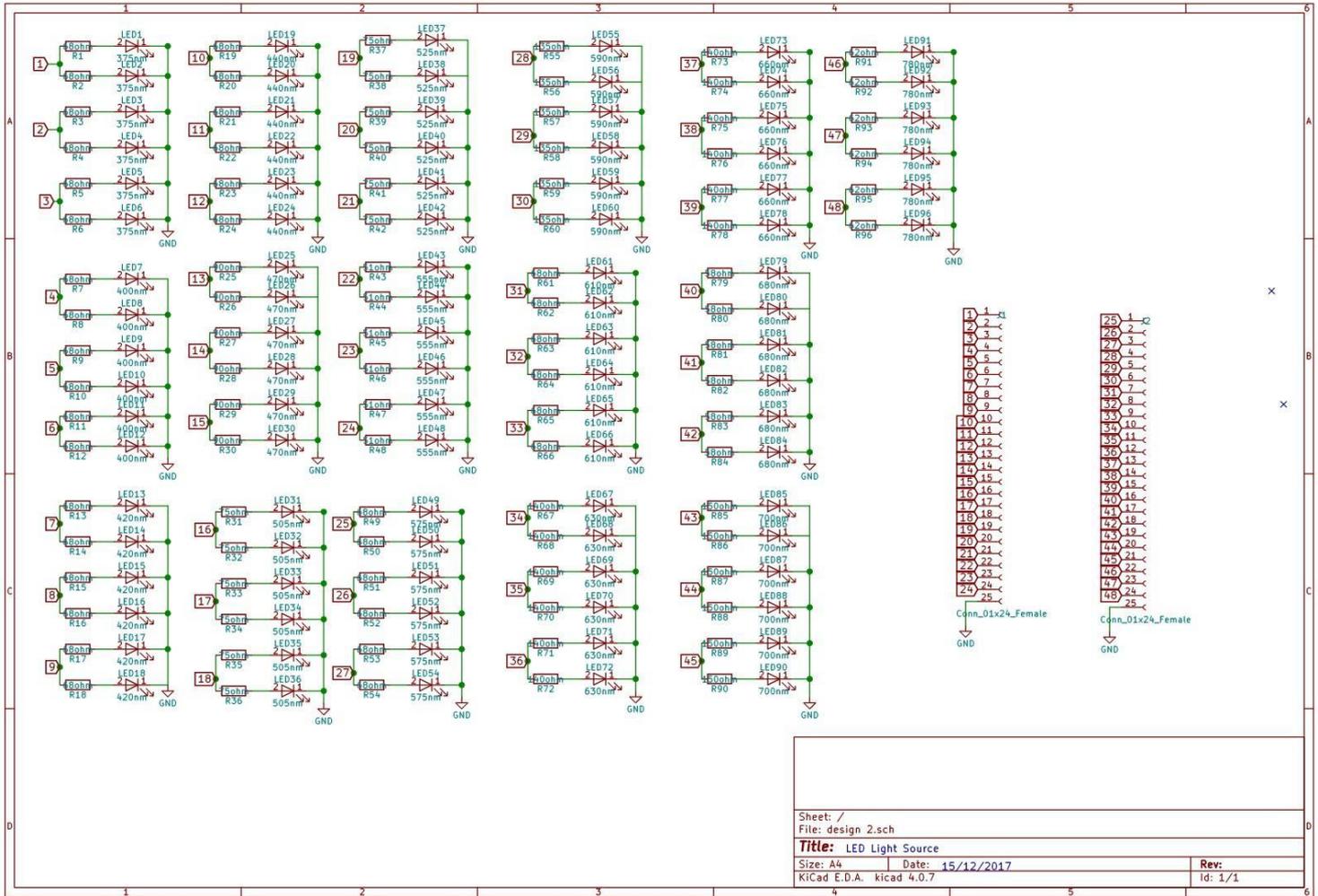


APPENDIX A.1: First Design PCB Layout



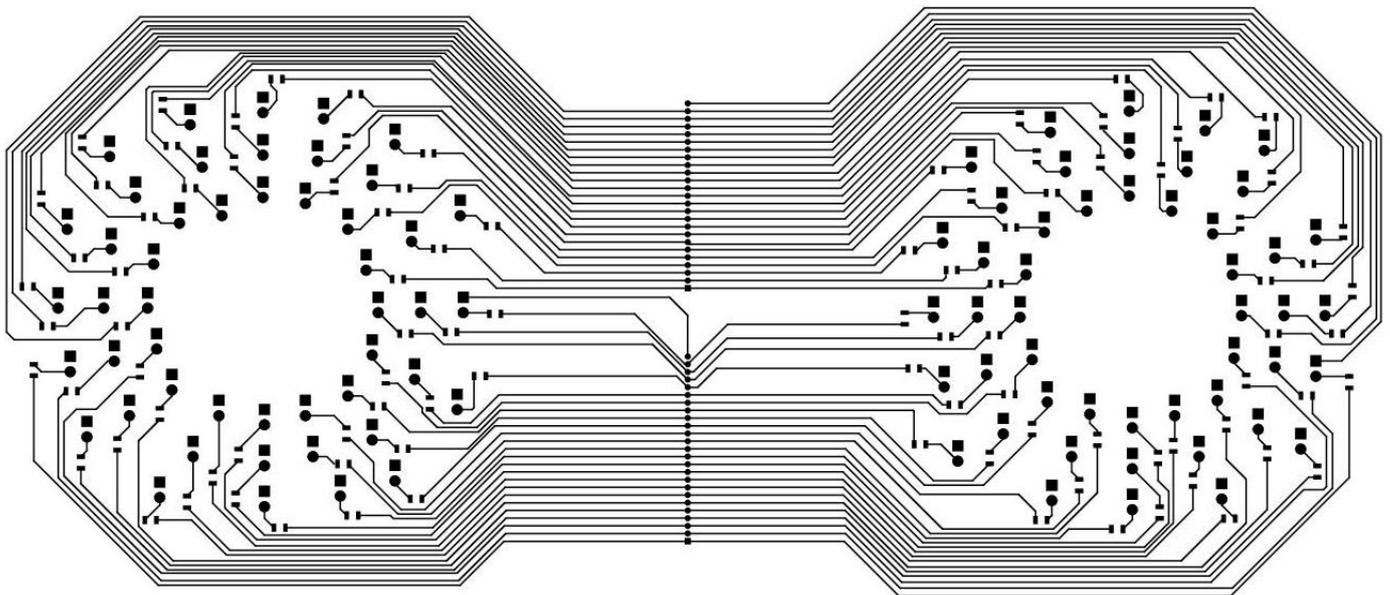
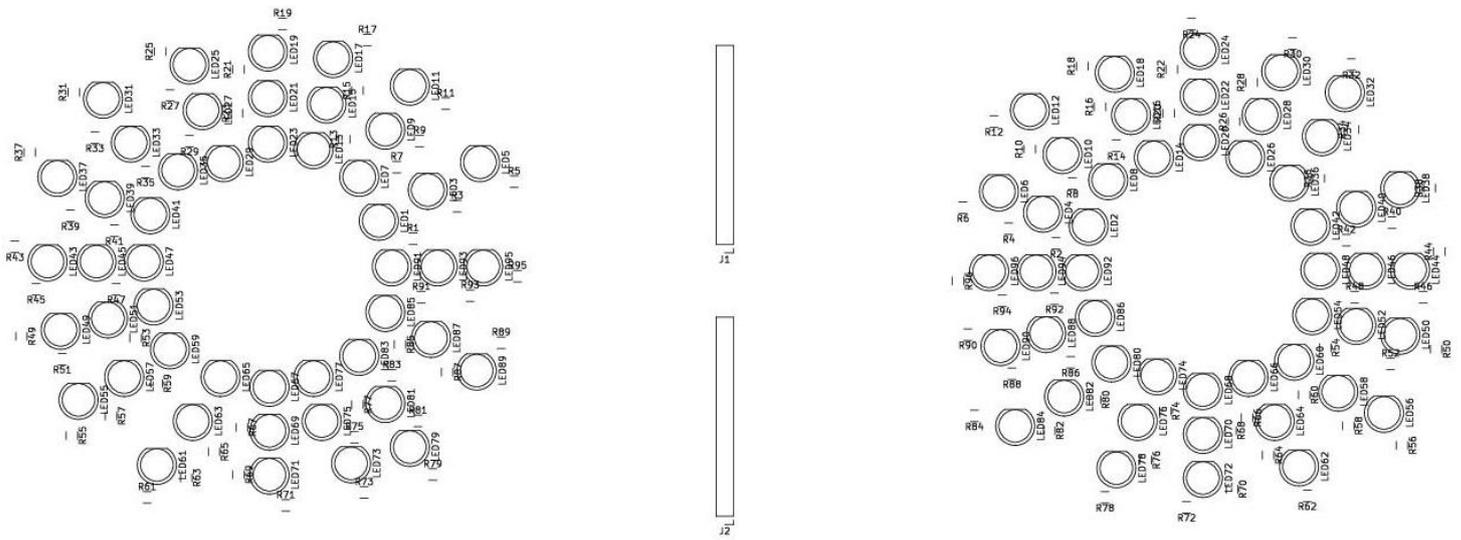


