FISH n CHIPS

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Abstract

Human immunodeficiency virus (HIV) is a global health problem that affects millions of people worldwide. Early, strain-specific diagnosis can reduce transmission of the disease as well as help initiate appropriate rapid antiretroviral therapy (ART) for more effective treatment. Individuals who reside in resource limited settings are unable to receive timely diagnosis due to the inaccessibility of laboratory equipment. We propose a microfluidic based point-of-care diagnostic to tackle this challenge. Our system, if fully functioning, is designed to analyze patient blood to determine two readings: a binary disease status and an indication of the general HIV strain. This is done by using fluorescence in situ hybridization (FISH) imaging with probes complementary to different viral strains of HIV. An innovative, inexpensive optical setup to read fluorescence is essential to making this technology adaptable to a point-of-care setting. Thus, the final product combines a disposable microfluidic FISH device with a portable fluorescence microscopy system along with accompanying analysis software. Such a product has the potential to allow for more informed treatment of HIV in the developing world. Additionally, it is a platform technology that can be applied to any virus by simply changing the FISH primers used. We have separately proven the ability of our imaging components to a) resolve cellular scale molecules and b) distinguish between fluorescent and non-fluorescent material. We created a novel microscope chassis integrating these two aspects of our project into a system which can image cellular fluorescence in a microfluidic device, but were unable to complete sufficient testing due to the University's move to an online presence in response to the coronavirus outbreak.

Background/Introduction

Human immunodeficiency virus (HIV) is a severe condition that leads to immune failure, acquired immunodeficiency syndrome (AIDS), and death by opportunistic infection if left untreated. Of the over 30 million HIV positive people worldwide, approximately 70% live in sub-Saharan Africa.[1] While a great deal of new therapies have been developed, in large part, they have not reached the developing world. This is due to a lack of early screening for the virus and ability to determine the specific strain. The latter is of high importance as different drug combinations work well for different strains of HIV. This results in delayed treatment which results in the high rate of transmission and mortality. Therefore, there is a need for low-cost and reliable point-of-care HIV diagnostics devices to achieve early diagnosis and effective treatments in these low-resource communities.

Globally, at the end of 2018, approximately 770,000 people died of HIV-related illnesses.[2] There is a growing demand within the global health community to improve the diagnosis of HIV so that transmissibility is reduced and effective treatments are delivered.[3] Testing is critical in both control and prevention of the disease. Unfortunately, the majority of testing options available are laboratory-based platforms with sophisticated instrumentation which is very expensive.[3] This leaves many rural and low resource regions neglected as testing is not accessible. POC tests are useful in these settings where there is a lack of well-trained laboratory technicians, poor physical infrastructure, extremes of climate, and lack of uninterrupted power supply, all of which impact the use of laboratory technologies.[4]

The only current in home HIV diagnosis that exists are rapid test devices such as Oraquick In-Home HIV Test. The test uses an oral swab to detect antibodies for HIV. However, these devices are less sensitive and specific than lab-based ELISAs.[4] Our proposed project will be different because it will be able to differentiate the strains of HIV present as it will detect the virus itself **(Table 1).** It will also have a lower cost, making it an ideal test for use in the developing world. Our optical setup will additionally allow convenience in imaging and increase the reach of the product.

Table 1: HIV POC Diagnostic Product Comparison

Product	**Price**	**Technique**	**Benefits**
	$29.99 per test	Antibody testing	Only at home test available in the market, proven technology with sufficiently high specificity and sensitivity
	$0.03 - 0.06 per test	Microfluidic assay using the FISH	Significantly lower cost, differentiation between various strains of the virus to inform treatment decisions

Business Analysis

Value Proposition

Our product is a rapid, low cost HIV diagnostic that, unlike our competitors, is also strain specific, and can be used in low resource settings.

Stakeholders

Key stakeholders in the provision and use of our device include regulatory agencies such as the FDA, various national regulatory bodies in Europe, and the NMPA in China, that are responsible for ensuring our device is of sufficiently high safety and accuracy to warrant sale, global NGOs that have the knowledge and networks to ensure that the device is given to the appropriate communities, patients that are unsure about their HIV status, and the community health workers responsible for operating the system.

Market Research and Competition

Our system has the opportunity to become a widely used HIV diagnostic in low resource settings. We hypothesize that a community health worker in these places with minimal training can operate the device.

We have identified several rapid diagnostic tests that are currently used in these settings, according to the World Health Organization, including the OraQuick Rapid HIV-1/2, HIV 1/2 STAT-PAK, Determine HIV-1/2, Uni-Gold HIV, INSTI HIV-1/HIV-2 Antibody Test, SD BIOLINE HIV-1/2 3.0, DPP® HIV 1/2 Assay, VIKIA HIV ½, and Reveal Rapid HIV Antibody Test.[5] All of these tests utilize similar diagnostic modalities, testing for the presence of HIV antibodies or HIV p24 antigen.[5] While internet shopping prices are not necessarily representative of the prices paid by NGOs for these diagnostics, a typical price for a diagnostic test is around $30/use, such as that for the OraQuick device. However, none of these options provide strain-specific information, which can inform which drugs an HIV-positive patient should be taking to most improve their outcome.

Given the number of people living in low resource nations newly diagnosed with HIV every year (~1.5 million),[6] and the cost of a typical low cost diagnostic test (~$30/test), and

given the assumption that one in 215 people that take an HIV test in these places have the disease (in 2009, 1 in 1500 Americans tested for HIV were positively diagnosed,[7] and given the much higher prevalence of HIV in low resource countries, we divided this number by 7), we estimate this market to be worth approximately $10 billion annually. We expect this market to grow proportionally with the growth in those positively diagnosed with HIV. As this number has been decreasing by about 2.5% per year since 1997,[6] we expect our market to have a long-term CAGR of ~-2.5%.

Cost and Revenue Model

As described later in this report, the cost of our device is approximately $100 up front, and each single use microfluidic chip and their associated reagents costs about $0.10.

Given that our system will be most useful in low-income settings where sophisticated laboratory technology is unavailable, and that our primary aim is, rather than to maximize profits, to ensure that as many impoverished places with a high incidence of HIV have access to our technology as possible, we will be selling our system to global NGOs at cost.

Intellectual Property

We do not believe any of our technology is patentable.

Project Overview

Objectives, Design Goals and Impact

Our product aims to provide an accessible HIV diagnostic device to low resource regions. End users will be community health workers. These workers will be trained in the use of the device and able to test community members in a high throughput manner. We propose a system that incorporates a disposable microfluidic device that performs a FISH assay to fluorescently

label a blood sample with a binary disease status and an indication of the general HIV strain. To decrease the cost of this assay, we will develop an inexpensive fluorescence optical system to image the microfluidic device. Our goal is to optically image the cells in HIV-infected blood samples and label such cells as infected with classification specificity of 0.95 and sensitivity of 0.95 (See Specifications table for a tabular view of these objectives). The assay should take no longer than 10 minutes. We also expect the cost of the imaging system to be about $250, while the disposable microfluidic devices will cost ~$0.05. We expect to analyze around 100 cells in the field of view. This is based on the work of the Issadore group and on the area that we are imaging. We believe that over repeated images (we aim for 30), this will be sufficient to determine if a sample is HIV+ or HIV-. This product is novel because no low-cost, rapid, strain-specific HIV diagnostic currently exists. If our product were commercially successful, it would dramatically change how HIV is diagnosed around the world, and would open the door to strain-specific treatment of HIV in even the most underdeveloped places.

The long term impact of such a device is extremely profound. This platform technology can be adapted to detect the presence of any virus by changing the FISH primers used. The technology could be used for the Flu or even in a pandemic outbreak such as COVID. The novelty in the design lies in the combination of technologies. We are striving to combine molecular scale imaging with a smartphone based system that is cheap and portable. As an additional layer, we aim to integrate microfluidics into our design to improve the workflow and cost.

While the table below gives a good idea of the trade-offs in the design, we have found one recurrent trade-off between cost of optical equipment and quality. This has been a large

design consideration and we ultimately found that we cannot achieve our goal with cheap plastic lenses. Rather, we had to use a slightly more expensive USB microscope to achieve the proper resolution.

