

Tissue Biopsy Dissociation

BME 301

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Abstract

Biological research often requires the study of individual cells to gain a better understanding of processes within the human body. The client conducts asthma research by obtaining lung tissue samples before and after an asthmatic response. Individual cells are then dissociated away from the tissue for study via flow cytometry. The current device being used for this process is the gentleMACS™ Dissociation Device, which does not allow for use of small tissue sample sizes. A small tissue sample size, 1-2 mm³, is desired to reduce the recovery time and pain of the patient. This design team was tasked to create a dissociation device that could successfully dissociate small tissue samples and yield viable cells that can be used in flow cytometry. To be considered successful this device must yield 10,000 cells from the tissue. To accomplish this task, a microfluidic device that utilizes shear force was 3D printed. Preliminary testing conducted on this design has not yielded cells from tissue, but has shown improvement from previous work. Modifications to the design, fabrication, and testing of this device will continue to move us toward our goal of dissociating lung tissue, which will allow our client to accurately study the asthmatic response and improve patient lives.

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I. Introduction

Motivation

Many doctors and scientists seek to understand different types of medical problems in a greater level of detail than what is currently known. Through careful experimentation and data collection there have been significant gains in knowledge pertaining to different diseases, conditions, and effective treatments.

Biological research often requires the analysis of cells to obtain new knowledge of specific cellular processes. Cells provide structure and function for all living things and house the biological machinery that make the proteins, chemicals, and signals for everything that happens within the body. There are about 200 major types of cells and they all function in different ways. Biologists rely on different types of tools to examine these cells and gain a better understanding of how they function. Learning more about how cells work, and what happens when they do not work properly, is imperative in understanding the biological processes that keep humans healthy [1].

The client for this project studies the effect of the asthmatic response on white blood cells, specifically eosinophils. The University of Wisconsin-Madison has a nationally known research facility for Asthma, Allergy, and Pulmonary Research. Asthma has been studied at UW Madison for over 30 years, producing over 400 research studies. These studies have investigated the role of genetics in asthma, treatment of asthma in children, and how colds affect asthma. This asthma research has significantly contributed to the development of new asthma medications and guidelines for treatment [2].

Asthma affects nearly 1 in 12 people in the U.S. alone, with the number of affected individuals is rising each year, according to the CDC [3]. A disease this widespread leads to huge economic costs with approximately 56 billion dollars a year in medical expenses, lost work days, and premature deaths caused by asthma [3]. If this project can help the client better understand the mechanisms behind the asthmatic response, then it may lead to better treatments and prevention methods for asthma. Understanding and elucidating the causes of this disease are important for our society to live longer, healthier, and more productive lives.

Dr. Mathur's research group at UW-Madison currently uses tissue dissociation as a method of studying individual cells to gain a better understanding of an asthmatic response. Lung biopsy procedures must be performed to obtain the desired tissue samples. After a biopsy procedure, the patient may experience pain at the site of biopsy during recovery. A project like this relies on volunteers for their study, so patient comfort and recovery is very important to continue having willing subjects. It is therefore desired to take the smallest biopsy possible to increase patient comfort while maintaining accurate and reliable results. By taking the smallest possible biopsy, the patient's pain, discomfort, and recovery time will be reduced [4]. In order to

make this a viable option, these smaller samples must be able to be dissociated into individual cells while maintaining the integrity of the cell, which is the goal of this design project.

Existing Devices and Current Methods

Tissue dissociation is commonly performed in two different ways. The first of which is chemical dissociation. There are several different protocols for chemical dissociation that vary with tissue type, but they usually follow the same set of steps. First, the tissue is placed in a specific concentration of collagenase and enzyme solution to break down the extracellular matrix of the tissue. Next, the solution is heated to an optimal temperature and incubated with gentle vortexing or mixing. Once this is complete, a filter is used to “strain” the solution and obtain certain types of cells. This is a very common method, but there are some drawbacks, mostly with inconsistency of cell yields and disruption of cell characteristics due to the enzymes used. What many researchers do instead is to use mechanical dissociation, or a combination of both chemical and mechanical dissociation. A handful of mechanical dissociation products are outlined below.

A well known mechanical tissue dissociation device is the gentleMACS™ Dissociator (Figure 1). This benchtop instrument performs a semi-automated dissociation of tissues into single-cell suspensions. It can process one to two samples at a time. There are two types of unique tubes that can be used with this instrument and each tube has a rotor that moves over teeth on the stator to perform mechanical grinding with rotation (Figure 2).



Figure 1. gentleMACS™ Dissociation Device. A dissociation tube is placed in the opening at the top, an automatic cycle is then selected with the buttons and display at the bottom.



Figure 2. Conical Tube used with grinding teeth used in the gentleMACS™ system to dissociate tissue.