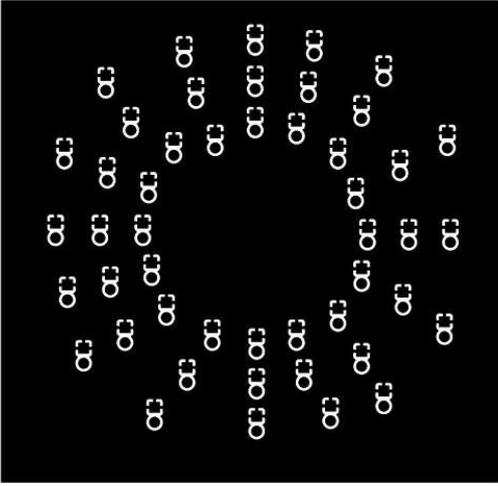
APPENDIX B: Final Design Schematic



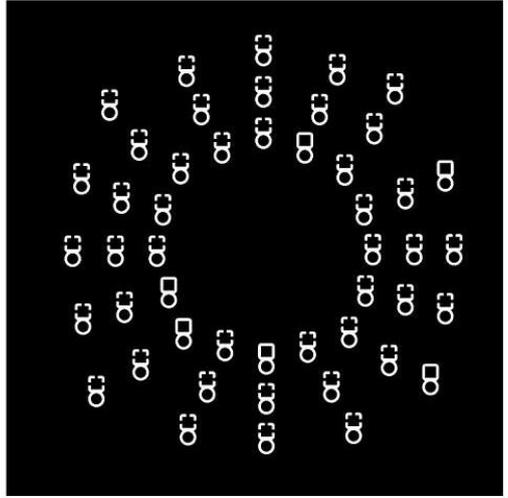
Sheet: /
 File: design 2.sch
Title: LED Light Source
 Size: A4 Date: 15/12/2017 Rev:
 KiCad E.D.A. kicad 4.0.7 Id: 1/1

APPENDIX B.1: Final Design PCB Layout





.....



APPENDIX C: Matlab Algorithms

APPENDIX C.1: reference.m

```
% open reference data

folder_name = inputdlg({'Please enter the name of the folder:'},...
    'Folder Name',1,{''},'on'); % input dialog to read name of folder with all the information
from user
raw_data = csvread(strcat(folder_name{1,1},'/raw1.csv'),1,0); % read raw data from the
folder, the raw data
% should extracted from a csv file

% select regions for reference
image = imshow(strcat(folder_name{1,1},'/image.png')); % show png image to users
selected_position = ginput(1); % use ginput to let user pick up a point on screen and
record the coordinate
close all; % close the image shown on the png image

% put reference data into 3D matrix

max_y = max(raw_data(:,1)) + 1; % first column in raw file records y-coordinate for
each pixel (row number)
max_x = max(raw_data(:,2)) + 1; % second column in raw file records x-coordinate for
each pixel (column number)
size_data = size(raw_data); % re-measure the size of the raw file for future use of
extracting pixel spectrum
raw_data_cell = cell(max_y,max_x); % create empty cell with the same size as the
image, each pixel has one spot
for i = 1:max_y
    for j = 1:max_x
        position_in_data = (i-1) * max_x + j; % calculate each pixel's row number in raw file
        raw_data_cell{i,j} = raw_data(position_in_data,3:size_data(2)); % extract each pixel's
data into its position in
% empty cell, the spectrum data is from third
column
% to the the end in the raw file
    end
end

% collect average data for reference

selected_region = raw_data_cell(round(selected_position(2))-1:...
    round(selected_position(2))+1,round(selected_position(1))-1:...
    round(selected_position(1))+1); % for better accuracy, get data from surrounding
point as a 3x3 square with
% center point of the point chosen and try to find out the average
spectrum
number_of_channels = size(selected_region{1,1}); % measure the amount of channels
```

```

selected_region_matrix = zeros(9,number_of_channels(2));    % create empty matrix to
record the pixels with close value
count = 0;    % create mechanism to count number of pixels with close value, initial value =
0

for i = 1:3
    for j = 1:3
        result(i,j) = acos((sum(selected_region{2,2}.* selected_region{i,j}))/...
            ((sqrt(sum(selected_region{i,j}.^2))) * ...
            (sqrt(sum(selected_region{2,2}.^2))));    % use spectral angle mapper in
collecting reference
                                % with single spectrum
        if result(i,j) <= 0.15    % means that pixel has same specrum with the point collected
            count = count + 1;    % add a number of pixel qualified
            selected_region_matrix(count,:) = selected_region{i,j};    % put all data in matrix
        end
    end
end

if count >= 6    % means the color of the selected region is quite constant
    reference_curve = zeros(1,number_of_channels(2));    % empty array for average data
    selected_region_matrix = selected_region_matrix(1:count,:);    % remove empty matrix
    for i = 1:number_of_channels(2);
        reference_curve(i) = mean(selected_region_matrix(:,i));    % get average data
    end
    reference_name = inputdlg({'Please name this reference:','Save
Reference',1,{'','on'}});    % % save reference into list
    save(strcat('reference\',reference_name{1,1},'.mat'),'reference_curve');    % test,
should save in another folder later
    open(strcat('reference\',reference_name{1,1},'.mat'));    % test
    msgbox('Reference spectrum complete')
else
    errordlg('Please retake reference spectrum, spectrum not constant')
end

```

APPENDIX C.2: check_dye.m

```
function [value] = check_dye()
    check_box = inputdlg({'Is there any other dye being used? (Enter 1 for yes and 0
no)'}, 'Enter Dye', 1, {''}, 'on'); % dialog box
    if check_box{1} == '1'
        value = 1;
    elseif check_box{1} == '0'
        value = 0;
    else
        disp('Typing error')
        check_dye()
    end
end
```