Use Process and Specifications

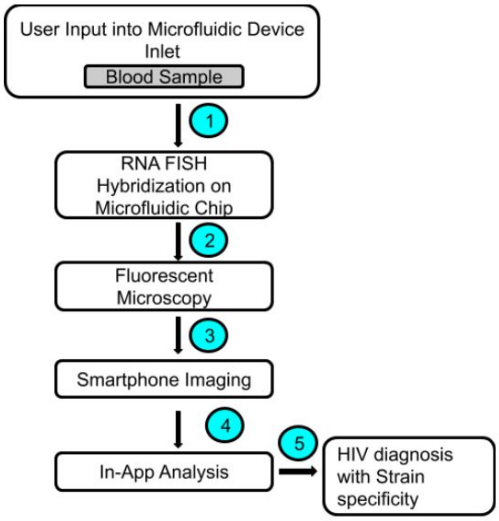


Figure 1: Overview of use process

Table 2: Final specifications for the HIV strain-specific diagnostic system

Category	**Specification**	**Value Units**	**Uncertainty/Tolerance**
Objectives	- Specificity of 0.95 - Sensitivity of 0.95 - Assay takes less than 10 minutes - Imaging system cost around $250 - disposable microfluidic devices cost ~$0.05		

Sample Format	**Consumable** **Volume of Sample**	1 mL test tube Thumb prick of blood solubilized in 200 µL PBS	+/- 5
Florescence in-situ Hybridization (FISH) Assay	**Blood Sample Volume** **Flow Rate** **Temperature of Assay** **Volume Wash Buffer** **Volume Hybridization Solution** **Time of Hybridization** **RNA FISH Probes**	200 µL 100 µL/min 37 °C 200 µL 80 µL 5 min Alexa Fluor 488 nm	+/- 5 +/- 5 +/- 1 +/- 1 +/- 1

Microfluidic Device	**Material Used**	Double Sided Adhesive Mylar (100 µm thickness)	
	Main Channel Width	2 mm	+/- 0.1
	Mani Channel Length	4 mm	+/- 0.1
	Inlet/Outlet Diameter	2 mm	+/- 0.1
	Whatman Polycarbonate Etched Filter pore size	5 µm	+/- 0.2
	Imaging Area	7.10 mm^2	+/- 0.1
Optical Encasing	**Material**	Polylactic Acid Filament (PLA)	
	Encasing Diameter	10 cm	+/- 0.05
	Encasing Height	6 cm	+/- 0.05

Fluorescence Imaging	**USB Microscope** **Illumination Mode** **Excitation Wavelength** **Emission Wavelength** **Emission Filter** **Magnification Lens**	250x Digital USB Microscope Fluorescence 470 nm 510 nm 515 nm bandpass Focal Length of 4.60 mm N.A. 0.40	 +/- 20 +/- 5 +/- 15 +/- 0.10 +/- 0.01
Image Processing	**Platform** **Target Binary Classification Accuracy** **Target Specificity** **Target Sensitivity**	MATLAB Application 95% 0.95 0.95	 +/- 0.5% +/- 0.1 +/- 0.1

Tolerance/Error is derived from the manufacturing methods used (ex. 3D Printing) or the manufacturer's website.

Regulations

There are two main governing bodies that curate the standards and regulations that apply to our project. The first governing body involved is the Food and Drug Administration (FDA). The FDA applies to our project through the subdivisions of the Center for Devices and

Radiological Health (CDRH) and additionally the Center for Biologic Evaluation and Research (CBER). The second governing body, the Centers for Medicare & Medicaid Services (CMS), is also part of the Department of Health and Human Services. Relevant legislation includes the Federal Food, Drug, and Cosmetics Act, which describes some of the necessary steps and testing required to bring a in-vitro diagnostic product to market (clause 201(h)), clause 351 of the Public Health Service Act, the Medical Device Amendments of 1976, which suggests our device is a Class III medical device and describes the required premarket application (PMA), as well as the Clinical Laboratory Improvement Amendments of 1988, which stipulates how premarket testing must be performed. As far as using our device abroad, the World Health Organization has set standards that must be followed. These standards matchup very closely with those in the United States. Ultimately, if approved by the FDA, the device will likely require a short, expedited follow up from the World Health Organization affiliate regulatory bodies before being used abroad.

Design and Development

Fall 2019 Semester

This semester, we took on an ambitious project to resolve fluorescent cellular scale features using low cost optical equipment. Given the lofty goals of the project, we ran into numerous challenges during our design iteration and testing. Nevertheless, during this semester, we have separately resolved fluorescence signal and cellular scale features. Our first design was to use bright field microscopy to image cheek cells. This served as our proof of concept that we could, in fact, resolve cellular scale features with an inexpensive optical setup (Figure 2). An image of this first version is shown below.

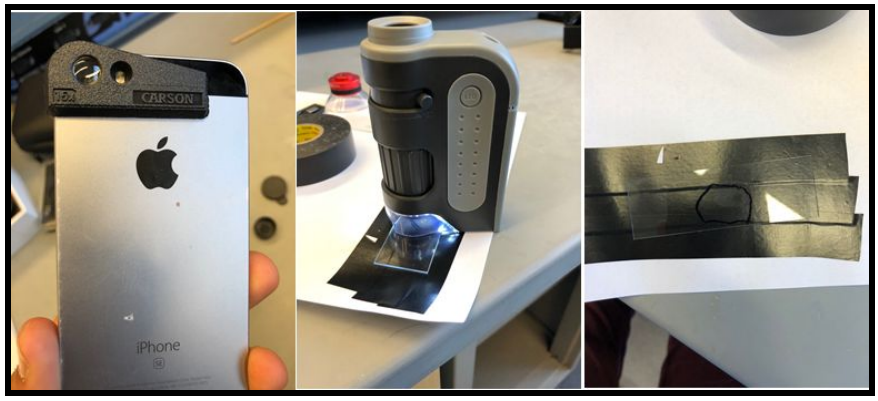


Figure 2: Proof of concept optical setup for smartphone imaging

The above figure shows the first iteration of lenses that were used. In series, we used a 15x IPhone SE lens coupled with a 65x pocket microscope. On the left of the figure, the slide that was used for imaging cheek cells is also shown. While this setup was sufficient for resolving cellular scale brightfield features, it consisted of numerous moving parts and also was quite bulky. This motivated us to further scale our design to optimize for the combination of fluorescence and cellular scale imaging. To do this, we made use of a 4.60 mm focal length laser pointer lens that could resolve cellular scale features.

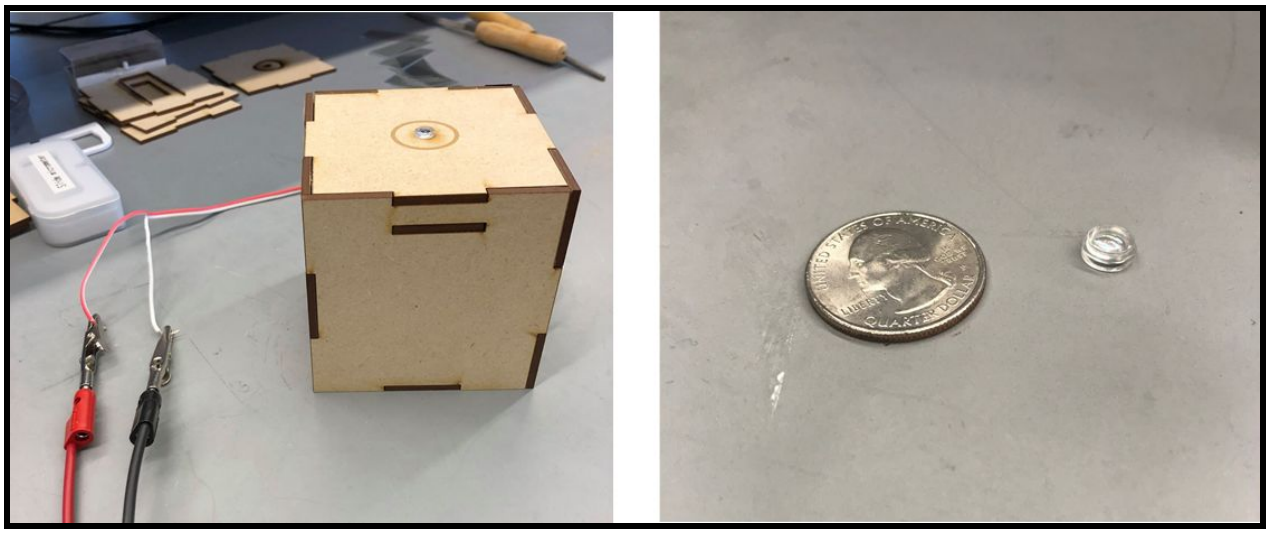


Figure 3: (left) Overall imaging setup (right) laser pointer lens

The above figure shows the laser pointer lens that was used (right) and the overall imaging setup (left). We were able to create a laser cut housing for optical imaging with medium-density fibreboard (MDF). This material was useful for our application because it does not reflect or scatter light, it is inexpensive, and it is consistent throughout. This minimizes ambient light and provides stabilization for moving parts in the system. These are important considerations in attempting to resolve cellular scale features simultaneously with fluorescence. Thus, we hypothesized that this key design iteration would help us meet our goal by increasing the compact, stable, and efficient nature of our imaging system.

