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UNIVERSITY OF WISCONSIN–MADISON

Miniature Microscope for FRET Microscopy

Preliminary Report

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1. Abstract

Microscopes are essential for understanding the structure of cells, microorganisms, and other molecular structures. Many educational institutions and scientists rely on these devices for important research, yet modern microscopes, while available to well-financed labs, are often not an option for a classroom. Therefore, many students are unable to use these devices to practice processes that they are expected to understand later. A typical epi-fluorescent microscope can cost over $100,000, which far exceeds a typical course budget. The client, Professor Matthew Merrins, teaches a human biochemistry lab at the University of Wisconsin-Madison. His lab currently uses Laconic, a Fluorescence Resonance Energy Transfer (FRET)-based biosensor to detect the presence of Lactate in cells. Ideally, this lab will allow students to learn about microscopy through experimentation, but with the cost constraint of the course, a "typical" microscope is out of the question. The goal of this design is to build an affordable, FRET-capable microscope that can be repeatedly manufactured for his students. The current proposed design involves a simplified microscope with a sample stand, a LED light source, an objective platform with a filter cube and filter-switching interface, a tube lens, and a camera. The data collected from the camera will be submitted to a proper software service for data analysis and extraction. Current design plans include assembly and testing of the excitation source.

2. Introduction

2.1. Problem Statement

The client, Professor Matthew Merrins, teaches human biochemistry lab at the University of Wisconsin-Madison. The course focuses on the enzyme lactate dehydrogenase, which produces lactate from pyruvate. Currently, his lab utilizes Laconic, a Förster Resonance Energy Transfer (FRET)-based biosensor. This biosensor detects the presence of lactate in healthy, living cells, but the fluorescence must be monitored over a period of time using an expensive microscope. This microscope excites the lactate biosensor using a complicated system of LEDs and filters. The fluorescence emission at two different wavelengths is recorded and a FRET efficiency ratio is calculated. Since the current microscope in his lab in extremely expensive, the goal is to simplify the microscope and build a low-cost alternative specific to the Laconic biosensor. The client would like a working prototype by the end of the fall semester.

2.2. Project Motivation

Current microscopes are extremely expensive due to their broad functionality. Even though this can be beneficial in a research lab, the client does not require as much flexibility for his simplified microscopes. The client would like to measure FRET, but with a specific focus on a single metabolic enzyme, lactate dehydrogenase. Ideally, he will have multiple devices for his class to maximize his students' educational experience. The design should be reproducible so that in the future he will have six to eight microscopes for his class.

2.3. Background

2.3.1 FRET

Fluorescence Resonance Energy Transfer (FRET) is the transfer of energy between two light-sensitive molecules. These molecules are known as chromophores, and they are referred to as the donor and the acceptor. FRET is a measurement of the different intensities of emission in order to determine the proximity of the two chromophores [1]. This is done by using a light source (usually an LED or laser) that will excite the donor chromophore. As the donor chromophore gets excited, it emits photons and transfers energy to excite the nearby acceptor chromophore; the transfer efficiency is a function of chromophore proximity. Usually the intensity of these sources is mapped using an absorption/emission spectrum, and a ratio of acceptor to donor emission intensity is obtained [2]. Many dynamic processes, such as protein-protein interactions, can be identified with various FRET biosensors. FRET is a popular method because of its ability to measure low concentration of molecules, and it has the capability to determine molecular dynamics of a given complex over time [3].

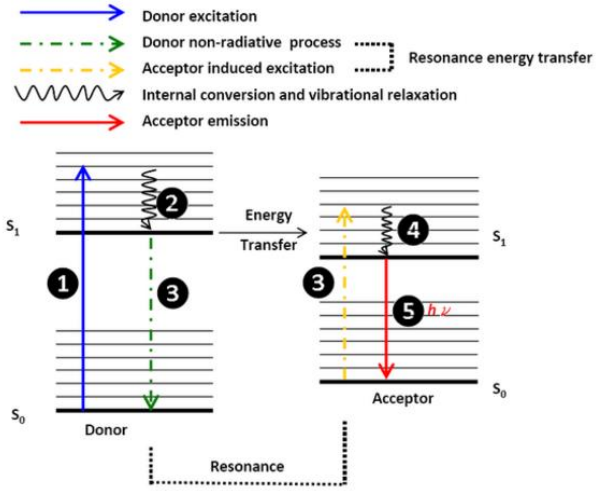


Figure 1. Schematic of Fluorescence Resonance Energy *[2]*. This image was obtained from the University of California, Davis, and shows of visual representation of FRET. S_0 represents the ground state, whereas S_f represents the lowest excited state after donor.

2.3.2 Laconic: Lactate Biosensor

Lactate is produced from pyruvate by the enzyme lactate dehydrogenase (LDH) in mammalian cells [1]. LDH is found in almost all body tissues, and it is vital in cellular respiration, signaling, and metabolic processes in healthy tissues [4]. In addition, if lactate is not regulated properly, this can lead to various risks to a person's health. Frequently, tumor cells have high rates of lactate production when oxygen is present [5]. As a result, many studies have been trying to further understand this process in living cells.

Professor Merrins' Lab specifically focuses on the nutrient metabolism in pancreatic islet beta cells. His goal is to further understand the cause of insulin release and how to cause cell proliferation as soon as insulin is needed. By using rodents that are obese or have diabetes, Professor Merrins is able to use FRET in order to monitor metabolite production in different cells types, such as in yeast and cancer cells.

The biosensor that Professor Merrins hopes to use in his lab course is a protein that consists of two connected fluorophores and a lactate binding site [1]. This biosensor can be used

to quantify lactate levels between 1 uM an 10 mM based on the FRET efficiency. Without lactate, the proteins are positioned well to allow for energy transfer between mTFP and Venus. The donor fluorophore is teal fluorescent protein (generally called CFP), which is excited by 430 nm light and fluoresces at 470 nm (Figure 2). The acceptor fluorophore is Venus (generally called YFP), which is excited by light around 470 nm and fluoresces at 535 nm (Figure 3). Professor Merrins plans to run various experiments in his biochemistry class that will cause different behaviors of these fluorophores. One of his experiments involves subjecting the cells to high and low concentrations of glucose. High glucose concentration causes an increase in lactate production [6]. As lactate production increases, this causes the intensity of the CFP channel to increase, and the intensity of the YFP channel to decrease. Therefore the FRET ratio will become smaller. When the cells are subjected to low concentration of glucose, CFP decreases and YFP increases which results in a bigger FRET ratio. Professor Merrins' ideal device will be able to detect the difference in intensity between the two fluorophores, and overall determine the FRET ratio between experiments.

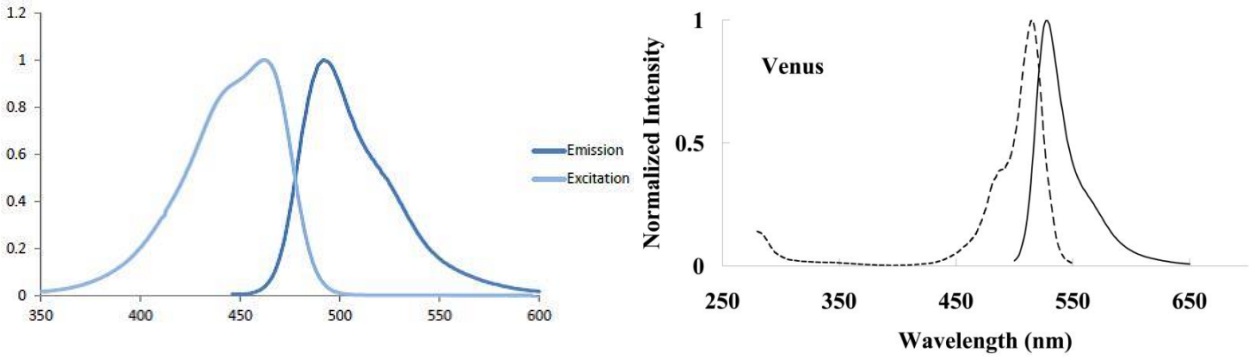


Figure 2. Left: Excitation and emission curves for mTFP with normalized intensity on the y-axis and wavelength (nm) on the x-axis *[15]*. Right: Excitation (dashed line) and emission (full line) curves for Venus *[16]*.

2.3.3 Client Background

Professor Matthew Merrins is an assistant professor in the Biomolecular Chemistry Department with a laboratory under the Department of Medicine at the University of Wisconsin School of Medicine and Public Health. His research is focused on nutrient metabolism in pancreatic islet beta cells using biochemistry, patch clamp electrophysiology, and quantitative imaging. Professor Merrins received his B.A in Chemistry and Biology at Oberlin College and his PhD in Physiology from the University of Michigan. He teaches Human Biochemistry Lab (BMC504) at the University of Wisconsin-Madison, where his lab uses an epi-fluorescent microscope to image cells.

2.3.4 Competing Designs

This project will specifically target the research done in Professor Merrins' human biochemistry lab. As a result, there is no current device on the market that caters to a low cost device that perfectly meets his lab's requirements. However, there are many similar devices on the market that could be modified for his needs.

The Dino-Lite is a small fluorescence microscope that is able to filter a specific wavelength of light [7]. In addition, it can be designed for the different fluorophores used. A

Dino-Lite fluorescence microscope can cost between $948-$968 which is dependent upon the type of LEDs needed and emission filters. In addition, software is included and comes with a stand with additional features for adjustments. Even though this device is low-cost, which is what the client requires, this device is not ideal for FRET since FRET requires the use and detection of two fluorophores and their emission wavelengths. As a result, the device would need to be modified to compensate for this.

Figure 3. Dino-Lite Fluorescent Microscope *[7]*. A small handheld microscope that connects to your computer.