APPENDIX C.3: check_dye.m

```
function current_dye = input_dye(list_of_dye, current_dye)
    length_current_dye = length(current_dye);    % current dye is the number of dye we are
writing
    enter_reference = inputdlg({'Please enter name of dye being used:','Enter
Dye',1,{'','on'});    % get a reference
    if any(strcmp(list_of_dye, enter_reference{1}))    % check if we have the dye in the
current reference list
        if any(strcmp(current_dye, enter_reference{1}))    % check if the reference is already in
the list we are writing
            disp('Dye exists')
            value = check_dye();
            if value == 1
                current_dye = input_dye(list_of_dye, current_dye);    % restart the function
            else
            end
        else
        end
        current_dye[length_current_dye + 1] = enter_reference{1};    % add the name of the
dye in the list
        value = check_dye();    % use check_dye to check if more dyes are coming
        if value == 1
            current_dye = input_dye(list_of_dye, current_dye);    % restart the function
        else
        end
    end
    else
        disp('Dye doesn exist')    % tell the user we dont have reference for this dye
        value = check_dye();    % use check_dye to check if more dyes are coming
        if value == 1
            current_dye = input_dye(list_of_dye, current_dye);    % restart the function
        else
        end
    end
end
end
```

APPENDIX C.4: spectral_angle_mapper.m

```
% preparation
folder_name = inputdlg({'Please enter the name of the folder:','Folder Name',1,{'','on'}}); %
input dialog for name of the folder

list_dye_struct = dir('reference\*.mat'); % open all existing reference in reference folder
number_of_current_dye = length(list_dye_struct);
list_dye = cell(1,number_of_current_dye);
for i = 1:number_of_current_dye
    list_dye{i} = list_dye_struct(i).name;
end

list_selected_dye = {}; % create empty cell for list of dyes should be selected
list_selected_dye = input_dye(list_dye,list_selected_dye); % add all dyes selected into
the cell
reference_data = cell(1,length(list_selected_dye)); % create empty cell for the data of
selected dyes
for i=1:length(list_selected_dye)
    reference_data{i} = open(strcat('reference\',list_selected_dye{i})); % input the selected
dyes into the file
end

raw_data = csvread(strcat(folder_name{1,1}, '\raw1.csv'),1,0); % read the 3D matrix of
the file in the folder
max_y = max(raw_data(:,1)) + 1; % measure the size of the image
max_x = max(raw_data(:,2)) + 1;
size_data = size(raw_data); % measure the amount of pixels

raw_data_cell = cell(max_y,max_x); % create empty cell, each represent one pixel
for i = 1:max_y
    for j = 1:max_x
        position_in_data = (i-1) * max_x + j; % find the position of the data in raw matrix
        raw_data_cell{i,j} = raw_data(position_in_data,3:size_data(2)); % put all data in the
cell
    end
end

% algorithm
image = imread(strcat(folder_name{1,1},'/image.png')); % show png image to users
red = image(:, :, 1); % RGB
green = image(:, :, 2);
blue = image(:, :, 3);
bright_rgb_list = {
    [255,255,0],... % bright yellow
    [135,206,235],... % sky blue
    [255,97,0],... % orange
    [0,255,0],... % bright green
    [255,0,0],... % bright red
    [160,32,240],... % bright purple
    [227,207,87],... % banana yellow
    [255,192,203],... % pink
    [139,69,19],... % horse brown
```

```

[255,0,255],...    % purple red
[34,139,34]      % forest green
};                % list of colors, currently have 11, can be added with more

if length(list_selected_dye) == 1    % if only one dye, run spectral angle mapper directly
instead
    dye_judge = zeros(max_y,max_x);    % create empty matrix to decide if the pixel contain
that dye
    color_index = randi([1,length(bright_rgb_list)]);
    color_selected = bright_rgb_list{color_index};
    for l = 1:max_y
        for m = 1:max_x
            result = acos((sum(reference_data{1}.reference_curve.*raw_data_cell{l,m}))/...
                ((sqrt(sum(raw_data_cell{l,m}.^2)))*...
                (sqrt(sum(reference_data{1}.reference_curve.^2)))));    % spectral angle mapper
equation
            if result <= 0.35    % test value
                dye_judge(l,m) = 1;
                red(l,m) = color_selected(1);
                green(l,m) = color_selected(2);
                blue(l,m) = color_selected(3);
            end
        end
    end
    % test sum(sum(dye_judge))+
else
    dye_judge = zeros(max_y,max_x);
    color_index = randperm(length(bright_rgb_list));
    for m = 1:max_y
        for n = 1:max_x
            result = zeros(1,length(list_selected_dye));
            for o = 1:length(list_selected_dye)
                result(o) = acos((sum(reference_data{o}.reference_curve.*raw_data_cell{m,n}))/...
                    ((sqrt(sum(raw_data_cell{m,n}.^2)))*...
                    (sqrt(sum(reference_data{o}.reference_curve.^2)))));
            end
            [value, index] = min(result);
            if value <= 0.35
                red(m,n) = bright_rgb_list{color_index(index)}(1);
                green(m,n) = bright_rgb_list{color_index(index)}(2);
                blue(m,n) = bright_rgb_list{color_index(index)}(3);
                dye_judge(m,n) = index;
            end
        end
    end
end
end

new_image = cat(3,red,green,blue);    % get the new image
imshow(new_image)    % show the image