As mentioned earlier, for our proof of concept, we used our first optical setup to image cells under bright field microscopy. The goal was to prove that cellular scale features could be resolved with an inexpensive optical setup. In theory, this proof extends to fluorescence. The results of our proof of concept were extremely encouraging and gave us evidence that resolving cellular features was feasible.

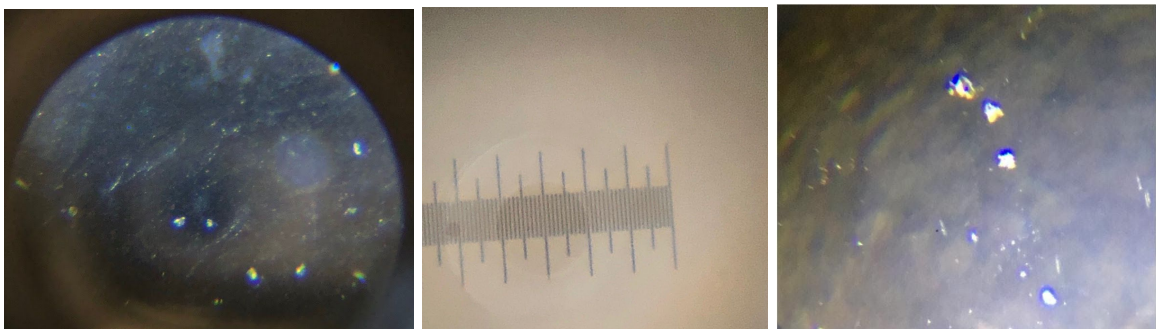


Figure 4: Cheek cells and a slide micrometer imaged with our optical setup

Above, cheek cells are seen from a non-digital zoom perspective under the initial optical imaging setup (left). For the proof of concept, we also provided an image of a slide micrometer (middle), which allowed us to resolve the 10 um delineations without digital zoom. Lastly, we

provided a digitally zoomed image of cheek cells (right). Thus, from the proof of concept, we are clearly able to resolve sub 10 um features and cells. To formally test our proof of concept, we aimed to show that we could program an algorithm to automatically segment the cells in the images obtained using MATLAB.

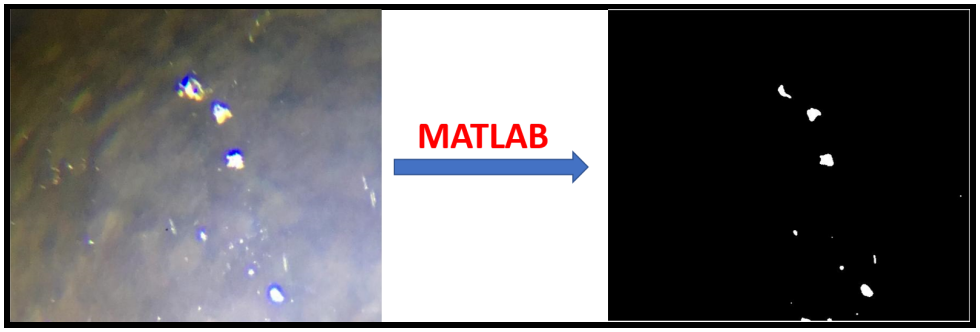


Figure 5: MATLAB imaging segmentation

Above shows the results of our algorithm. Here, we see that we are successfully able to segment 100% of the cells in the sample image shown. We processed the noise in the segmentation by thresholding based on the continuous area of the segmented spots. We repeated this process for five other images of cells and found an overall segmentation accuracy greater than 95%. This was an encouraging step, as through quantitative testing, we showed successful imaging and segmentation of cells with target accuracy above 95%. These experiments composed our proof of concept.

Moving forward from the proof of concept, we created the MDF laser-cut optical housing discussed earlier. Below in an engineering diagram from our final mechanical design for the ugly working prototype.

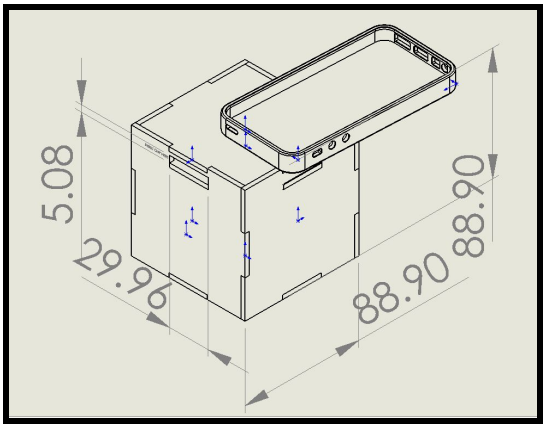


Figure 6: Engineering drawing of the minimum value product optical encasing.

The final device is a laser-cut, press-fit cube with a central lens hole with a diameter of 5 mm (not shown, underneath iPhone case), slit for the insertion of a microfluidic FISH device and a shelf to hold said device, and a 3D-printed iPhone case adhered to the top. The dimensions were optimized to prevent ambient light from entering the device and to minimize the number of moving parts. On the inside of the device, we had to incorporate a number of elements to allow for fluorescence microscopy. This involved picking the right LED illumination, optical filters, and optimization of the working distance. To pick the proper filters, we iterated over a number of choices. A few of the approximately dozen filters that we iterated through are shown below.

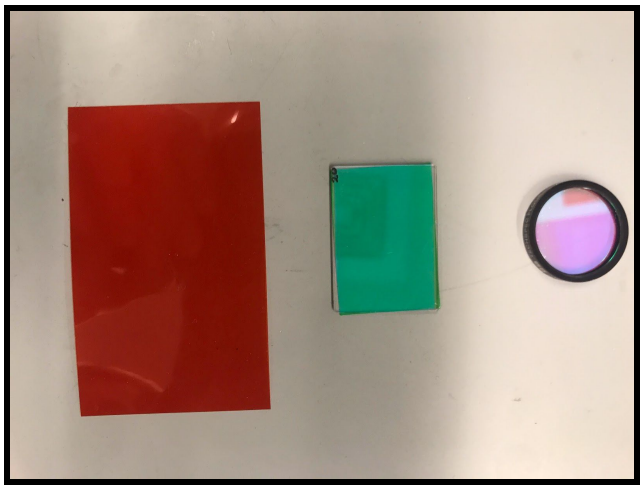


Figure 7: Shown are the filters that were tested in our iterative design process

On the left we see a simple film filter of bandpass 480-520 nm. The middle shows a dichroic filter of similar bandpass. Lastly, on the far right is a laboratory grade filter of bandpass 500-550 nm. We found this lens to be ideal and used it in our detection of fluorescence moving forward. For LED illumination, we first attempted to use a simple LED resistor circuit to illuminate. However, we found this to be insufficient in generating enough light to excite fluorescence probes.