The Lumascope 620 uses FRET to image living cells. This microscope is one of the most powerful devices on the market, and has a 5-filter set with 3 exciting filters, 1 triple-band emitting filter, and 1 beam-splitting filter. Utilizing confocal microscopy, one can obtain nanoscale resolution of specimens. In addition, the cells remain alive because this microscope minimizes photobleaching. The microscope also features different configurations of the objective lens, multiple laser options, filters, and detectors [8]. Even though the client would be able to use this microscope for his research, it is too expensive to obtain for a classroom setting because of the microscope's broad capabilities. The team requested a quote of the microscope, and the CEO of Etaluma Inc., Chris Shumate, said it is depended upon the chassis, filter and lens, illumination source, electronics, and type of camera. Many of the current designs with this type of equipment cost over $100,000, based on the variable cost within the quality of the parts requested for the microscope.

Figure 4. The Lumascope 620 *[8]* is an expensive option that does more than FRET analysis.

The Nightsea converts a stereo microscope into a simple fluorescence system. The product accomplishes this by using an attached filter and an external excitation source. The light source and the filter are assembled to be used with specific fluorophores. The Nightsea comes with one-color complete setup, modular excitation/emission sets, and a modular white head for a total cost between $1,880-$2,180. Although this device is relatively cheap, this device is not ideal for FRET. This is because FRET uses two fluorophores and would thus require swapping two filters relatively quickly. There is no current data acquisition system as well, which would need to be integrated in the design in order to extract relevant data for determining the FRET ratio [9]. Additionally, as can be seen in Figure 6, the sample is illuminated and detected from above. This can affect the wavelength of the light that reaches the cells and that is detected, skewing the data.

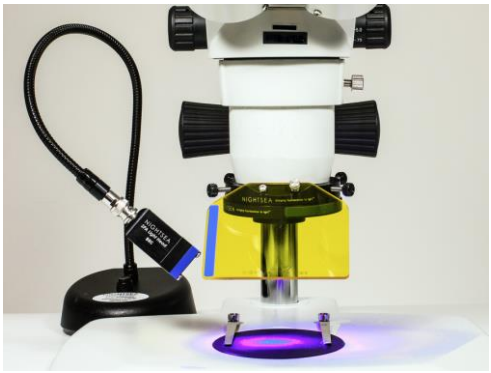


Figure 5. The NightSea Model SFA *[9]* consists of an excitation source and a filter that attaches to stereoscope.

The OPN-Scope is a low cost, open source epifluorescence microscope that is used in classrooms and teaching labs in colleges [10]. The first design was built to be added to a compound microscope while a second design was built as a standalone product. This device has two different designs that incorporate 3D printed parts and parts that can be purchased at local hardware stores or from websites like eBay. The six 3D printed parts include an outer shell, drawer that contains the filter cube, handle to pull the drawer in and out, tube to hold the eyepiece and tube to hold the light source. The filter cube holds the excitation and emission filter, along with the dichroic mirror. Some of their materials include: a 2000 lm tactical LED flashlight with a lens for focusing the beam as their excitation source; a Lenovo ThinkPad T430s; a 14-MegaPixel OMAX A3514OU color camera, and ToupView 3.7 software for controlling the time and gain of the OMAX camera. Most of the optical components they acquired were used to control cost.

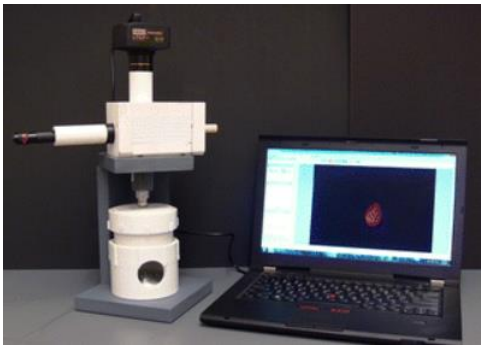


Figure 6. OPN-Scope Open Source Fluorescence Microscope System

2.4. Product Design Specifications

The final product will be a simplified single prototype microscope that will allow the client's students to measure FRET in a classroom setting. This device will be similar to his lab's microscope, as it will contain an excitation source at 430 nm, two different filters for the FRET response (one at 470 nm for the donor emission and the other at 535 nm for the acceptor emission), and a camera. The camera will capture the images of the specimen in the solution chamber and upload them to a compatible computer for image analysis. The goal of the device is to extract accurate acceptor-donor FRET ratios from the images collected. This accuracy does not have to be research-grade, but the microscope should be accurate enough that students can detect a change in lactate expression.

Along with this, the device must be intuitive to use, and the students should have to put in minimal work to obtain the image outputs. The students are not expected to have an extensive microscopy background; therefore, they should have to do little to no image processing. The final product must be under $2,000 so that the lab would be able to purchase at least one device annually with its current budget. To accomplish this goal, most unnecessary/excessive parts of a microscope, such as eyepieces and other components, will be eliminated in this prototype. However, this semester the client expanded the budget to $4,000 for the initial prototype in order that a working prototype is successfully developed. An estimate of the size of the microscope is a 20 cm by 30 cm base with a height less than 45 cm. If additional software for image analysis is needed, the software used must be free and capable to pair with the microscope to reduce cost. The client requires that the microscope be inverted and that a degree of versatility be present in the design for future applications. A full list of specifications can be found in the PDS in Appendix A.

3. Designs

3.1 Previous Design Work

The design of this miniature microscope for FRET microscopy was started in Spring 2017. During this semester the team was working with a $2,000 budget, limiting the options for excitation sources and optics. The team considered three cost saving designs (Appendix B.), all of which required the same excitation source. The overall final design is pictured below (Figure 7).

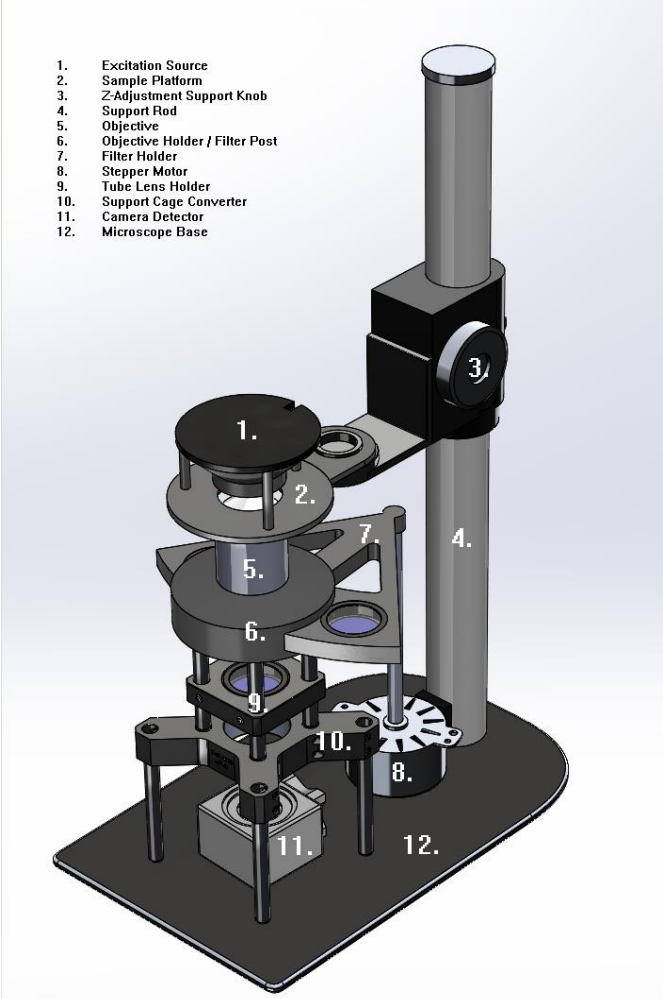


Figure 7. Overview of proposed microscope structure. A legend is included to identify all important components of the design.

Last semester was primarily spent trying to optimize that excitation source, which eliminated the need for a dichroic mirror and an excitation filter, reducing the strain on the budget. The team designed a 430 nm, 10-LED array that sat like a crown over the top of the objective to work as the excitation source. First, a circuit to supply equal amperage at exact time intervals to each LED was devised using a shift register, Arduino UNO, and potentiometer (Appendix C). Next, the team went through a few design iterations for the LED holder, attempting to excite the sample from a crown around the objective (Figure 8).

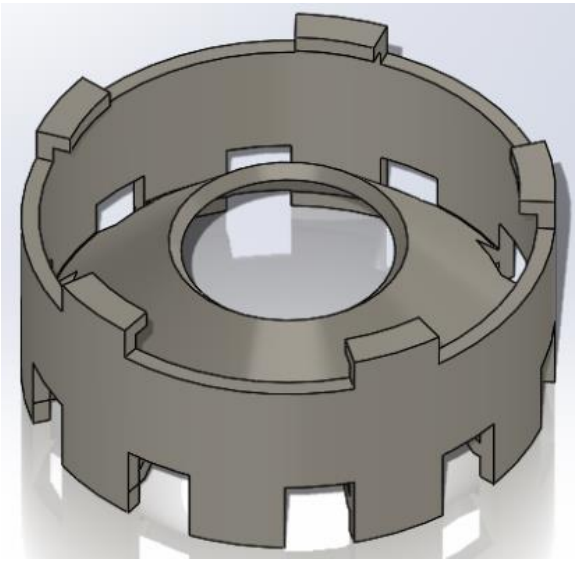


Figure 8. Final LED sample holder from last semester

The LED array was tested with the client's camera (Orca Flash 4.0) as well as the prototype camera (DMK42BUC03 - The Imaging Source). Reasonable results were obtained with the client's camera during the initial testing, but the prototype camera was not sensitive enough to be combined with the prototype excitation source. At a secondary testing with the final LED holder, there was interference from the LED holder in changing the wavelength of excitation light, effectively blocking any signal to the prototype camera or the client's camera. Therefore, the team chose to pursue a different direction for excitation.

One piece of the design that the team hopes to keep or modify for use in the final prototype is the filter-swap mechanism. The current design involves a stepper motor, custom semi-circular filter holder, and a filter post in the beam path of the microscope (Figure 9).