```

APPENDIX C.5: linear_unmixing.m

% preparation

```
folder_name = inputdlg({'Please enter the name of the folder:','Folder Name',1,{'','on'}}); %  
input dialog for name of the folder
```

```
number_cores = inputdlg({'How many cores do you wanna use?','Core  
Option',1,{'','on'}}); % let user choose how many pools they want to use  
number_cores = str2double(number_cores{1});  
parpool(number_cores);
```

```
list_dye_struct = dir('reference*.mat'); % open all existing reference in reference folder  
number_of_current_dye = length(list_dye_struct);  
list_dye = cell(1,number_of_current_dye);  
for i = 1:number_of_current_dye  
    list_dye{i} = list_dye_struct(i).name;  
end
```

```
list_selected_dye = {}; % create empty cell for list of dyes should be selected  
list_selected_dye = input_dye(list_dye,list_selected_dye); % add all dyes selected into  
the cell  
amount_dye = length(list_selected_dye);  
reference_data = cell(1,amount_dye); % create empty cell for the data of selected dyes  
for i=1:amount_dye  
    reference_data{i} = open(strcat('reference\',list_selected_dye{1})); % input the selected  
dyes into the file  
end
```

```
raw_data = csvread(strcat(folder_name{1,1}, '\raw.csv'),1,0); % read the 3D matrix of the  
file in the folder  
max_y = max(raw_data(:,1)) + 1; % measure the size of the image  
max_x = max(raw_data(:,2)) + 1;  
size_data = size(raw_data); % measure the amount of pixels
```

```
raw_data_cell = cell(max_y,max_x); % create empty cell, each represent one pixel  
for i = 1:max_y  
    for j = 1:max_x  
        position_in_data = (i-1) * max_x + j; % find the position of the data in raw matrix  
        raw_data_cell{i,j} = raw_data(position_in_data,3:size_data(2)); % put all data in the  
cell  
    end  
end
```

% algorithm

```
image = imread(strcat(folder_name{1,1},'/image.png')); % show png image to users  
red = image(:,:,1); % RGB  
green = image(:,:,2);  
blue = image(:,:,3);  
bright_rgb_list = {  
    [255,255,0],... % bright yellow  
    [135,206,235],... % sky blue  
    [255,97,0],... % orange  
    [0,255,0],... % bright green
```

```

[255,0,0],...    % bright red
[160,32,240],... % bright purple
[227,207,87],... % banana yellow
[255,192,203],... % pink
[139,69,19],... % horse brown
[255,0,255],...  % purple red
[34,139,34]     % forest green
};              % list of colors, currently have 11, can be added with more
dye_color = zeros(amount_dye,3);
color_index = randperm(length(bright_rgb_list));
for dc = 1:amount_dye
    dye_color(dc,:) = bright_rgb_list{color_index(dc)};
end

if amount_dye == 1    % if only one dye, run spectral angle mapper directly instead
    dye_judge = zeros(max_y,max_x);    % create empty matrix to decide if the pixel contain
    that dye
    result = zeros(max_y,max_x);
    reference_1 = reference_data{1}.reference_curve;
    parfor l = 1:max_y
        for m = 1:max_x
            result(l,m) = acos((sum(reference_1.*raw_data_cell{l,m}))/...
                ((sqrt(sum(raw_data_cell{l,m}.^2)))*...
                (sqrt(sum(reference_1.^2)))));    % spectral angle mapper equation
            if result(l,m) <= 0.1    % test value
                dye_judge(l,m) = 1;
                template_red = red(l,m);
                % template for saving red channel value, then converting
                % red->green, green->blue, blue->red template so the
                % color is different from before and background noise
                red(l,m) = green(l,m);
                green(l,m) = blue(l,m);
                blue(l,m) = template_red;
            end
        end
    end
    % test sum(sum(dye_judge))
else
    number_channel = length(raw_data_cell{1,1});

    if number_channel > 400
        cutting_index = ceil(number_channel/400);
        parfor m = 1:max_y
            for n = 1:max_x
                focus_pixel = raw_data_cell{m,n};    % pixels by pixels, one by
one
                cut_channels = floor(number_channel/cutting_index);    % matlab unable to
go through equations that is too large (set maximum as 400)
                sum_square = @(c) 0*c(1);    % set an equation with
unknown variable but equals to zero for the variable so
equation, this variable will be used to mount    % it can go into unknown solving
                                                    % the sum of square of mins in each