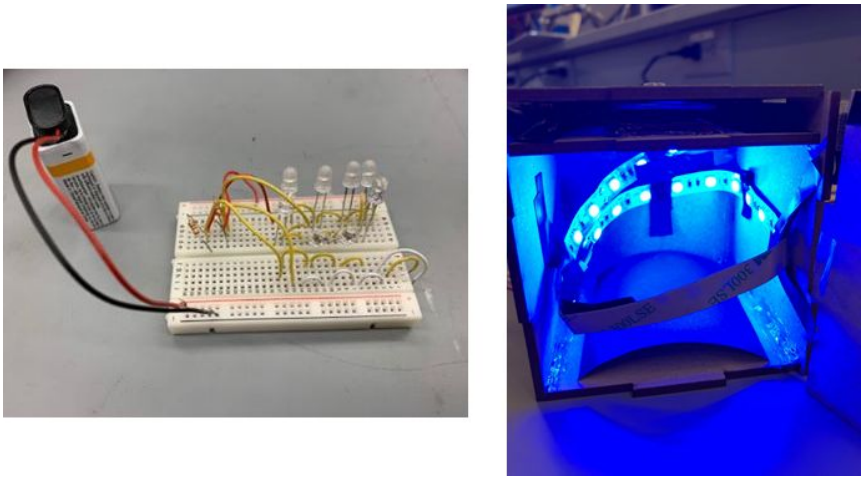


Figure 8:Shown are the LED circuits tested. In our final design we used a total of 30 LEDs as part of a strip configuration. This generated sufficient lighting.

The above figure shows the old LED circuit (left) consisting of simple LEDs and 680 k ohm resistors. Given that it failed to generate sufficient illumination, we decided to use powerful strip LEDs (right) that illuminate at 470 nm. These selection iterations for filters and LEDs led us to our final optical, fluorescence imaging design.

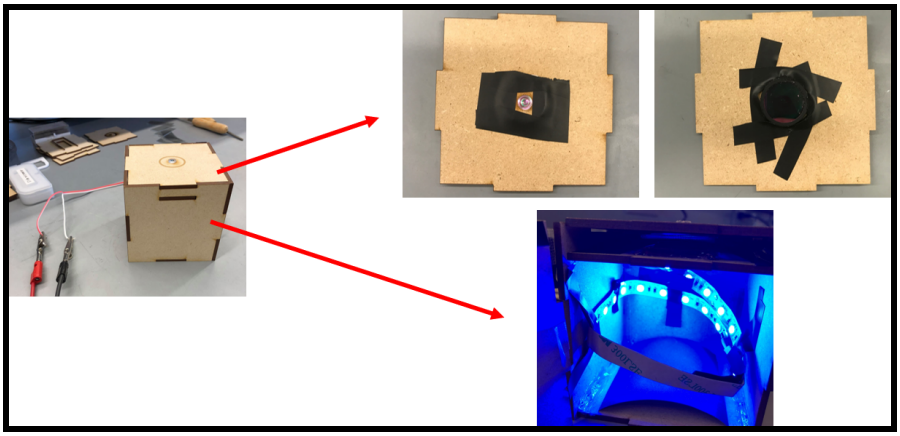


Figure 9: Shown is our optical setup, including the lens, filter, and LEDs used

The above figure shows that under the top of our imaging encasing the lens and optical filter were placed. Under the lens setup, there is a shelf to rest the specimen being imaged. Lastly, under the shelf we have our strip LEDs to illuminate the specimen.

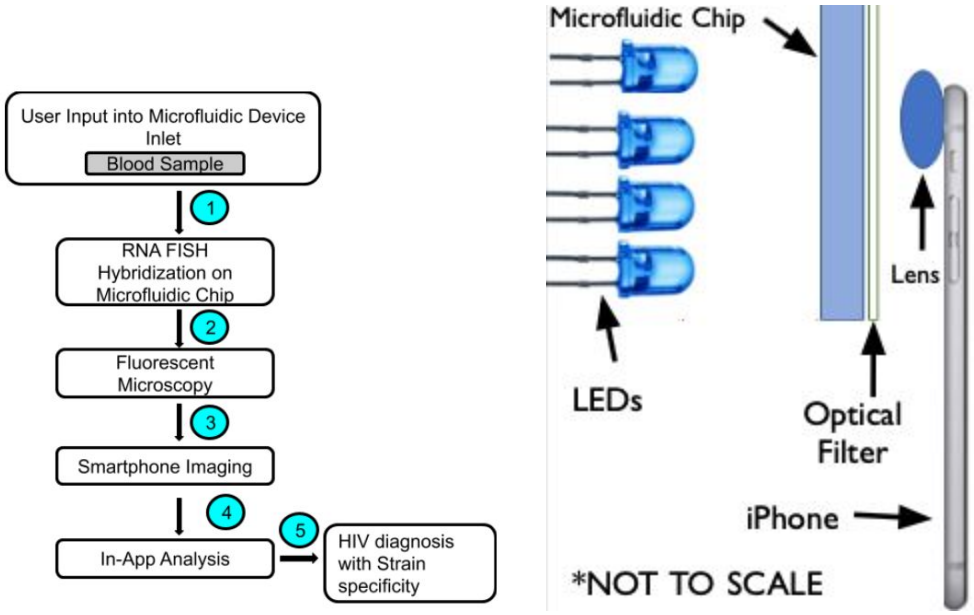


Figure 10: Engineering schematic of user process and fluorescence excitation

The above figure shows the overall workflow and optical setup. Starting with the optical setup (right) we successfully engineered a setup consisting of blue LEDs, a green bandpass filter and a lens (blue oval) adjacent to the phone. The blue block represents the microfluidic chip. In

the overall workflow of the device, we next turned our attention to creating a microfluidic device capable of housing FISH assays.

To create such a device we underwent laser cutting training in the Issadore lab and used adhesive mylar and acrylic material.

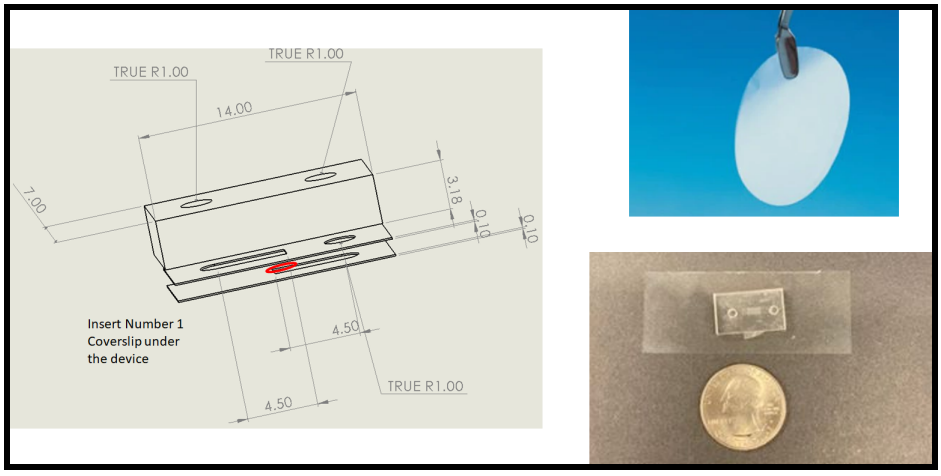


Figure 11: Engineering drawing and image of our microfluidic device

Overall, the device consists of a top acrylic layer with two inlets. The next two layers were designed to allow cells to be trapped above the polycarbonate filter (red and top right). The solution can then exit via the right outlet holes. Thus, the device will trap cells on a polycarbonate filter while hybridization and wash buffer can be pumped through the device. An additional advantage is that imaging can occur directly in the device on the polycarbonate filter. This again allows for minimal moving parts and in situ imaging on the device. Together the synthesis of the microfluidic device and development of the new optical encasing, filters, lenses, and LEDs constituted the minimal viable product.

To test the minimal viable product, we first tried to simultaneously resolve both fluorescence and cellular scale features. Unfortunately, we were not able to do this, likely due to

the small working distance of the laser pointer lens being used. Moving forward, we sought to show that we could resolve cellular scale features and fluorescence separately in our final minimal viable product environment.

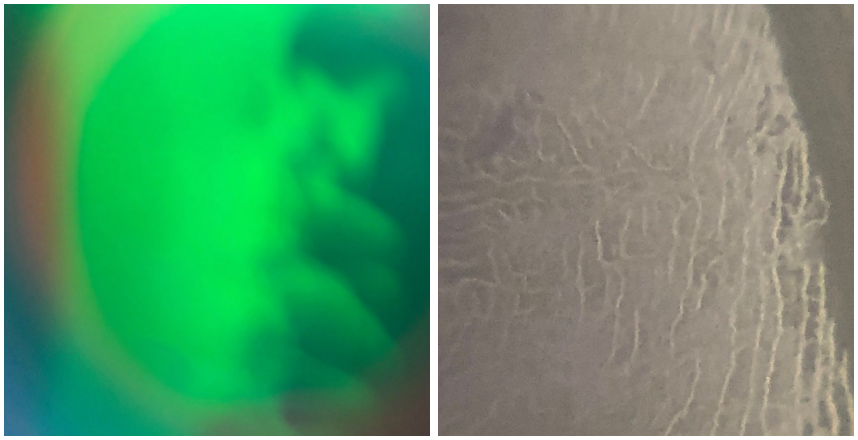


Figure 12: We can resolve a difference between fluorescent and non-fluorescent material

The above images show that we were able to resolve a difference between fluorescent and non-fluorescent material under our imaging setup. Here, we were imaging *E. Coli* bacterial colonies that had been transfected with green fluorescent protein. In similar experiments to the proof of concept, we were also able to show that under the MVP optical setup, we were able to image cheek cells and see the slide micrometer down to 10 um resolution.