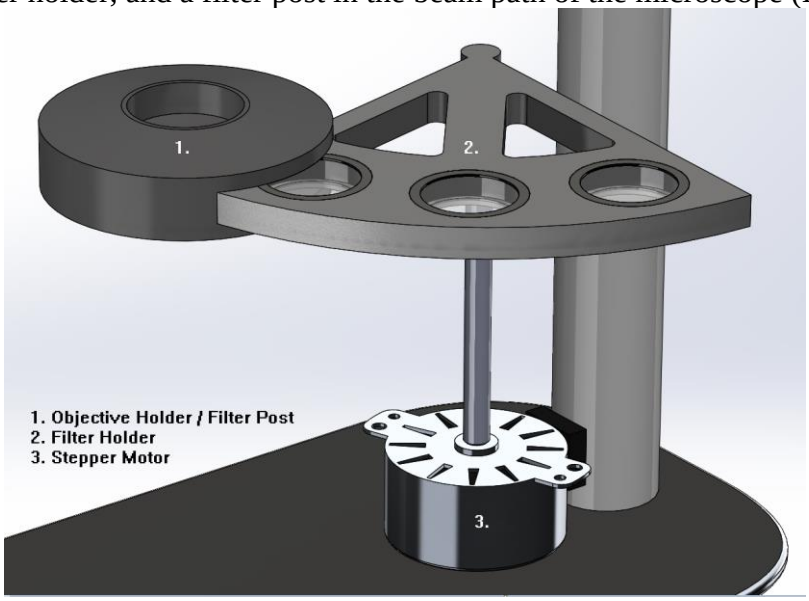


Figure 9. An exploded view of the filter swapping mechanism. This system shares the role of also supporting the objective (not shown). The stepper motor attached to the microscope base will provide the input to switch filters between images.

A stepper motor with sufficient speed is used to switch between the 470 nm and 535 nm emission filters accurately, and it can be remotely controlled by experimenters to coincide with excitation illumination and image capture. A custom-printed filter holder will house the needed emission filters and allow fast, compact, and simple swapping of filters by allowing the stepper motor to introduce a small amount of torque that shifts the needed filter into position behind the objective. Movement to the previous filter means that a similar amount of torque is needed in the opposite direction by the stepper motor. In this manner, filter swapping can be accomplished by prompting the stepper motor to rotate in 'positive' and 'negative' directions.

3.2. Design Framework

The team decided on three potential designs for this simplified epi-fluorescent microscope for FRET imaging with various pros and cons. All designs will achieve the same goal of imaging cells expressing Professor Merrins' biosensor as well as outputting a FRET ratio. The designs ideas differ in their mechanical components, software, and mechanism to swap emission filters. All the designs have the same camera, excitation source, optical filters, a objective, and tube lens.

The camera for each design idea will be using is the DMK42BUC03. The camera's specifications can be seen in Appendix D and common camera specifications are defined in Appendix E. The excitation source that will be used is described in section 3.7. Professor Merrins provided optical filters to be used. The objective to be used is Nikon CFI S Fluor 20x. The specifications of the objective are in Appendix F, definitions of objective specifications can be viewed in Appendix G. The tube lens to be used is yet to be determined; however, the tube lens will need to mimic that of Professor Merrins' microscope, the Nikon TI Eclipse [11]. By mimicking the optics of the Nikon TI Eclipse, it can be used as a reference when determining the quality of the prototype. The Nikon TI Eclipse has an internal magnification of 1.5x resulting in overall magnification 30x when a 20x objective is being used. Therefore, a tube lens that provides a magnification of 1.5x will be required.

3.3. Design One: ThorLabs Assembly

The ThorLabs assembly is the first proposed scope design based on the Design Framework. This scope is assembled with parts that would be purchased from ThorLabs; a company that sells a variety of parts for optical devices. This company is able to provide all necessary parts needed to build a FRET microscope, which creates a high compatibility between parts, while also minimizing compounding error. The potential disadvantages to this scope include the price and the limitations on customization. ThorLabs parts can cost several hundred dollars, which will push the budget near its limit. The scope is also limited based on the parts that ThorLabs sells, so there is little room for creativity from the team with this scope.

3.4. Design Two: Artisan Assembly

The artisan assembly is the second considered implementation of the previously defined design framework. The primary objective of this design is to personally manufacture the majority of parts in order to minimize the monetary impact of each part on the budget, and to allow for total customization. While more technical parts such as the camera, excitation source, optical filters, objectives and the tube lens will still be purchased, all mechanical components of the

design including the filter swap would be fabricated by the team. Additionally, software will be developed to control and interface the excitation source, the camera, and compiling the data in a .csv file for the user. As previously mentioned, this kind of autonomy on many aspects of the design permit a much wider scope of design possibilities and customization while utilizing less of the budget, allowing for more expensive optical lenses or cameras to be purchased. Conversely, the fabrication of many parts requires much more expertise and time in comparison to other designs, both limiting resources for this project. Small precision errors in each part are inherent in the process of fabrication, especially when expertise and proper machinery are not available. This compounds with each self-manufactured part included in the design, leaving users with a greater level of uncertainty in the robustness of data gathered, and overall loss of precision in the images collected by the microscope.

3.5. Design Three: Combo Assembly

The combo assembly is the third and final design proposed to meet all the requirements of the Design Framework found in section 3.2. This assembly is a combination of the two designs above, in that it takes advantage of some of the parts that ThorLabs offers, in addition to allowing the team to design some of the parts to the scope. This design creates a compromise on cost; by manufacturing some of the parts instead of buying them from ThorLabs, there will be some budget cuts on the scope. Furthermore, this allows for more creativity and customization with the scope. The disadvantages to this design include concerns with tolerance stacking and scalability. Since there is a combination between ThorLabs parts and fabricated designed parts, there is a risk for tolerance stacking with the scope, leading to less precision. The scope must also have the capability to be manufactured over eight times, which is another potential concern with this assembly.

The combo scope also takes advantage of software that will be written by the team. This software will be focused specifically to run all the tasks necessary for the FRET microscope. This software may be time consuming, however, as there could potentially be a lot of debugging and testing required.

3.6. Design Matrix

After thoroughly researching these three designs, the team created a design matrix to rank them against one another in order to determine which should be pursued. The team considered five different categories in order to determine the best option: image quality, manufacturability, cost, operability, and reliability. Considering the advantages and disadvantages of each option, the team collaborated to give each design idea a ranking out of 5 for each component of the design matrix. Design scores highlighted in blue won their category (or tied for the top) and the total highlighted in green is the score for the design idea the team chose.

Table 1 represents the design table matrix for the three different design ideas. The highest scoring design(s) for each respective criterion is highlighted in blue, and the highest scoring design total is highlighted in green.

Criteria (Weight)	ThorLabs Assembly		Artisan Assembly		Combo Assembly	
Image Quality (30)	4/5	24	4/5	24	4/5	24
Manfacturability (20)	5/5	20	3/5	12	4/5	16
Cost (20)	3/5	12	5/5	20	4/5	16
Operability (15)	4/5	12	3/5	9	4/5	12
Reliability (15)	5/5	15	3/5	9	3/5	9
Total (100)		**83**		**74**		**77**

3.7 Design Criteria

Image Quality was selected as the most important design criterion because it is critical that the camera receives enough signal from the fluorophores to create a useful image. This means that the intensity of the donor and acceptor wavelengths should be detectable and small changes will need to be discerned as well. All of the designs scored equally in this category because all three use the same camera, the DMK42BUC03. This camera was already tested this semester and evaluated. Current tests have proved its quality to detect and discern the two wavelengths. This proven camera gave the team confidence that it would work with any of the assemblies considered.

Manufacturability was tied for the second most highest design criterion because it is important for the scopes to be easily built and assembled based on the designs. This category considers scalability because our client wishes to ultimately have multiple scopes. This category also considers aligning all of the components so that the image is focused onto the detector of the camera as well as the manufacture of any circuitry used to power the design. If there is anything unusual about the stand set-up, it is also included in this category. ThorLabs scored the highest, with a 5/5 because the team would be able to make a list of parts that are purchased from ThorLabs and write a protocol to assemble to scope accordingly. With the other two designs, some manufacturing is required. Since the combo scope still contains some ThorLabs parts, it scored a 4/5, while the completely independent artisan scope scored a 3/5.

Cost was also tied for the second most important design criterion because our client gave us a strict budget, which was doubled to $4,000 for prototyping. The additional flexibility with the budget allows the team to spend more this semester, but it is important to consider that the ultimate goal is to build a scope that costs less than $2,000 since the client wants 8 total scopes. The client's plan is to purchase one or two scopes per year based on this $2,000 budget. However, when evaluating the assemblies this semester, the $4,000 budget was evaluated. The artisan scope scored the highest with a 5/5 because most of the parts would be designed by the team themselves. When purchasing parts from companies like ThorLabs, the prices are oftentimes more expensive because these companies also cover the manufacturing and documenting of the optical pieces. The combo assembly scored second highest with a 4/5 because some of the parts in this design are purchased from ThorLabs. Finally, the ThorLabs assembly scored lowest with a 3/5 because of the priciness of some of the ThorLabs parts.

Operability is meant to quantify user-friendliness of each design. Therefore, the team

considered how much image processing would need to be done for each design and whether or not it would be easy for a student to use. The ThorLabs assembly and the combo assembly both scored the highest at 4/5. These were chosen to have a great operability because they are the easiest scopes for the students to work with. The most significant advantage in operability with this scope is the automatic filter swapper. This would be much more easily controlled when purchased by ThorLabs. ThorLabs also offers its own software, which would likely be easier to use than the team's independently designed software. All three designs require the same image processing due to the optics used.

Reliability is the final design criteria considered because the design should be able to withstand student use for as long as possible. For a cost effective microscope, ideally the client should not need to purchase new parts or new devices for as long as possible. The ThorLabs scored the highest with a 5/5 because of the quality of the parts that ThorLabs has to offer. The team believes that these professionally manufactured parts will likely be more reliable than parts manufactured by the team. The other two designs scored 3/5 for this reason as well.