```

```

channel

    for i = 1:cut_channels
        ind_square = @(c) 0*c(1) + focus_pixel(i*cutting_index-1);    % set up the
initial S value so that we can write min = S - C1S1 - C2S1 .....    % also with known variable equal to
zero so it can go into unknown solving equation
        for j = amount_dye
            ind_square = @(c) ind_square(c) -
c(j)*reference_data{j}.reference_curve(i*cutting_index-1);
                                                                    % - CNSN one by one dependin on
the amount of dye
            end
            sum_square = @(c) sum_square(c) + (ind_square(c))^2;    % add up the
square of mins
            end

            c0 = [0,amount_dye+1];    % theoretically, Sigma(c)
could be equal to the number of dye,    % if there are two dyes being used, and
both C1 and C2 are greater than 1, in order to    % let the program still be able to detect
the dye without causing error an space must    % be provided, this value could be re-
adjusted in the future

            % therefore assume the maximum is the number of dye
            c = fminsearch(sum_square,c0);    % try to solve for the
closest solution for unknown set of Cs
            color_cal = zeros(1,amount_dye);    % used to calculate color
            for l = 1:amount_dye
                if c(l) >= 0.2
                    color_cal(l) = c(l);    % set c value for future use if c is
greater than 0.2 (means probably there is this kind    % of dye here)
                end
            end

            sum_c = sum(color_cal);    % algorithm to add
up color
            new_raw_rgb = zeros(1,3);    % what i currently
use is by using the concentration we achieved from
            new_rgb = zeros(1,3);    % as the
concentration of the RGB readings R = R1C1 + R2C2.... (take red
            for rrgb = 1: amount_dye
                new_raw_rgb = new_raw_rgb +
dye_color(rrgb,:)*color_cal(rrgb)/sum_c;    % as an example), and add up RGB
readings with corresponding weiting for
            end
                                                                    % a concentrated RGB mixed
value, if the value exceed 255, it reaches maximum
            for nrgb = 1:3
                if new_raw_rgb(nrgb)<= 255
                    new_rgb(nrgb) = new_raw_rgb(nrgb);
                else