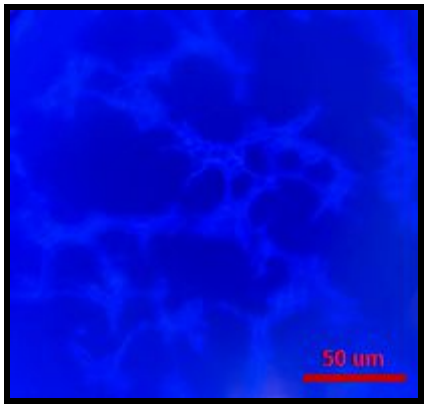


Figure 13: HEK (Human Embryonic Kidney) cells imaged using our device. Cells were

harvested at confluence and prepared on a microscope slide.

The figure above shows an additional experiment that we conducted. We imaged Human embryonic kidney cells (HEK293), an immortalized lab cell line, under our setup. Here, we can clearly see that we are able to resolve cellular scale features with our device. This being said, we were not able to combine the resolution of cellular scale features with fluorescence. This limited the amount of testing that we were able to do with our device. Overall our testing of the minimal viable product was limited to qualitative readout. We took several images of fluorescent material (similar to those shown above) and visually compared with the non-fluorescent counterpart. We could visibly see in all cases a clear difference in material. One way that we thought to quantitatively check this was to take the mean green voxel intensity in the image. What we found was that, in the case of fluorescent imaging, there was a significant increase in the green voxel intensity of the images ($P<0.05$). This data is not shown as it was from the first semester, but it was performed over 10 images taken with iPhone. This leads us to believe that our minimal viable product is able to successfully discriminate between fluorescent and non-fluorescent material. Similar to the proof of concept, we were able to algorithmically segment cheek cells at an accuracy greater than 95%. These results coupled together allow us to demonstrate the ability of our device to separately visualize fluorescence and cellular features.

In the future, numerous additional testing measures and changes to the device must be made. Ultimately, our goal is to detect cells that have been labeled with fluorescent RNA FISH probes. Thus, we must be able to simultaneously see fluorescence and cellular scale features. In the future plan, we discuss our plan to do this in more detail. Once this bar has been met, the ugly working prototype can undergo further testing. As a first test, we aim to fix 10 uM green

fluorescent cells or beads into our microfluidic device. Then we will use our imaging algorithms to segment the cells and compute the percent accuracy. This experiment will provide proof that we can simultaneously look at both parameters. Moving forward, we also hope to test 10 labelled and 10 unlabeled solutions of cells and use our device and algorithms to generate a binary classification. We aim to achieve 95% accuracy, .95 specificity, and .95 sensitivity under these testing conditions. This is something we aimed to show at the end of the second semester. However, courses moving to virtual instruction prevented us from testing our final prototype. Lastly, by the end of the semester, our final testing goal is to perform a FISH experiment inside the device, image the device with our fluorescence microscope and computationally determine the amount of fluorescent vs non-fluorescent cells in the sample as a proof of concept for this imaging platform. This would provide validation of the entire user process of our device.

In these testing experiments, a few specific readouts are being evaluated. The most important being fluorescence. This is our ultimate output measure that we want to test for. Another specific readout is the signal to noise ratio. We want to make sure that we maximize this value to clearly see individual cells and minimize the background signal. Another readout will be the resolution. Here, in our testing we aim to keep the resolution around 10 um, so that a wide frame is captured while ensuring cells are visible. Lastly, we are interested in the concentration of probes needed to achieve sufficient signal for our device. During our final testing, we will vary the concentration of probes that we are labeling cells with to find a minimal amount necessary. This will help with cost considerations.

With regard to the end user of the device, we anticipate a slight calibration process to be necessary. Each user may have a slightly different cell phone and the staining might occur with

small variation between users. For these reasons, we plan to add a calibration aspect to our device. During the second semester, we plan on adding features to allow the user to move the imaging platform in the vertical and horizontal directions. This will allow the user to focus the imaging apparatus on the cells to provide the highest resolution. To help the user find the optimal z slice for imaging we may provide a calibration slide. Additionally, the software will aid in calibrating the fluorescence aspects of the device. In the app, we plan to allow the user, on the image, to select areas that are background and visibly not cells to get an idea of what our algorithms should filter out. Overall, these calibration steps should be intuitive for the user and aid in the procurement of accurate data.

Table 3: Fall 2019 Semester Budget

Item	**Cost**
¼ in. MDF	$6.99
Colored film filters	$1.50
Laser pointer lens	$7.00
LED strip	$5.88
Edmund Optics filters	$60.71
DCKina 30x Super Microscope Lens	$25.00
Arducam MI2 Lens Set	$50.00
Carson MicroBrite Pocket Microscope	$15.00

Spring 2020 semester:

In the spring 2020 semester, we made considerable changes to our device design. We successfully achieved our two main goals for the optical setup. First we created an optical encasing that had a large enough working distance to accommodate the filters and image the

cells. Next, we used a USB microscope to allow for fine focus on our target cells. This would allow for fine movements of the sample closer and further from the camera and tuning of the focus. These changes brought us one step closer to performa FISH experiment inside the device, as well as conducting computational analysis of these imaged cells. Given the problems faced in the first semester, we created a design where no parts were loose, no ambient light could enter, and a flexible working distance of the lens was possible. To this end, we decided to redesign our optical setup based on an online tutorial in fluorescence microscopy.

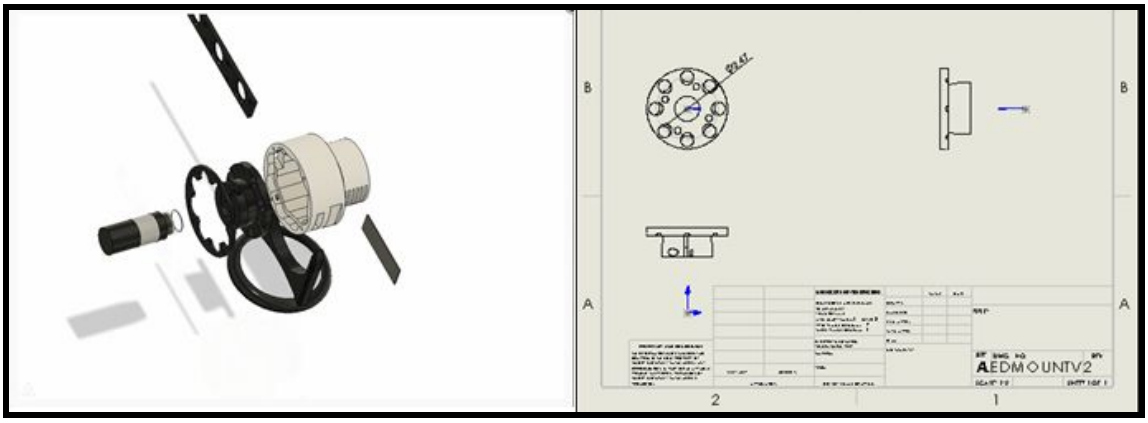


Figure 14: Shown is an overview of the new schematic we used to design our optical encasing. Notice the engineering diagram on the right and high level overview of the parts on the left.

From this diagram (Fig. 14) we can see a good overview of the device. The device consists of a USB lens that has up to 200x magnification. This lens is plugged into the computer and uploads its image to MATLAB. There are then a few mechanical parts that hold the rest of the setup. The beige piece in the diagram forms the base of the device. It also has two slits for the filter wheel to be inserted and the microfluidic device to be inserted. The black piece directly

above was printed to have holes for insertion of LEDs. This is where the optical excitation will come from. The last piece is an adaptor between the lens and the LEDs.