3.8 Power and Control of Excitation Source

The team chose to use three Vollong 3W high power LED's in parallel to excite the sample. These LED's operate with a forward voltage of 3.6 V at 750 mA [12]. These LEDs are held in parallel with 5 ohm resistors such that a 3.7 V rechargeable lithium battery can power the whole circuit for 2.6 hours. Each LED provides 200 lumens of light, for a total of 600 lumens. The circuit is controlled by a Raspberry Pi 3 microcontroller which opens and closes an npn BJT transistor. When the Raspberry Pi switches "on", the transistor is powered and enters the active region, such that the circuit can run. When the Raspberry Pi switches "off", the transistor does not receive power and thus the LEDs will be turned off. This circuit is estimated to cost $67.88.

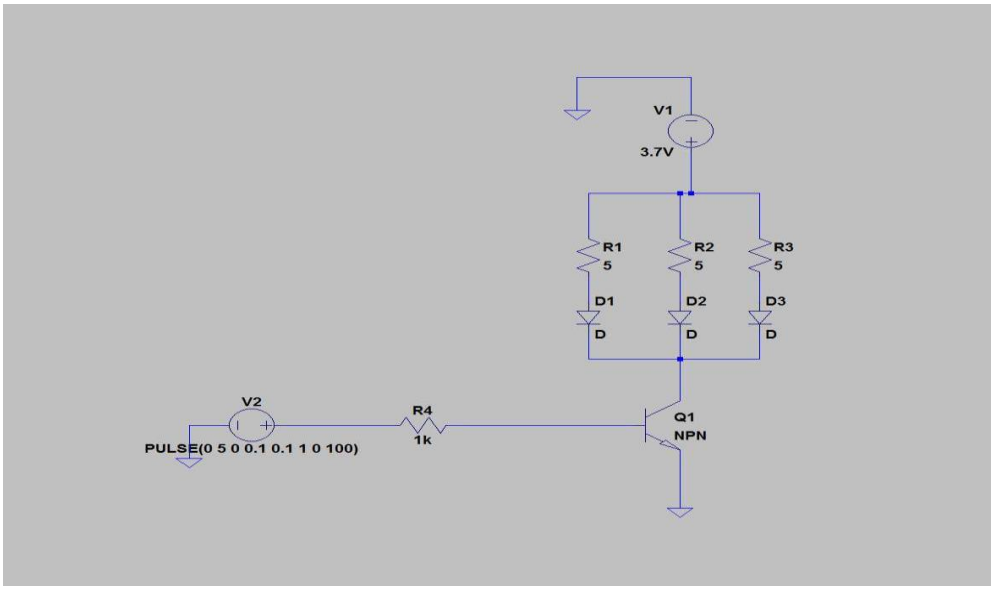


Figure 10. Schematic of circuit for excitation source.

In Figure 10, the Raspberry Pi 3 is modeled by a Pulse Signal, which fluctuates between 5 V on period and a 0V off period. The three LED's are represented by diodes with a forward voltage of 3.6 V. The rechargeable lithium battery is modeled as a 3.7 V voltage source.

The client has also provided the team with a Sutter Instruments TLED system to provide excitation for the system. The TLED provides 430nm high power LED light at a forward voltage of 6 V. This LED system is controllable manually via a toggle switch or through a TTL connection to a computer [13]. This system is advantageous in that it can be connected directly to the microscope because it is built by Sutter Instruments. However, this TLED system adds a significant amount of cost to our system, which must be considered with scalability of the device. The TLED system is currently listed at $1,975 [13].

These two systems can be compared to the client's $100,000 scope based on the brightness the light source provides. The SOLA light source that client's scope uses is capable of providing 750 lumens of light at 100% output [14]. This is comparable to the proposed circuit design, which is able to provide 600 lumens. Additionally, during testing the team did not need more than 50% output from the client's microscope. The exact CREE LED used in the TLED scope is unknown; however, most CREE XHP-50 LEDs are capable of providing at least 700 lumens of light. This makes the TLED comparable to the SOLA as well. The scope will be tested with both light sources, in order to test the quality of the proposed scope in consideration for scalability of the scope next semester.

4. Experimental Set-up

4.1 Previous Experiments

In order to understand the experimental setup of the project this semester, it is necessary to understand the testing and results from the previous semester. In preliminary testing, the team compared the LED-array excitation source with the Lumencor SOLA of the client as well as the prototype camera with the ORCA-Flash 4.0. This testing showed that the prototype camera could detect the biosensor with the Lumencor SOLA, and the ORCA-Flash 4.0 could detect the biosensor with the prototype camera at 535. However, the ORCA-Flash 4.0 could not detect the biosensor at 470, and the combination of the prototype camera and LED array proved ineffective at detecting the biosensor at either wavelength.

Using a two-tailed T-test, the team compared the prototype camera to the ORCA-Flash 4.0 in detecting the 535 nm fluorescence of the biosensor. Using this analysis, it was shown that there was no significant difference in the signal to background ratio of the two cameras, indicating that the prototype camera could be used as an alternative to the client's in the prototype (Appendix H.)

4.2. Experimental Overview

4.2.1 Preliminary Camera Experimentation 1

The BME design team decided to do a quantitative comparison of FRET detection of the current camera purchased (DMK42BUC03) compared to the client's camera (ORCA-Flash 4.0). This test was performed in order to determine if there was difference of the detection of FRET between the two cameras. This test would be able to confirm whether the DMK42BUC03 could be used for accurate FRET results. As a result, both cameras were attached to the client's microscope, and the same FRET protocol was performed.

Throughout the experiment, pancreatic islet cells were used, and the software Nikon Elements was used to control the microscope and light exposure. Images from the purchased

camera were taken using IC Capture. Images were taken using a 40x objective lens.

The goal of this experiment was to determine if the camera purchased would be able to detect the FRET efficiency when subjected to high and low concentrations of glucose. Therefore, the client's camera was used as the control which would later be compared to the FRET efficiency obtained from the purchased camera.

4.2.2 Preliminary Camera Experimentation 2

Since a 40x objective lens was used for the first experimentation, the field of view was extremely small using the prototype camera. In an actual experiment that biochemistry students will be doing, they will be using yeast cells which will be much smaller than islets, so this would likely be fine for the desired application. However, in order to compare the images, a second experiment was completed using a 20x objective lens. As a result, the following images taken and analyzed were from the second experiment.

4.2.3 Preliminary Camera Testing

With no light exposure present, the pancreatic islet cells were first subjected to a high concentration of glucose (16.7 mM) for a duration of 10 minutes. During the high glucose wash, a time-lapse was used in order to detect the FRET ratio over time. Every six seconds, two images were captured and the two emission filters were switched out instantly. A 20X objective lens was used for the remained of the experiment. Once the cells were washed for approximately ten minutes, the time-lapse was stopped, and a variety of images were captured. A selection of these photos of the time-lapse were chosen to be representative of all of the images.

5. Testing and Results

5.1. Preliminary Results

The images were first captured with the client's camera using the 430-470 nm and 500-535 nm emission filters, and the client's excitation source, the Lumencor SOLA. For each emission filter, two images were captured; one image using 10% SOLA and one image using 50% SOLA. The team first took images at high glucose with the DMK42BUC03 and the 20x objective. Since initial images taken at 10% SOLA showed that the camera was not able to detect the islets (Figures 11A and 11C) the SOLA was increased to 50%, where it appeared that the islets could be detected for each of the desired wavelengths (Figure 11B and 11D).

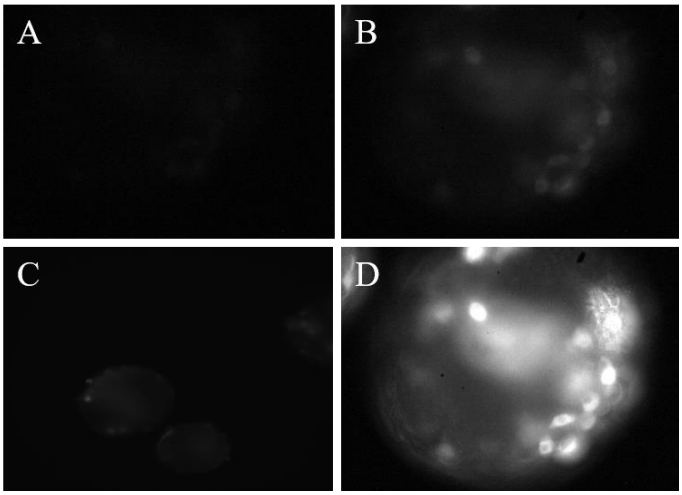


Figure 11. Fluorescence microscope images at high glucose concentration with 20x objective and DMK42BUC03. (A.) 470 nm emission filter with 10% SOLA (B.) 470 nm emission filter with 50% SOLA (C.) 535 nm emission filter with 10% SOLA (D.) 535 nm emission filter with 50% SOLA

After images were obtained using the DMK42BUC03, the team captured more images using the client's camera at high glucose. The same objective, excitation intensities and emission filters were used, starting with a 10% SOLA (Figures 12A and 12C), but they were also imaged at 50% SOLA (Figures 12B and 12D). The image taken at 535 nm appeared to saturate, however, indicating a lower SOLA power would be required.

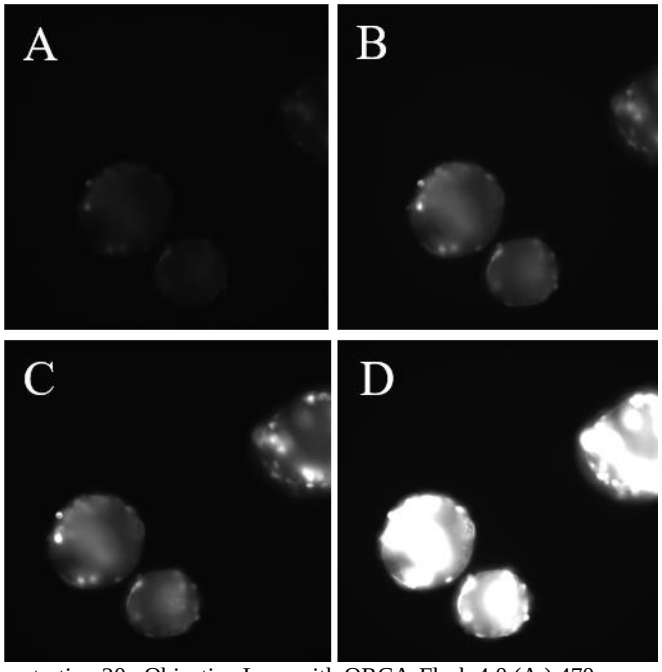


Figure 12. High Glucose Concentration 20x Objective Lens with ORCA-Flash 4.0 (A.) 470 nm emission filter with 10% SOLA (B.) 470 nm emission filter with 50% SOLA (C.) 535 nm emission filter with 10% SOLA (D.) 535 nm emission filter with 50% SOLA.