```

```

        new_rgb(nrgb) = 255;
    end
end

    image(l,m,:) = new_rgb;
end
end
else
    parfor m = 1:max_y
        for n = 1:max_x
            focus_pixel = raw_data_cell{m,n}; % same algorithm but
            % designed for image with less than 400 channels already, all the algorithms
            % are the same

            sum_square = @(c) 0*c(1);

            for i = 1:number_channel
                ind_square = @(c) 0*c(1) + focus_pixel(i);
                for j = amount_dye
                    ind_square = @(c) ind_square(c) - c(j)*reference_data{j}.reference_curve(i);
                end
                sum_square = @(c) sum_square(c) + (ind_square(c))^2;
            end

            c0 = [0,amount_dye];
            c = fminsearch(sum_square,c0);
            color_cal = zeros(1,amount_dye);
            for l = 1:amount_dye
                if c(l) >= 0.2
                    color_cal(l) = c(l);
                end
            end

            sum_c = sum(color_cal);
            new_raw_rgb = zeros(1,3);
            new_rgb = zeros(1,3);
            for rrgb = 1: amount_dye
                new_raw_rgb = new_raw_rgb + dye_color(rrgb,)*color_cal(rrgb)/sum_c;
            end

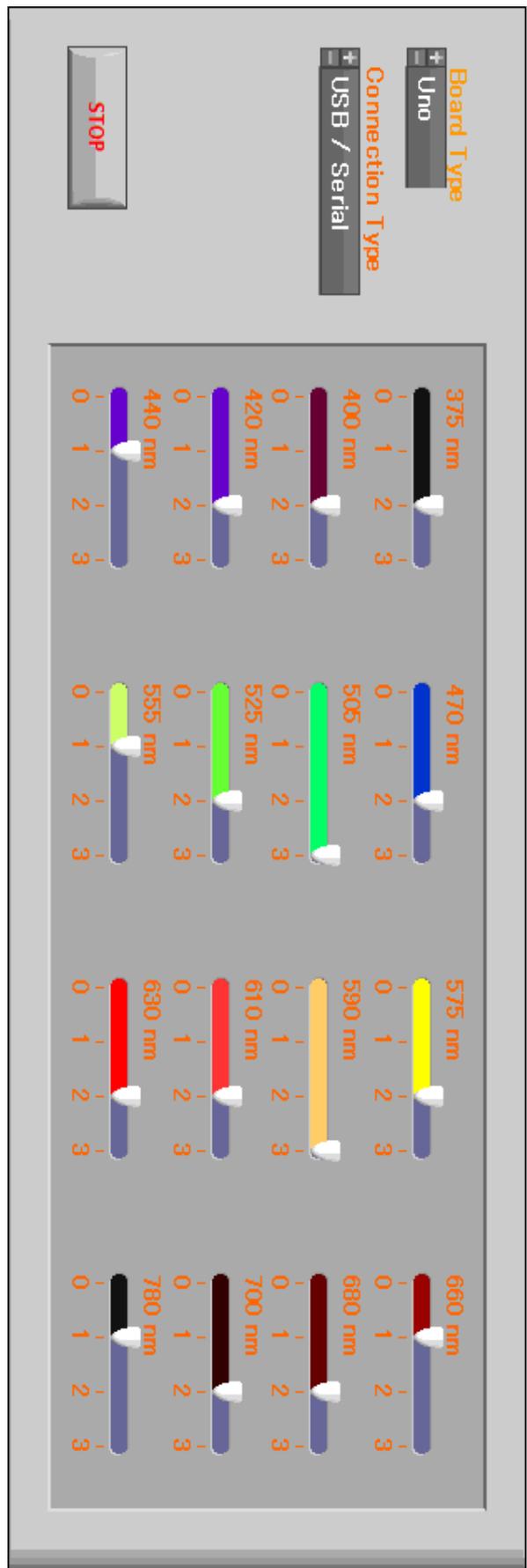
            for nrgb = 1:3
                if new_raw_rgb(nrgb)<= 255
                    new_rgb(nrgb) = new_raw_rgb(nrgb);
                else
                    new_rgb(nrgb) = 255;
                end
            end

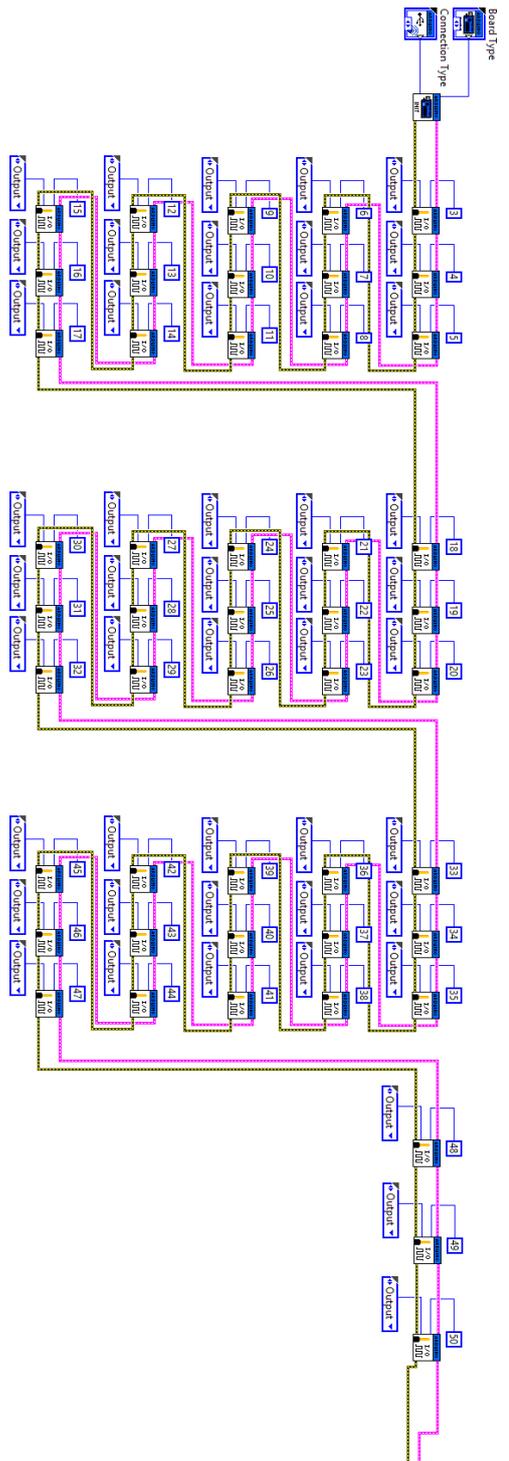
            image(l,m,:) = new_rgb;
        end
    end
end
end

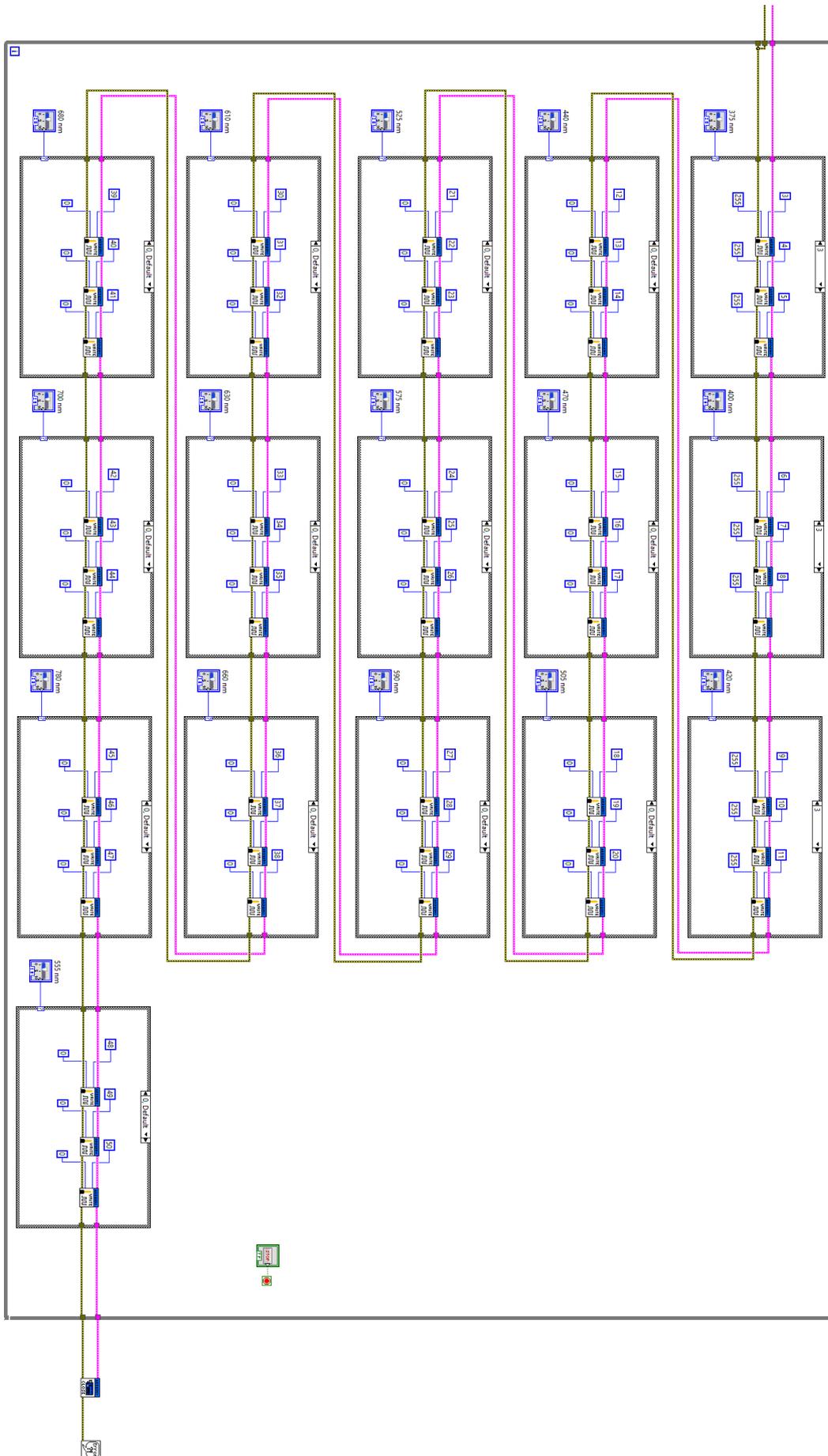
imshow(image) % show the image