Figure 15: Shown is our 3D printed optical encasing. Here we can see the USB microscope, holes for the LEDs and where the microfluidic slide is inserted.

Above (Fig. 15) we can see how the pieces of our device begin to fit together. Here we see how the microfluidic device can be inserted and imaged on a laser cut tray. We also see the tight fit of the USB microscope, minimizing the ambient light entering the device.

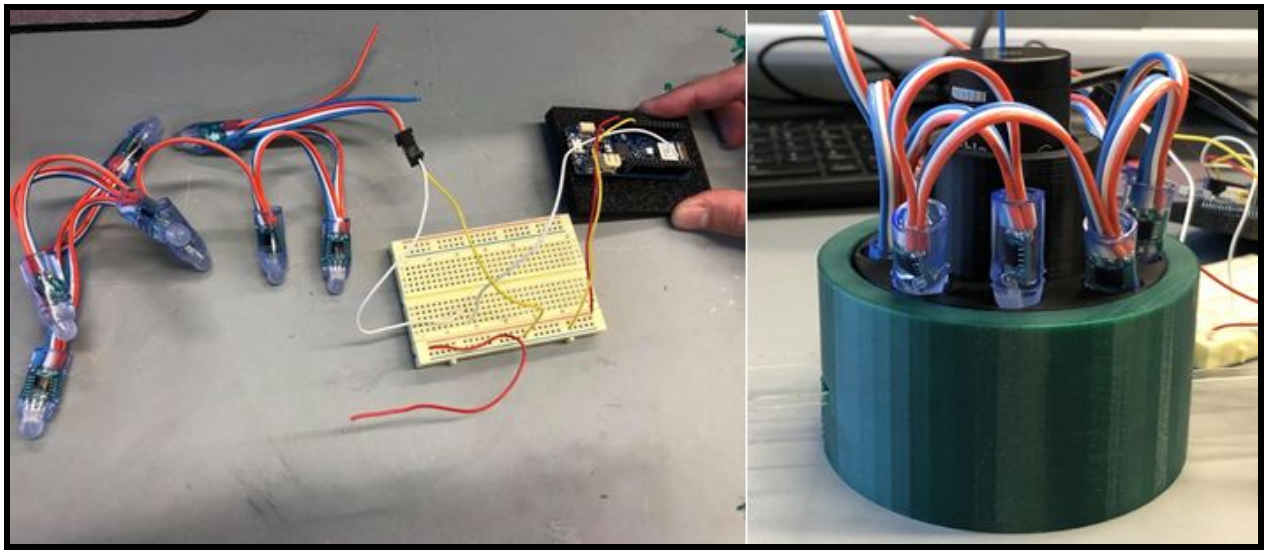
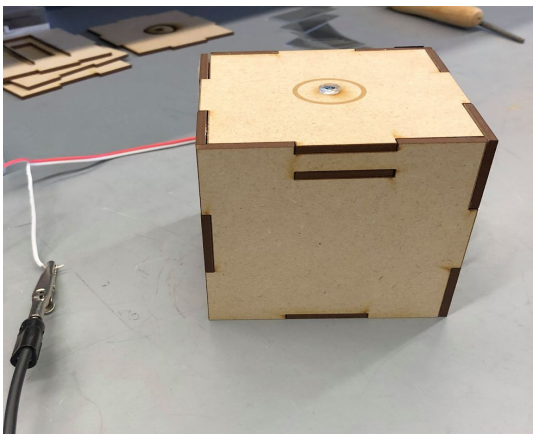
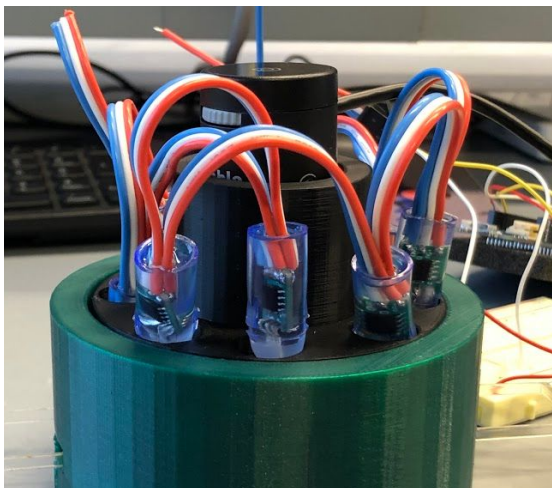


Figure 16: Shown here is the circuit used for the LEDs. We also show how the LEDs fit into the overall device.

Above (Fig. 16) we can see how the device appears when the LEDs are integrated into the setup. They fit well into the 3D printed holder and are slightly angled to provide optimal light on the sample. This represents the final mechanical and optical design of our device. It is also important to note that on the filter wheel we used: Longpass Filter, Cut-On Wavelength: 500 nm. Overall, our new device set up was much more effective at imaging and much easier to adjust and replicate (Table 3).

Table 4: Improved System Set Up

Fall 2019 Set Up	Spring 2020 Set Up

	
• Parts were loose • Let ambient light through • Could not detect fluorescence • Relied on phone camera	• Fixed working distance • Reproducible • Blocked more light • Upgraded camera

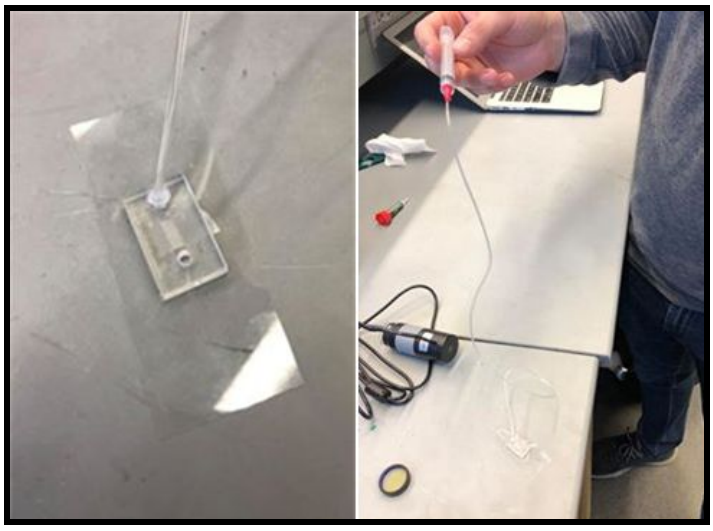


Figure 17: Shown is a use case of the microfluidic device.

After re-designing the optical encasing, we next turned our attention to the microfluidic aspect of the device. We cut tubing that would fit with our device and attached this to a syringe

to pass fluid through the device. Overall, we demonstrated that we were able to pass specimen through the device. However, we were not able to test this due to moving online.

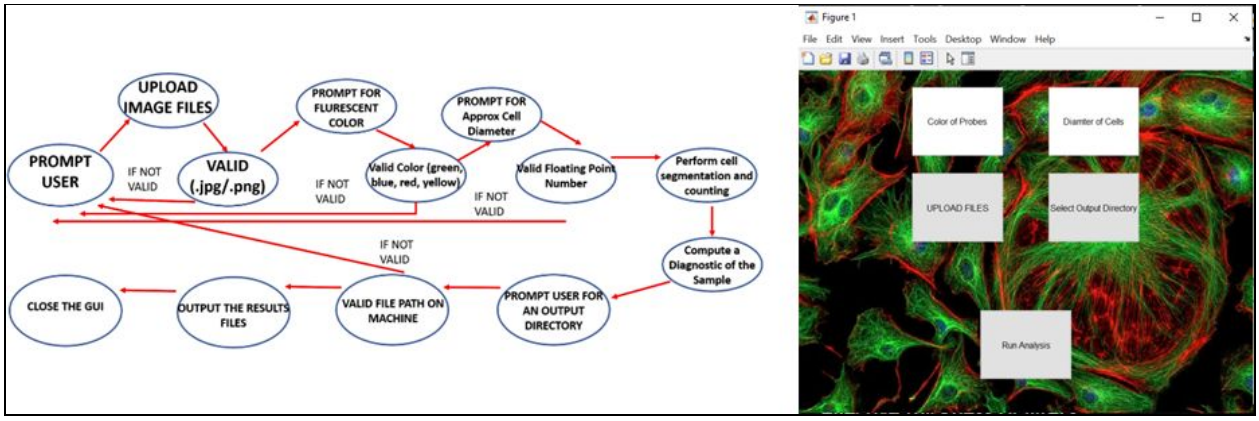


Figure 18: Shown is an overview of the software that goes with our device. This software is used to segment cells in images produced on the device.