After all images were obtained, the time-lapse was started, and low glucose concentration (2 mM) was added over a period of 10 minutes, taking photos with the Orca Flash 4.0 and 20x

objective during the transition. Then, photos were taken at low glucose with the DMK42BUC03 and 20x objective, using both 10% SOLA (Figures 13A and 13C) as well as 50% SOLA (Figures 13B and 13D) for the experiment. It clearly showed that 50% SOLA is the better option for imaging the cells, and it also demonstrated that a higher intensity would not necessarily be needed for the team's prototype.

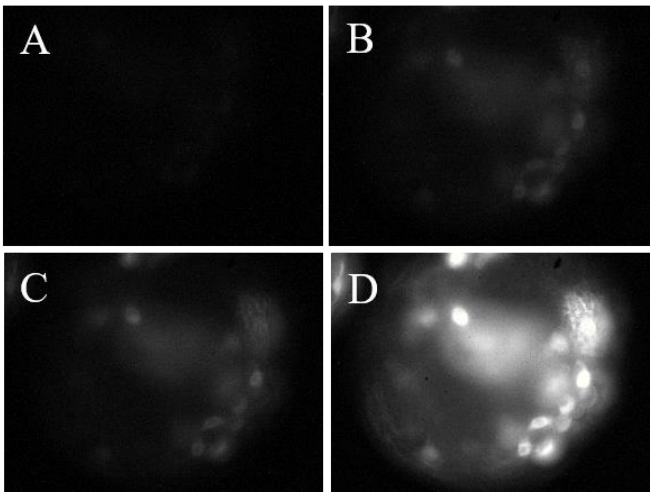


Figure 13. Low Glucose Concentration 20x Objective Lens with DMK42BUC03 (A.) 470 nm emission filter with 10% SOLA. (B.) 470 emission filter with 50% SOLA. (C.) 535 emission filter with 10% SOLA. (D.) 535 emission filter with 50% SOLA.

Finally, low glucose images were taken with the Orca Flash 4.0 and the 20x objective lens. These showed that for the client's microscope 50% SOLA (Figures 14B and 14D) saturates the image taken at 535 nm (Figure 14D). However, 10% SOLA (Figures 14A and 14C) did not appear bright enough; therefore, the ideal brightness is likely somewhere in-between to compromise between the low 470 nm fluorescence and the high 535 nm fluorescence, something the prototype microscope will need to keep in mind as well.

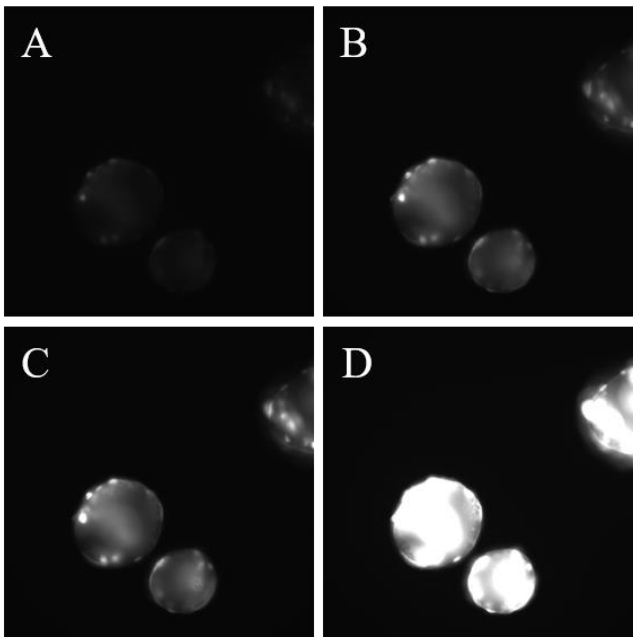


Figure 14. Low Glucose Concentration 20x Objective Lens with ORCA-Flash 4.0. (A.) 470 nm emission filter with 10% SOLA. (B.) 470 emission filter with 50% SOLA. (C.) 535 emission filter with 10% SOLA. (D.) 535 emission filter with 50% SOLA.

The team also imaged the islets using the client's ORCA-Flash 4.0 and the 40x objective to get a similar field of view as when using the 20x objective with the prototype camera. These images showed that at 50% SOLA and 535 nm (Figures 15B and 16B) there are no discernable islets. Even at 10% (Figures 15C and 16C) the camera blurs the islets together. This shows that perhaps by increasing the magnification of the prototype scope the team will not need to worry about using such a bright excitation source.

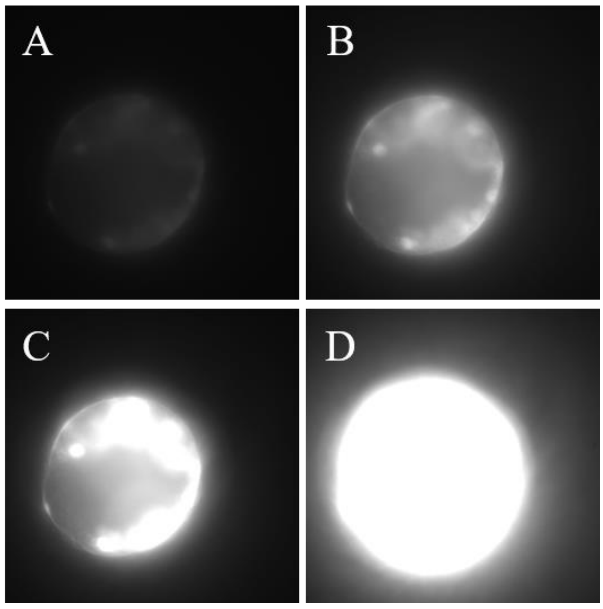


Figure 15. High Glucose Concentration 40x Objective Lens with ORCA-Flash 4.0 (A.) 470 nm emission filter with 10% SOLA. (B.) 470 emission filter with 50% SOLA. (C.) 535 emission filter with 10% SOLA. (D.) 535 emission filter with 50% SOLA.

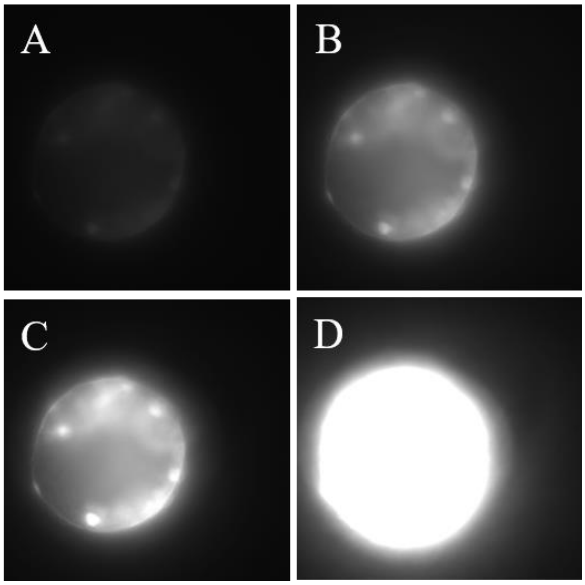


Figure 16. Low Glucose Concentration 40x Objective Lens with ORCA-Flash 4.0. (A.) 470 nm emission filter with 10% SOLA. (B.) 470 emission filter with 50% SOLA. (C.) 535 emission filter with 10% SOLA. (D.) 535 emission filter with 50% SOLA.

5.2. Summary of Results

5.2.1 Time-Lapse Data

A time-lapse of the CFP and YFP intensities were graphed over a period of 20 minutes. The graph, seen in Figure 17, represents the time-lapse data taken after equilibration. The pancreatic islet cells were first subjected to a high concentration of glucose (16.7 mM) for a duration of 10 minutes, and then subjected to low glucose (2 mM) for ten minutes. Every six seconds, two images were captured swapping out the 430-470 nm and 500-535 nm emission filters. This graph shows that the camera is able to successfully detect the difference between the CFP and YFP channel. In addition, this graph shows that the FRET ratio, which is the YFP/CFP intensity, decreases.