```

APPENDIX D: LabVIEW Graphical User Interphase







APPENDIX E: Antibodies

The following antibodies were used in this experiment:

Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 350 from Thermo Fisher Scientific, catalog # A-11045, RRID AB_2534100.

Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 405 from Thermo Fisher Scientific, catalog # A-31553, RRID AB_221604.

Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 430 from Thermo Fisher Scientific, catalog # A-11064, RRID AB_2534111.

Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 from Thermo Fisher Scientific, catalog # A-11008, RRID AB_143165.

Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 514 from Thermo Fisher Scientific, catalog # A-31555, RRID AB_2536171.

Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 532 from Thermo Fisher Scientific, catalog # A-11002, RRID AB_2534070.

Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 546 from Thermo Fisher Scientific, catalog # A-11003, RRID AB_2534071.

F(ab')₂-Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 555 from Thermo Fisher Scientific, catalog # A-21430, RRID AB_2535851.

Goat anti-Mouse IgG1 Cross-Adsorbed Secondary Antibody, Alexa Fluor 568 from Thermo Fisher Scientific, catalog # A-21124, RRID AB_2535766.

Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 594 from Thermo Fisher Scientific, catalog # A-11032, RRID AB_2534091.

Rabbit anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 633 from Thermo Fisher Scientific, catalog # A-21063, RRID AB_2535727.

Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 635 from Thermo Fisher Scientific, catalog # A-31574, RRID AB_2536184.

Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 from

Thermo Fisher Scientific, catalog # A-21235, RRID AB_2535804.

Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 660
from Thermo Fisher Scientific, catalog # A-21055, RRID AB_2535722.

Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 680
from Thermo Fisher Scientific, catalog # A-21058, RRID AB_2535724.

Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 700 from
Thermo Fisher Scientific, catalog # A-21038, RRID AB_2535709.

Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 750 from
Thermo Fisher Scientific, catalog # A-21037, RRID AB_2535708.

Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 790
from Thermo Fisher Scientific, catalog # A11357, RRID AB_2534140.

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[19] The following antibodies were used in this experiment:

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Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 430 from Thermo Fisher Scientific, catalog # A-11064, RRID AB_2534111.

Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 from Thermo Fisher Scientific, catalog # A-11008, RRID AB_143165.

Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 514 from Thermo Fisher Scientific, catalog # A-31555, RRID AB_2536171.

Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 532 from Thermo Fisher Scientific, catalog # A-11002, RRID AB_2534070.

Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 546 from Thermo Fisher Scientific, catalog # A-11003, RRID AB_2534071.

F(ab')₂-Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 555 from Thermo Fisher Scientific, catalog # A-21430, RRID AB_2535851.

Goat anti-Mouse IgG1 Cross-Adsorbed Secondary Antibody, Alexa Fluor 568 from Thermo Fisher Scientific, catalog # A-21124, RRID AB_2535766.

Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 594 from Thermo Fisher Scientific, catalog # A-11032, RRID AB_2534091.

Rabbit anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 633 from Thermo Fisher Scientific, catalog # A-21063, RRID AB_2535727.

Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 635 from Thermo Fisher Scientific, catalog # A-31574, RRID AB_2536184.

Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 647 from Thermo Fisher Scientific, catalog # A-21235, RRID AB_2535804.

Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 660 from Thermo Fisher Scientific, catalog # A-21055, RRID AB_2535722.

Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 680 from Thermo Fisher Scientific, catalog # A-21058, RRID AB_2535724.

Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 700 from Thermo Fisher Scientific, catalog # A-21038, RRID AB_2535709.

Goat anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 750 from Thermo Fisher Scientific, catalog # A-21037, RRID AB_2535708.

Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 790 from Thermo Fisher Scientific, catalog # A11357, RRID AB_2534140.

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