It has several settings for different dissociation process cycles, and special protocols have been developed by the company for dissociation of specific tissues [5]. This device was

previously used in the lab to dissociate tissue samples, but due to the size of the tube and grinding teeth it is unable to dissociate tissue samples smaller than 3 mm^3 . The protocol used for this device can be found Appendix B.

To solve the issues of other dissociation devices, researchers have started creating their own microfluidic dissociation devices. The idea behind these microfluidic devices is to push a small amount of solution and tissue through a small space which creates shear force on the tissue which should take off individual cells.

The first microfluidic device examined for this project was created to dissociate tumor cell aggregates. Its novel design utilized a series of channels that halved in size until the smallest channel was $125 \text{ }\mu\text{m}$ in width. The walls of the channels have smooth constrictions that reduce liquid vortices that may trap cells (Figure 3). The device uses pressurized air to force tissue samples that are in solution through channels which causes gradients of velocity to form that produce shear forces strong enough to break apart cell aggregates. This device has been shown to work for tumor cells and cell aggregates [6]. This design still utilizes enzymes to help loosen cells, but once they are put in the device they are subject to maximum shear forces of 9 dyne/cm^2 found in the smallest channels. They found that the best method was to run their cells through the device 10 times in order to obtain the most individual cells. To create this product they used laser cutting to create seven layers and glued them together.

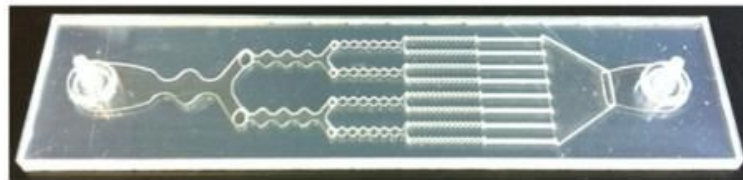


Figure 3. Microfluidic device for dissociation of tumor aggregates.

Another novel microfluidic device is shown below and was used to dissociate brain tissue into single neurons [7]. Once again, this device was created to surpass the uncertainty and non-standard protocols of pipetting and mixing that were used previously. Their solution was to create a device with a single small channel in the center (Figure 4). The researchers used two syringe pumps that were programmed to create an oscillating fluid flow that forced the tissue back and forth through the device to induce shear stress and force cells to enter this small channel. It successfully dissociated neurons. The device was fabricated using PDMS and pressed between pieces of acrylic to seal.

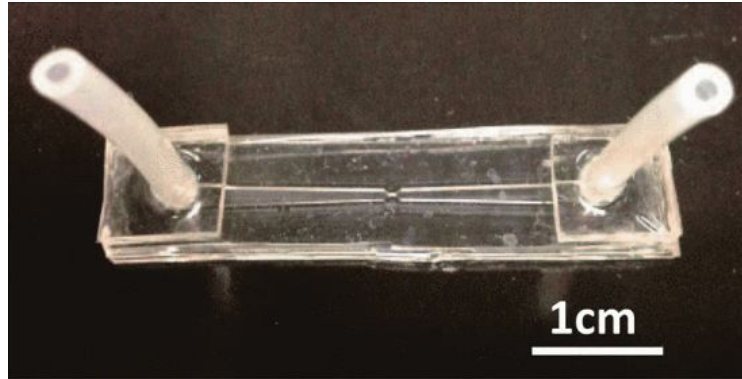


Figure 4. Microfluidic device for dissociation of neurons.

Problem Statement

Dr. Mathur's asthma research requires biopsies of lung tissue before and after an induced asthmatic reaction. The tissue needs to be dissociated so that changes in the cells can be studied using flow cytometry. The current method of dissociation requires a tissue sample size of 3-4 mm³ but the use of a smaller tissue sample size, 1-2 mm³, is desired. This smaller tissue sample is unable to break down and dissociate with the current dissociation method. Therefore, the team is tasked with creating a dissociation device that will successfully dissociate a smaller tissue sample and yield at least 10,000 viable cells to study.

II. Background

Relevant Biology

The purpose of the device is to successfully dissociate lung biopsy tissues for asthma research. Though there are different causes of asthma, the client focuses on allergic asthma. Normally harmless airborne allergens trigger an inflammatory response in airways of the lungs, called bronchial tubes. This response is initiated when mast cells release large amounts of histamine to flood the area with extracellular fluid and to attract eosinophils and neutrophils to the affected site (Figure 5). The inflammatory response is amplified by helper T-cell lymphocytes [8].