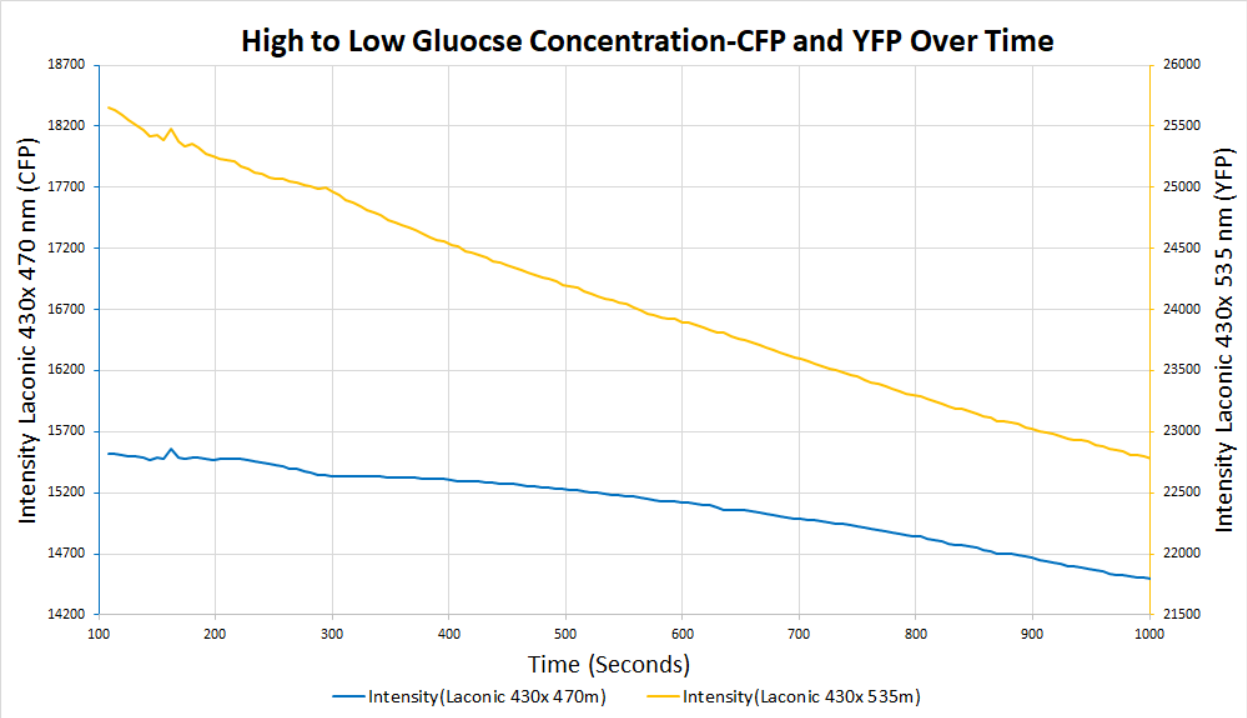


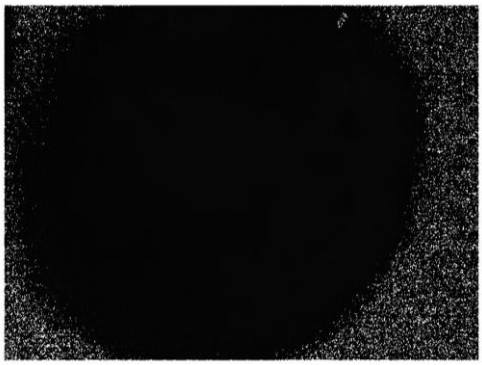
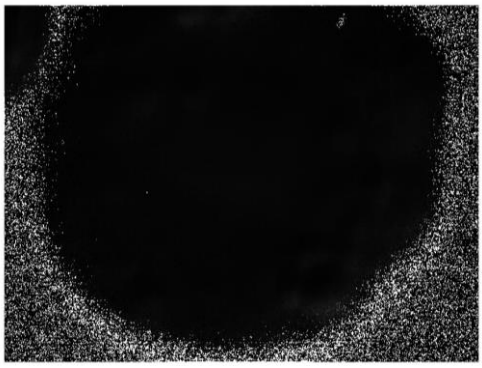
Figure 17. Time-lapse data of high to low glucose concentration of CFP and YFP intensity. This graph represents the CFP and YFP intensities over times. Pancreatic cells were subjected to a high concentration of glucose (16.7 mM) for ten minutes, and then subjected to a low concentration of glucose (2 mM) for ten minutes.

5.2.2 Summary of Camera Analysis

An analysis of the images was performed in MATLAB. The images taken with the DMK42BUC03 at high glucose with 50% SOLA were loaded into MATLAB. The 535 nm emission image was divided element wise by the 470 nm images to produce a ratio image. Using a built in MATLAB function, the average pixel value was recorded. This was repeated with the low glucose at 50 percent sola as well.

The ORCA-Flash 4.0 images were analyzed similarly except the image was cropped such was that only the islet was analyzed and not the background of the image. Once the image was cropped the 535 nm image was divided element wise by the 470 nm emission image to produce a ratio image. A built in function was used to determine the average pixel value in the ratio image. This was done for both the high glucose images and low glucose images. The ratio images and their average pixel values are displayed on the following page.

Table 2. The first column is the image when the YFP channel is divided element wise by the CFP channel. The average pixel value of the radioed image is displayed in the second column

Ratio Images	Average Pixel Value
 High Glucose Concentration, acquired with DMK42BUC03	12.9486
 Low Glucose Concentration, acquired with DMK42BUC03	21.4199
 High Glucose Concentration, acquired with Orca Flash 4.0	1.5629
 Low Glucose Concentration, acquired with Orca Flash 4.0	3.4551

6. Future Work

The next steps will be to choose a tube lens that mimics the tube lens of the Nikon TI Eclipse. Picking the correct tube lens will involve mathematical calculation to determine the necessary focal length of the tube lens. This will narrow down the list of tube lenses to choose from. Once the tube lens is chosen, the next step will be to build a prototype microscope in Professor Roger's lab. When the prototype is built, images of a bar chart will be acquired. The bar chart will be used to determine the experimental resolution, magnification and field of view of the optical system. These specifications can then be incorporated into the PDS.

The mechanical and control aspects of the design will be simultaneously worked on. Mechanically, the first step will be to determine what type of mounts will be needed for the Nikon S Fluor and tube lens. The next step will then be to purchase or manufacture a stage that allows control over the Z-axis motion. This will allow the user of the microscope to focus the image. A motorized filter swapping mechanism will be purchased from Thorlabs; however, the system for mounting the emission filters on the mechanism is currently unknown. Finally, the issue of mounting the excitation source to the rest of the optical system will be need to be resolved. Once all the above tasks have been completed, the final task on the mechanical side will be refining the alignment and reducing vibrations from the motorized filter swap.

Control over the optical system will done via laptop or raspberry pi. It would be easier to first implement the control system with the raspberry pi and then make the proper changes to allow a user to use the microscope via their own laptop. The first step in the control system will be to acquire images. The Imaging Source, the manufacturer of our camera, provides a software development kit, SDK, to control the camera. Using the SDK, the camera can be controlled. The next step will be to write software to control the filter swapping mechanism. Thorlabs provides an SDK for their filter swapping mechanism. The final step with regards to the control system will be to integrate the control of the camera with control of the filter swapping mechanism to acquire images when the correct filter is in place.

7. Conclusion

The team decided to work on building a Laconic FRET-based biosensor for their client Professor Matthew Merrins. The goal of this project is to build a single prototype that his students would be able to use in his biochemistry class. The microscope built should have similar features to his lab’s current microscope and include an excitation source, a camera, and a series of filters for under $4,000. After further research and various meetings with the team’s client and advisor, the team decided to first build the microscope out of Thorlabs components.

Before purchasing any Thorlabs equipment, the team tested to make confirm the camera purchased last semester sensitive enough to detect changes in fluorescence intensity. Upon performing image analysis in MATLAB it was determined that the camera was indeed good enough to detect these small changes. While performing tests and analyzing results, the light soruce was also redesigned to make it brighter and easier to control.

The next steps will be choose a tube lens such that the optics of the prototype microscope closely mimic that of the Nikon TI Eclipse. This allows the team to use properly compare the Nikon TI Eclipse to the prototype microscope. Additionally, the team needs to figure out how to use the Imaging Source software development kit to control the camera, as well as the the

Thorlabs software development kit to control the filter swapping mechanism.

The goal of the semester is to have a functional microscope and software that can acquire time series images, calculate and record the FRET ratio using yeast cells. The FRET ratio will be recorded into an excel spreadsheet, and then students can graph the FRET ratio as glucose levels change.

8. Acknowledgements

The team would like to thank their advisor Professor Jeremy Rogers and their client Professor Matthew Merrins for guiding them through the design process. In addition, special thanks also goes out the entire BME department for providing helpful resources for this design project.

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10. Appendices

Appendix A.

Product Design Specifications
Miniature Fluorescent Microscope

Team Members: Ben Ratliff, Ethan Nethery, Kaitlyn Gabardi, John Rupel, Kadina Johnston
BME 400
Client: Professor Matthew Merrins
Advisor: Professor Jeremy Rogers
Last Updated: September 20, 2017

Problem Statement: The client, Professor Matthew Merrins, teaches human biochemistry lab at the University of Wisconsin-Madison. The course focuses on the enzyme lactate dehydrogenase, which produces lactate from pyruvate. Currently, his lab utilizes Laconic, a Förster Resonance Energy Transfer (FRET)-based biosensor. This biosensor detects the presence of Lactate in healthy, living cells, but the fluorescence must be monitored in time lapse using a high cost microscope. This microscope excites the lactate biosensor using a system of high power LEDs and a filter wheel, and the fluorescence is recorded. The project goal to to build a lower-cost, limited alternative to the client's more expensive microscope

Function: The final design will be a single prototype device that will allow his students to measure FRET in his human biochemistry class. This device will be similar to his lab's microscope as it will contain an excitation source, two different filters for FRET, and a camera that will capture the images of the specimen in the solution chamber.

Client Requirements:

- Initial prototype built must be made under $4,000
    - Eventually build multiple devices around $2,000
- Compact and intuitive for student use
- Software used to process images must be free
- Easy to obtain FRET results
- Device should be able to detect yeast cells
- Result should be similar to results obtained from client's microscope
- Should be an inverted design
- Interchangeable filters and excitation source
- Device can be repeatedly manufactured with limited engineering experience required
- Microscope should have significant and detectable change in fluorescence upon stimulation between 470 and 535 nm from 430 nm excitation source

Physical and Operational Characteristics:

A. *Performance Requirements:* The designs must be able to accurately measure light intensities at 470 and 535 nm. These readings do not have to be simultaneous but must be close in time. An excitation source of 430 nm should induce this response, which will be recorded by a detector (camera) and uploaded to a freeware image analysis program

(ImageJ/MATLAB) on a compatible computer for analysis. The lactate level can then be extracted based on the ratio of 470 and 535 intensities.