We next developed a software package in MATLAB to go with our device. Essentially, the user interface (Fig. 18) prompts the user for the color of probes, diameter of cells, and to upload the image files to analyze. The software will then apply the MATLAB segmentation algorithms discussed earlier to each image in the stack and return whether the images represent infected samples.

See the appendix for more information about manufacturing of the device, including a build procedure, user manual, and bill of materials.

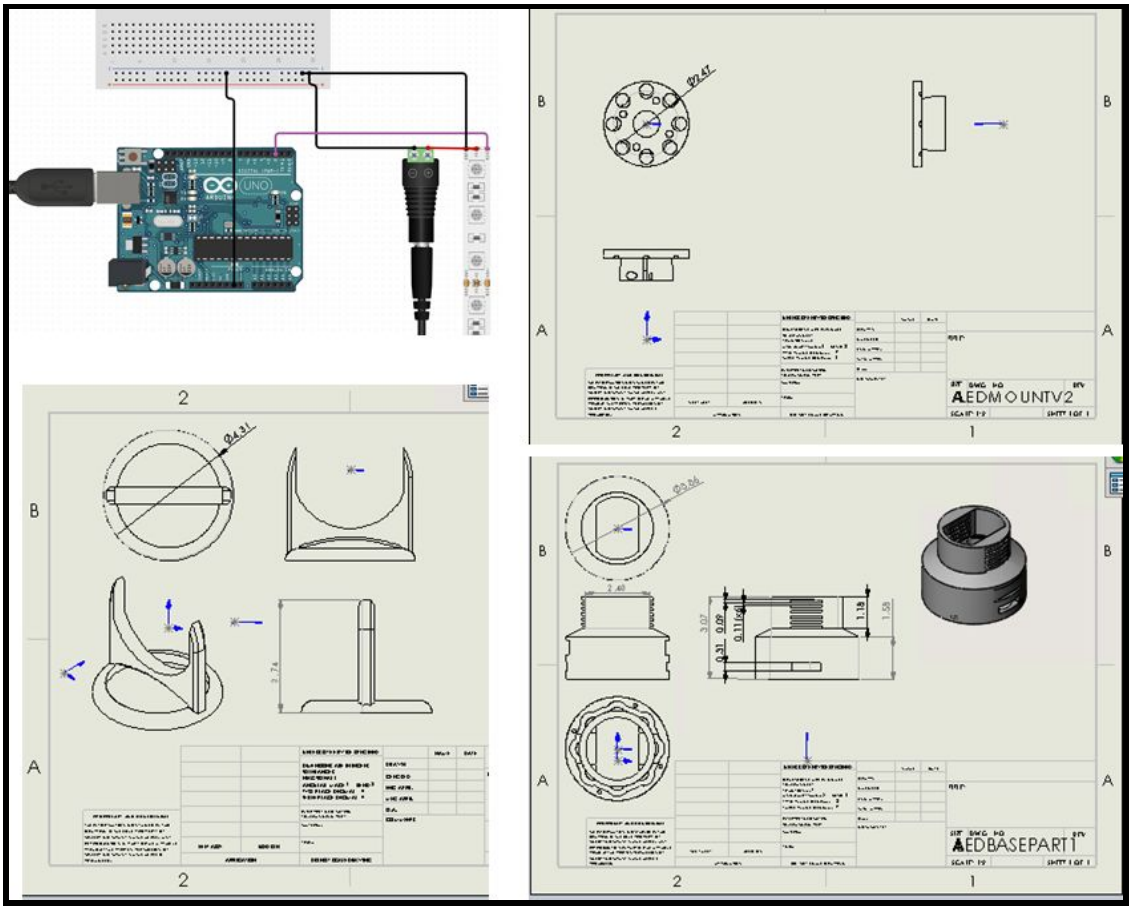


Figure 19: Shown are supplementary engineering drawings of a few mechanical parts and the circuit for reference.

Table 5: Spring 2020 Semester Budget

Item	Cost
Arduino	$33.00
LEDs	$12.50
Optical filter	$15.00
USB Microscope	$40.00
PLA filament	$10.85

Testing and Validation

Given more time for testing and validation, both during this semester and beyond, our first step would be the testing and validation of our microscope set-up. We would begin by evaluating the set-up's brightfield capabilities.Performance would be evaluated through the diagnosis of slides with stained cells. We would ensure that the microscope can resolve down to 5-10 microns , as this is the diameter of a cell, via a slide micrometer. Once this was confirmed, we would thenl test the fluorescent component of our microscope.

To test the fluorescent capabilities, we would use slides of stained cells. Cells would be stained with DAPI to observe the nucleus and then green fluorescent protein (GFP) would be used to differentiate the RNA FISH probe signals. The GFP staining would be used to exclude background autofluorescence that can occur in cells. Again, we would test these components to guarantee that the inclusion of fluorescence does not interfere with the visualization of the cells. The resulting images would then be compared to those of a commercial microscope. Validating this step will reduce error in the subsequent image analysis. An ANOVA test would be run to determine whether there are any statistical significant differences between the means of the three groups. We expect that there will not be a significant difference and confirm the ability for our device to accurately image fluorescence.

Table 6: Example of 10 sample images with manually counted cells. The number of fluorescent cells should be the same number of cells in the image.

Sample #	# of DAPI- stained cells	# of GFP-stained cells	Expected number of cells in image
1	20	20	20
2	34	32	35

3	25	24	25
4	44	45	45
5	40	42	42
6	26	26	26
7	29	30	31
8	31	32	32
9	36	36	37
10	37	37	38

Our next step in testing and validation would be with the image processing software. We need to ensure that the processing software is able to undergo image analysis to detect the cells that are positive or negative for HIV. The software would segment each cell by first detecting the nuclei through the DAPI stain. We would fix a known number of stained cells on a slide and analyze it under a lab-grade fluorescent microscope. We would count the cell concentration manually and this would be our control. Next, we would apply our algorithm to the resulting images and compare these results to the control. Based on this analysis we would validate that our software can segment the cells effectively. A two-sample t test would be used to determine if the mean population of the microscope and our device sample is equal. This would be applied to both GFP and DAPI stained groups. We would expect there to not be any statistically significant differences and confirm the validation of our device.

Table 7: The number of cells in the images can be compared by manually counting and by using the image processing software.

# of DAPI cells manually counted	**# of GFP cells manually counted**	**# of total cells counted by algorithm (via**	**# of GFP cells counted by algorithm**

		DAPI)	
25	25	25	25
36	36	34	34
26	26	26	26
42	42	39	37
23	23	22	20

Before the system can be employed on test samples, the minimum concentration of fluorescent cells in solution that can be detected must be determined. To do so, 10 dilutions of FITC-tagged 10um fluorescent particles (Sigma Aldrich #90287) would be created, ranging from 100 particles per microliter to 100,000 particles per microliter. Each dilution would be run through a microfluidic chip and the microscope system, and the number of fluorescent particles observed would be counted by the imaging processing software. This would be repeated five times for each dilution. The minimum concentration at which any number of particles could be detected in all five trials would then be compared to known physiological concentrations of T-cells (the cells infected by HIV) in blood when mixed with phosphate-buffered saline solution. If the minimum detectable concentration is less than or equal to approximately 80% of physiological T-cell concentration, then clinical trials would proceed. The number of replicates was chosen in order to balance the amount of time and resources utilized as well as the statistical power of our results.

Table 8: Example of testing for 100 pt/uL. Testing for 9 other concentrations up to 100,000 particles per uL would be similarly conducted for 5 replicates. If a concentration within 80% of the blood phosphate buffered saline solution is found, the system can successfully be used.