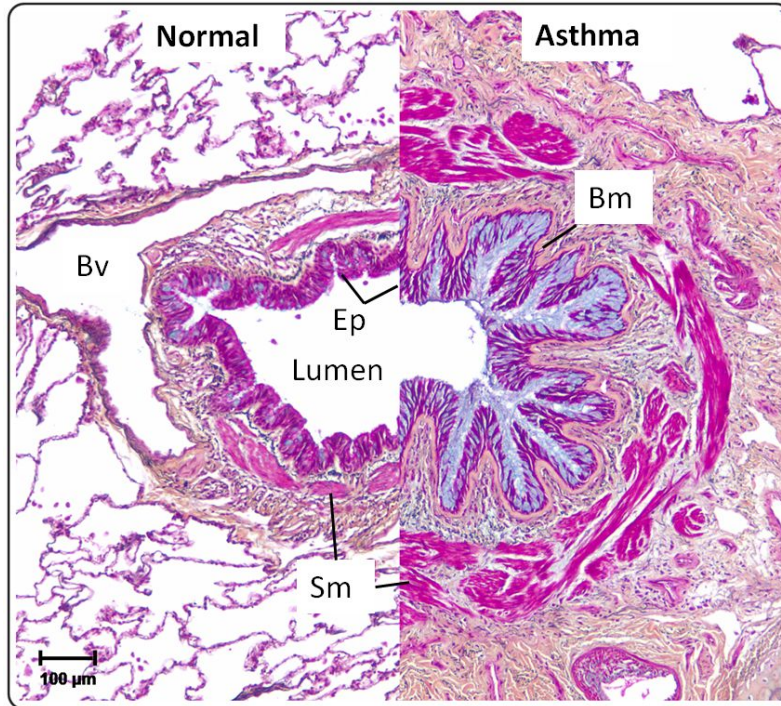


Figure 5. Asthmatic Bronchial Tissue. This image depicts the cellular level reaction of asthma in a bronchial tube cross section. In comparison to normal tissue, inflammation is clearly present in the asthmatic tissue.

To analyze changes in the lung tissue with the allergic response, a biopsy must be performed. There are several biopsy procedures to collect the tissue sample: open, needle, thoracoscopic, and transbronchial. The open biopsy is completed by making an incision in the chest to surgically remove tissue. Similarly, the needle process involves guiding a needle through the chest wall with a CT scan or fluoroscopy.

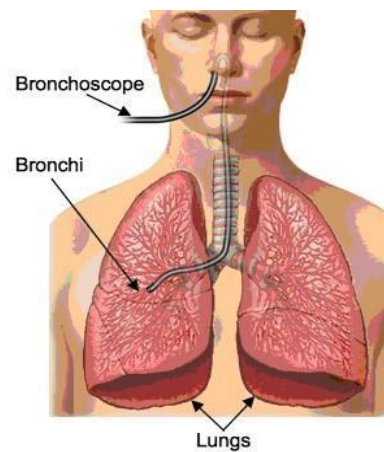


Figure 6. An example of bronchoscopy, a method used by the client to remove pieces of lung tissue for cellular analysis.

Thoracoscopic biopsies push an endoscope into the chest cavity, and then through the endoscope tools can be inserted to obtain tissue. Nodule removal or tissue lesion may also be performed. Lastly, the transbronchial biopsy, or bronchoscopy, guides a fiberoptic bronchoscope through the nose and into the bronchioles, where the device removes a 1-2 mm³ sample of tissue [9]. These techniques vary in invasiveness, with some requiring anesthesia. The lung biopsy procedure the client uses is the bronchoscopy (Figure 6).

Once the tissue sample is obtained, it must be dissociated into individual cells. Dissociation is the process by which single cells are liberated from a cell aggregate. To achieve this, the extracellular matrix (ECM) must be broken apart without lysing the cells themselves. Two main ways of dissociation include mechanically applying shear forces to the tissue and enzymatically breaking down the extracellular matrix. Unfortunately, many methods of mechanical and chemical dissociation disturb surface markers, nullifying data received from flow cytometry.

Flow cytometry is a method for analyzing the expression of molecules on the cell membrane and within the cell. These cells are fluorescently tagged for specific proteins and ligands on their surface using immunocytochemistry. This fluorescent intensity is then measured by the cytometer (Figure 7). The device has lasers that focus on single stained cells at a time and measure the light scattered and fluorescence emitted [10]. One particular measurement the client desires is the ability to analyze is the activity of eosinophils. Eosinophils are a type of white blood cell, and normally account for only 5% of all white blood cells. High eosinophil counts are related to asthma and allergies, and flow cytometry can detect the amount of these cells.