B. *Safety:* The design should minimize contact between the excitation source and user. This is due to the fact that the excitation source is near the UV light spectrum which is damaging to human skin tissue.
C. *Accuracy and Reliability:* This product should be accurate enough to determine the acceptor-donor ratio. FRET results should be similar to the results obtained from the client's microscope.
D. *Life in Service:* Product itself would last for years and system components should be easily replaced if broken or damaged.
E. *Shelf Life:* Shelf life would be 50 years. Optical filters and CMOS cameras will have lifetime guarantee as long as proper care is given to these components.
F. *Operating Environment:* The design must operate at room temperature.
G. *Ergonomics:* Product should be simple and intuitive for students to use. The image collection and accept/donor ratio calculation should be as simple as possible.
H. *Size:* Able to be used as a typical laboratory station on a lab desk (20 cm by 30 cm base), size similar to competing/conventional microscopes. All non-essential components for analysis should be discarded. Height of microscope < 45 cm.
I. *Power Source:* Device will be powered by a power outlet from the wall, thus eliminating the need for battery replacement.
J. *Weight:* 1 to 10 pounds
K. *Materials:* The device will have an internal circuit and will likely utilize a single, super-bright white LED, plastics, wires, optical filters, and a raspberry pi. A CMOS camera will be used along with a stepper motor for the mobile filter swap. Two emission filters and one excitation filter will be used in order to differentiate the 470 nm and 535 nm fluorescence. The final product will also include 3D printed parts such as the LED holder, filter swap platform, and stand.
L. *Aesthetics, Appearance, and Finish:* Simple aesthetics, appears intuitive to use, and simple finish.

Production Characteristics:

A. *Quantity*: One prototype with ability to be repeatedly fabricated over time with plans to have a total of six to eight devices would be implemented over an 8 semester period.
B. *Target Product Cost:* Max cost is $4,000.
    a. Goal is to make final product around $2,000

Miscellaneous:

A. *Standards and Specifications*: Should comply with current FRET analysis protocol and/or be adapted into a simple protocol for the client's human biochemistry class teaching lab analysis.
B. *Patient-Related Concerns*: Cost is the highest determinant in design. The functionality should be sufficient for teaching purposes on a budget of 1/60 of current device ($120,000 to $2,000-$4,000). Resolution is not a key concern, only that the difference in emission intensities can be accurately extracted from experimentation. The data

collection is the largest concern, and data analysis should be used by an easily accessible freeware service.

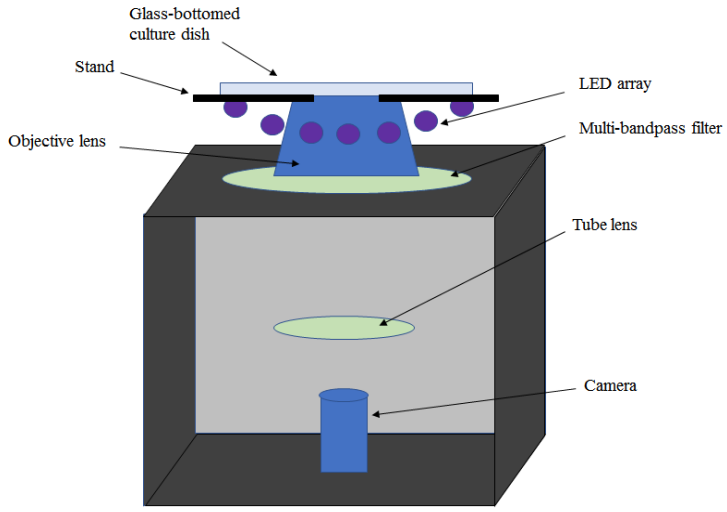
C. *Competition*:

a. Dino-Lite:
    i. This product is small fluorescence microscope where each type of microscope has a specific wavelength and filter designed for specific fluorophores. They are not ideal for FRET since fret requires the use of two fluorophores.
b. Lumascope 620:
    i. This product is for professional use. It can be used for a variety of fluorescence microscopy techniques. It is expensive due to its broad capabilities
c. 3D Printed OPN Scope
    i. This device uses 3D printing to make the outer shell, drawer for the fluorescence filter tude, and tube that holds the eyepiece and light source. A 3D printed device allows the capability to modify the structural parts of the microscope, in addition to making the device extremely cheap to fabricate as compared to customized part manufactured from different companies.
d. Nighsea:
    i. This product converts a Stereo microscope into a simple fluorescence microscope. Using an attachable filter and an external light source the microscope can detect light from fluorophores. The lens are designed for specific fluorophores and is not ideal for FRET.

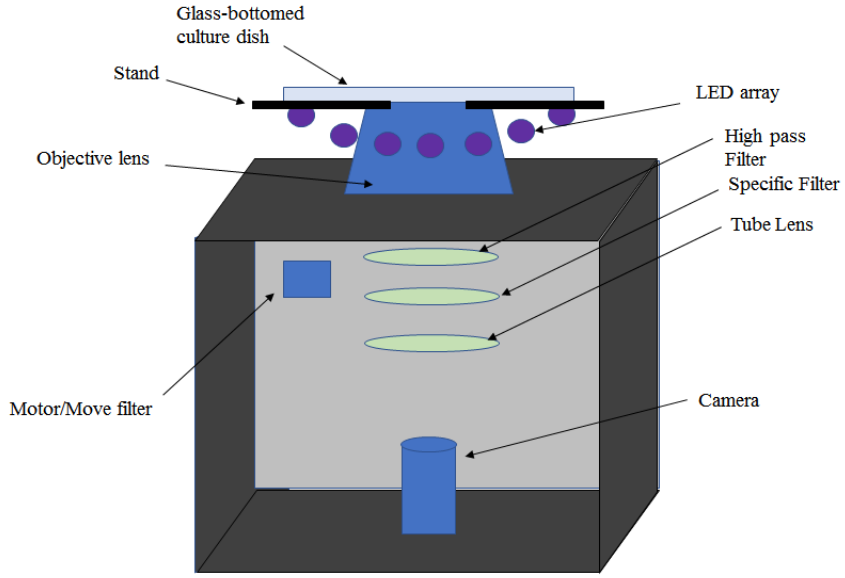
D. *Customer:* Human biochemistry lab (BMC 504) instructor and students.

Appendix B.

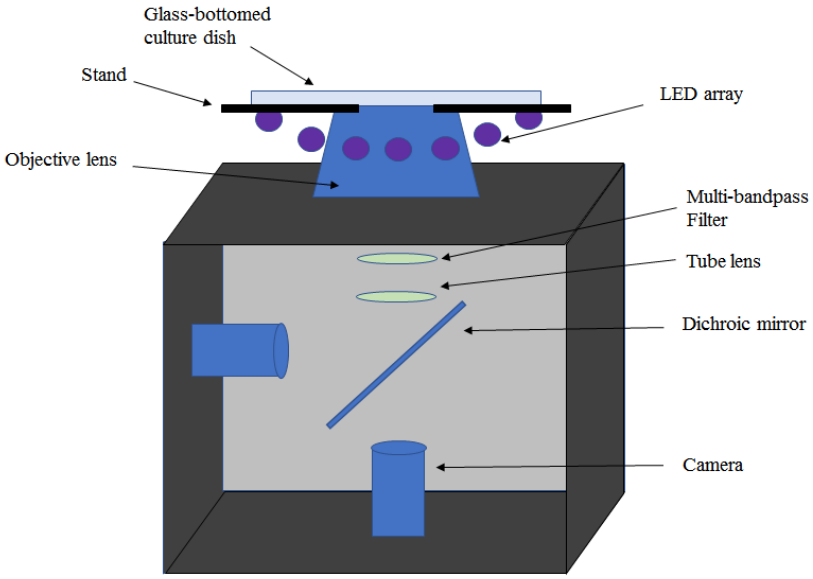
Single-Shot Design Schematic. This is the first design idea proposed and it consists of no moving parts.



Filter-Swap Design Schematic. This is the second design idea proposed and it consists of a motor that swaps out the filters.

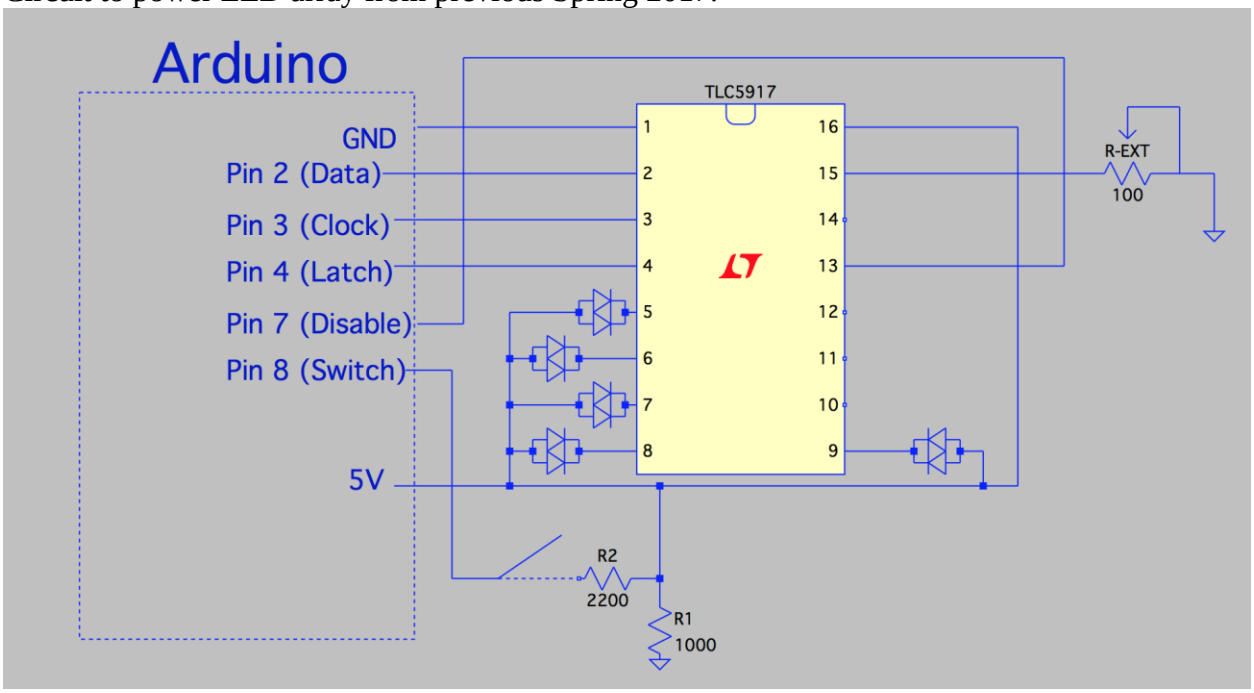


Beam-Splitter Design Schematic. This is the third proposed design and it consists of mirror that splits the two wavelengths of interest.