Concentration	Replicate	Cells Detected via

	Number	Algorithm?
100 pt/uL	1	no
100 pt/uL	2	no
100 pt/uL	3	no
100 pt/uL	4	no
100 pt/uL	5	no

Then, our device could proceed to clinical trials. This would consist of enrolling a total of 100 participants into a clinical trial. These patients would be split between 50 healthy controls and 50 patients infected with HIV (of various strains). Groups would be age and sex balanced. We would then collect blood samples (volume based on the tests described above) from each participant, solubilize the blood in phosphate-buffered saline and pass the samples through the microfluidic device following our FISH procedure. Next, we would take images of the cells caught on the imaging area of each device. We would separate each trial into a train and test set following an 80/20 split. Then, using the segmentation algorithm, we would calculate the average GFP fluorescence of each image. We would analyze each of the training images at various thresholds for defining HIV+ and determine the true and false positive rates. This would allow us to plot ROC curves for our results. From the ROC curves we would pick a suitable threshold with the notion that true positive accuracy is of the utmost importance. We would then be able to report a classification accuracy on the test set.

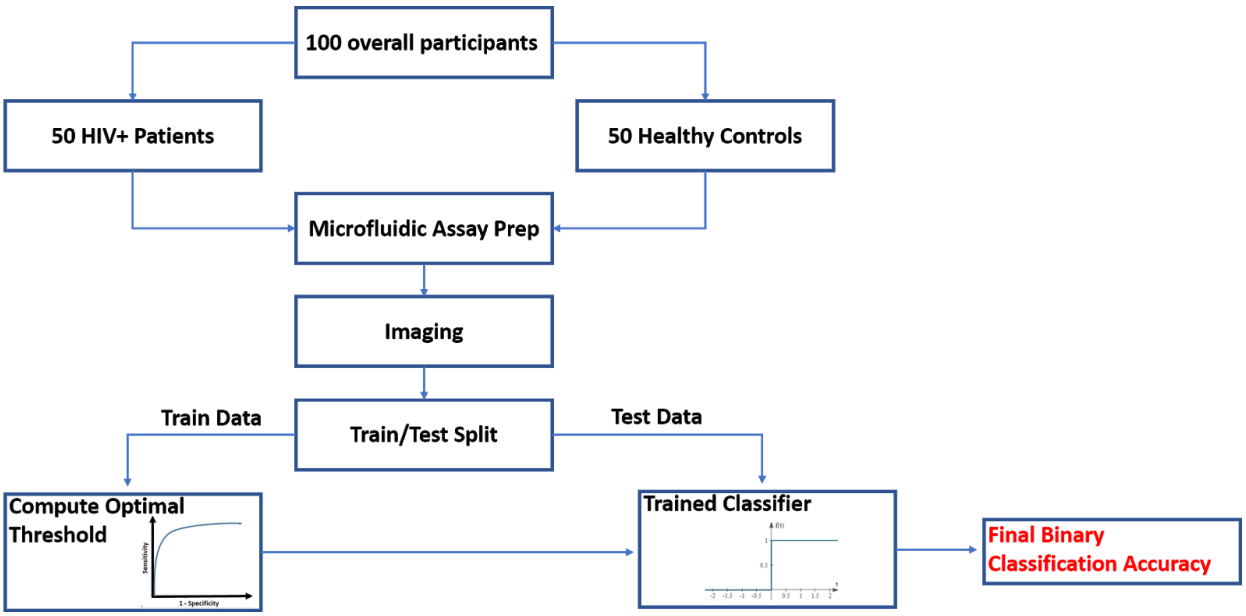


Figure 20: Shown is a schematic overview of the clinical trial.

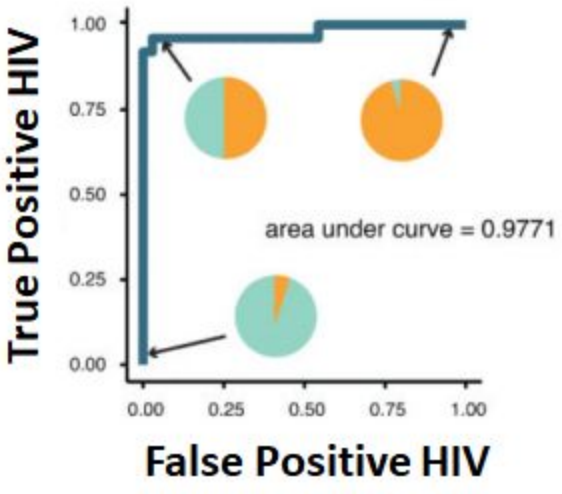


Figure 21: Shown is a hypothesized ROC curve adapted from Shaffer et al. Essentially, the area under this curve represents the accuracy of a binary classification. The shape of the curve represents the tradeoff between the true positive and false positive rate for using different thresholds for classification. Under this paradigm, each point on the ROC curve represents the use of a different fluorescence threshold.

Table 9: Average GFP fluorescence of cells in the image as determined by the cell counting software along with the true label of the image. This would be done for all 100 sample images.

Image #	Average GFP Fluorescence	HIV+ or HIV-
1	5.7	+
2	6.4	+
3	6.6	+
4	2.3	-
5	4.5	-

Table 10: The determined false and true positive rates are calculated based on the calculated average GFP fluorescence, their true labels and the given fluorescence threshold. From this, the optimal threshold can be calculated.The threshold would be used to determine the labels of the held-out testing set. The overall accuracy of classification can then be determined.

Fluorescence Threshold	False Positive Rate	True Positive Rate
1	0.85	0.89
2	0.76	0.85
3	0.63	0.83
4	0.50	0.80
5	0.36	0.73
6	0.26	0.64
7	0.19	0.56
8	0.12	0.40
9	0.06	0.04

We determined 100 participants to be a strong number for the patients in this trial. This

is based on previous literature and previous work done by the Issadore group. Furthermore, we see this as a realistic first test of the device. Depending on the strength of the results of the first clinical trial, more replicates may need to be performed. It is conceivable that this first trial would inform changes to the specifications of our device such as imaging area/volume of sample that we may need to change before performing another replicate trial. This iterative process would continue until we reach our target sensitivity and specificity of 0.95. We would judge this using the statistical technique of ROC curves based on different fluorescent intensity thresholds for a sample to be called HIV+ we will then select the optimal threshold. In addition, we would run a two sample t-test between the fluorescence values in the HIV+ and HIV- groups to gauge if our test can reasonably separate these two groups. We would also compute a confidence interval for the difference. These statistics would help us gauge device performance and provide evidence of the validity of our test.

Future

Moving online has left us with a great deal of future goals. First, we wish to test the setup we have created. We believe that we have constructed an optical imaging setup able to image cellular scale fluorescent features. In the future, we aim to test this imaging setup by passing 10 uM GFP beads through the microfluidic device. We then can determine whether our imaging setup can pick up these beads and whether our software can adequately perform segmentation. As another future goal, we would like to run a full FISH assay in our device and visualize RNA fluorescence with the optical imaging setup. We then also wish to determine whether the software can pick up these outputs. This leads us into our final goal of conducting a clinical trial with the device. Ideally, we would collect samples from patients with and without HIV and run a

full experiment to determine if the device can distinguish between the two populations. This would provide proof that our device may be used as a diagnostic. It is also important to note that there are numerous other applications of this device. It is a platform technology, able to be adapted to numerous different viruses. The only thing that has to change in the setup is the FISH primers used. With this small change, this device could be applied to the Flu or even COVID.

Reflection

If we were to do this again, we should look at the design more holistically rather than incrementally. While we were quickly able to resolve cellular level features in brightfield imaging, this occurred at a much smaller working distance than was necessary to work with the filters needed for fluorescence imaging. It would have been helpful to consider the challenges associated with fluorescence imaging earlier so that we could potentially find a lens with a larger working distance earlier. It also might have been helpful to perform a more thorough literature review before beginning the project in order to determine the challenges of building a fluorescence microscope from scratch. While we reviewed many previous setups that worked with brightfield imaging, we did not research similar fluorescence setups enough. This led to our pivot at the end of first semester.

Looking back, our biggest takeaway is to focus more on the upfront research and feasibility of a project. When we approached Dr. Issadore with the possibility of doing a senior design project that built off of the MicroFluFish from several years ago, he suggested a project focusing on the optical aspect of bringing this device to the point of care. We undertook this project with his suggestions. However, we should have spent more time as a team discussing the

feasibility of this project and determining our interest in this area. None of us had experience or particular interest in optics. Our interest was more in the FISH and microfluidic aspects of the project which were possible to work on after the completion of the optical model. With more preliminary background research, we may have been able to come to the conclusion that this project was not as feasible as it originally sounded.

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