Appendix C.

Circuit to power LED array from previous Spring 2017.



Appendix D.

The Imaging Source DMK42BUC03 Specifications

Sensor Type: CMOS

Sensor Model: Micron* MT9M021

Shutter: Global

Pixel Size: 3.75um X 3.75um

Sensor Size: 4.8mm X 3.6mm

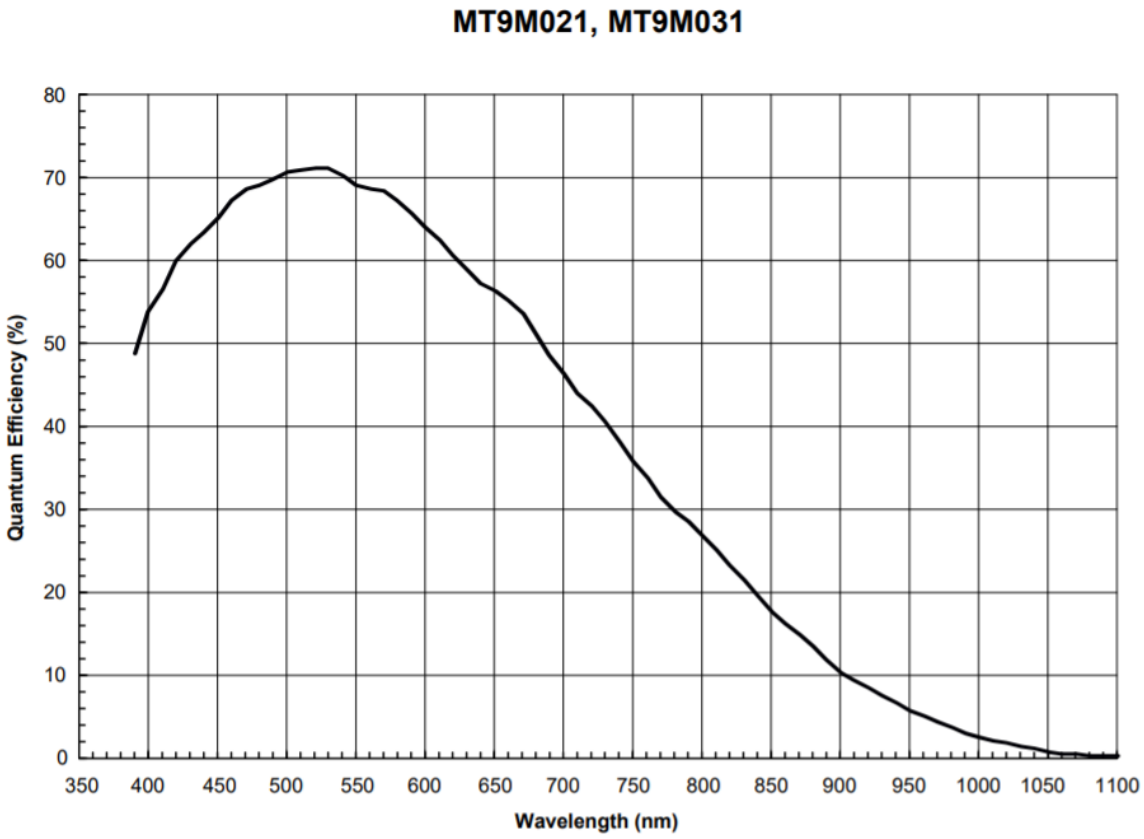
Signal to Noise Ratio: 38dB

Dynamic Range: 8 bit

Frame Rate:

Quantum Efficiency: Above 64% between 450nm and 600nm

*Micron was acquired by ON Semiconductor



Appendix E.

Glossary of terms that are used to describe optical sensors

CMOS: Complementary metal oxide semiconductor, is a type of otpical sensor. Typically cheaper than CCD sensors. Light is directly converted to electricity in every pixel

CCD: charged coupled devices, another type of optical sensor, Typically more expensive, but takes better quality images than CMOS sensors. CCD outputs the charge of each pixel, (charge is not the same as voltage), to a limited number of nodes and then the charge is converted to a voltage.

Shutter: A shutter determines how the pixels are activated. In a rolling shutter, a row of pixels is read out and then the next row and so on and so forth until the last row. A global shutter reads all the pixel values at once.

Pixel: The pixels are the elements that make up an image sensor. The pixel collects the charge of the light.

Signal to Noise Ratio: A ratio of the signal of interest to the noise of the camera. A large signal to noise ratio indicates the sensor has low noise. Usually measured in decibels. Accounts for both shot noise and temporal dark noise

Dynamic Range(definition one): The ability of the sensor to detect the difference in light intensity when the light intensity is very similar.

Dynamic Range(definition two): Ratio of signal to noise, but only accounting for temporal dark noise.

Quantum Efficiency: The percentage of photons converted to an electrical signal

Temporal Dark Noise(aka read noise): The amount of error associated with a signal

Shot Noise: The square root of the signal. Shot noise is the amount of noise in the light source itself.

Absolute Sensitivity Threshold: The number of photons needed to create a signal equivalent to the noise of the camera.

Saturation Capacity(aka Well Depth): The amount of electrons that can be stored in a pixel

Frame Rate: A measure of how fast a sensor can acquire and output an image. Measured in Hertz(Hz) or Frames per second (fps).

Appendix F.

Specifications of Prof. Merrin's Nikon Objective

Numerical Aperture: 0.75
Working Distance: 1mm
Magnification: 20x
Type: Super Flour
Mounting Threads: M25 X 0.75
Designed for 200mm tube lens

Appendix G.

Glossary of terms used to describe optical objective

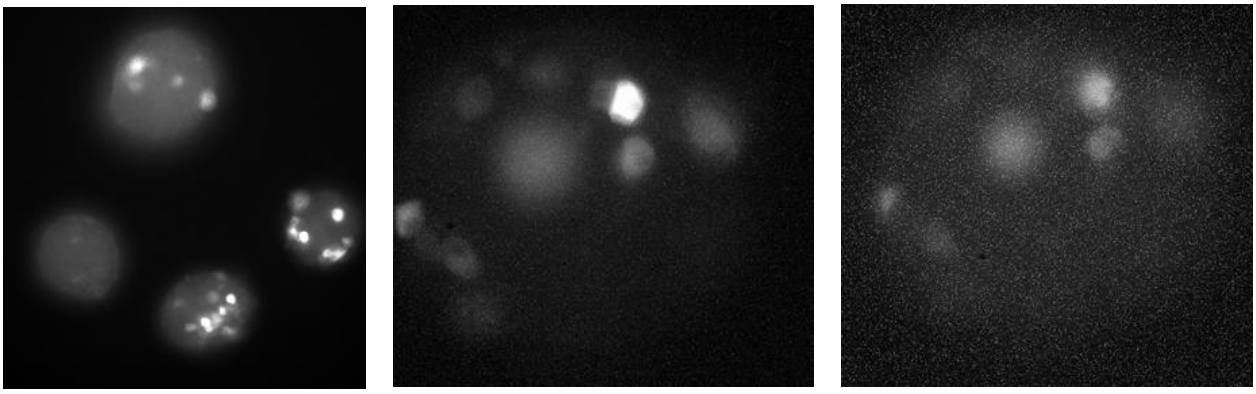
Numerical Aperture: Ability of microscope to collect light and resolve details. Unitless higher is better

Working Distance: Distance from the outer objective lens to the specimen's cover slip.

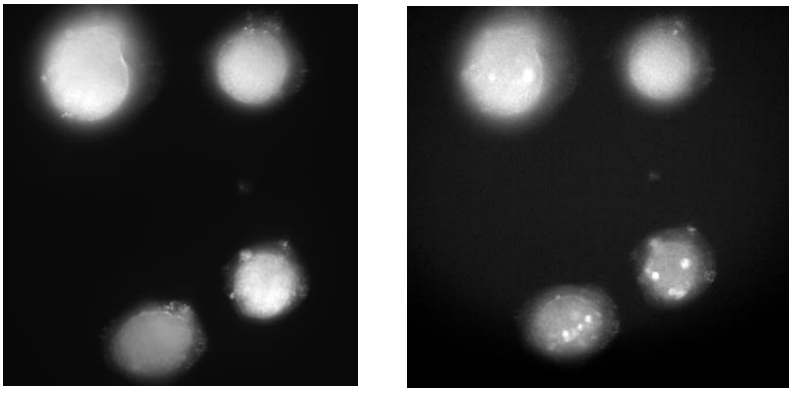
Achromatic: Most affordable objective, corrected for two wavelengths, Significant Abbreviations when using green light

Fluorite: Have higher Numerical Apertures then achromatic objectives. Made with special minerals to reduce auto fluorescence. Corrected spherically for two or three wavelengths of light. Corrected chromatically for two or three colors
Apochromatic: Most expensive and optically corrected objective type. Complicated lens system
PLAN: Simply means the objective corrects for curvatures in the image

Appendix H.
Imaging results from last semester
Left to right: Client camera and Lumencor at 535 nm. Prototype camera and client light source at 535nm. Prototype camera and Lumencor at 470nm.



From left to right: Client camera and prototype light source at 470 nm. Client camera and prototype light source at 535 nm.



From left to right: Prototype camera and light source at 470 nm. Prototype camera and light source at 535 nm.