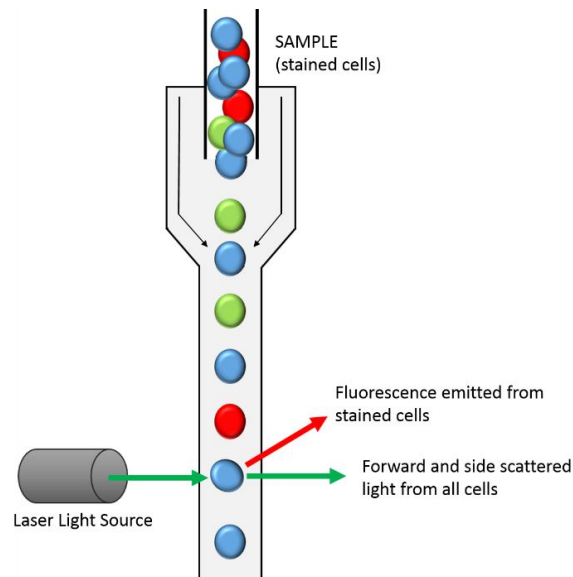


Figure 7. A flow cytometer measures the fluorescence of stained cells and sorts these cells based on fluorescent markers to allow for very specific measurement of the number and types of cells in a mixture.

When dissociating cells it is important to know the amount of cells expected to be in the tissue to know if the device is yielding the amount you want. There are some ways to test a device that will allow for the quantification of cells that make it through which is detailed later. Here we want to look at quantifying the amount of cells that can be obtained from lung tissue biopsies. Since testing is performed on both murine and human models, we look at both counting mouse and human cells in lung tissues. One study conducted found that the average lung volume was 8.6 cm³ with 86.8% of it being alveolar region and 7.25 x 10⁸ number of cells in that region [11]. Back calculating, this means there are 9.72 x 10⁷ cells per 1 cm³ of tissue. Our tissue is approximately 2 mm³, which means there are approximately 200,000 cells in a mouse sample of that size. This cell count is composed of all types of cells. A similar study looked at the cell count in a human lung [12]. It was found that there are on average 230 x 10⁹ cells per 4,341 mL of lung tissue. This translates to 5.29 x 10⁷ cells per 1 cm³ of tissue. Once again, assuming our sample size is 2 mm³, there are approximately 100,000 cells.

Client Information

The client, Dr. Sameer Mathur, is the director of Allergy and Immunology Clinics and the Chief of Allergy at the VA Hospital. He has interests in eosinophil immunoregulatory activity, and performs research on asthma, comparing biopsies before and after an induced reaction.

Product Design Specifications

The main specification for this project is the development of a device to successfully dissociate lung biopsy tissue samples which are 1-2 mm in size. There must be a minimum of 10,000 cells recovered with the cells being ideally white blood cells. Since the dissociated tissue will eventually be run through a flow cytometer for analysis, there should be no disruption to cell characteristics such as eosinophils. The device's cost should not exceed approximately \$10 per use, and if it is reusable, the material must be able to withstand sterilization procedures, either ethanol or autoclaving. A more complete list of design specifications can be found in Appendix A.

Previous Work

Last semester three members of the current design team worked to create a device that would dissociate cells from a lung biopsy sample. Three different design ideas were evaluated including a modification of the Miltenyi gentleMACS to fit smaller tissues, a microfluidic device with decreasing sized channels, and screw device that would use mechanical degradation to break apart the tissue. After evaluation, the team decided to pursue the microfluidic design. The design consisted of a single layer with channels that branched and got smaller to a final width of 0.6 mm. The team fabricated the device using the SLA 3D printers at the Makerspace. Four

rounds of testing were conducted with the device but there were some difficulties with the flow rate and with the device not being sealed properly. The team was unable to get a tight seal with using a acrylic top and rubber o-ring last semester which resulted in leaking of the fluid and an inconsistent flow rate. The peristaltic pump that was used last semester to drive the sample through the device was unable to achieve the flow rate that the team had predicted was needed based on flow analysis. Due to these problems the device failed and the tissue did not pass through the channels resulting in minimal cell recovery. The team believed that the design would work based on flow calculations if the problems were addressed.

III. Preliminary Designs - Fabrication Methods

Since the proposed final design is very similar to the design of the previous semester, the team determined that an evaluation of various fabrication methods to improve usability and fluid flow would be helpful. The team researched several possible fabrication methods for creating smaller channel sizes for our device. The fabrication methods were narrowed down to four options: 3D printing, PDMS photolithography, laser cutting, and micromilling.

Fabrication Method 1 - 3D Printing

3D printing is a process that makes solid three dimensional objects from a converted digital file using different plastics. The process involves creating the general base outline of the intended object and adding layer by layer along the z-axis. The device fabricated last semester was made using an Ultimaker 3 at the UW-Madison Makerspace. This type of printer uses fused deposition modelling. This technique uses thermoplastics that are melted and extruded through the nozzle onto the stage in layers. This option was utilized last semester because it was cheap, quick, and available, but it had some drawbacks. Specifically, this printer has layer thickness of 250 microns. It also had support material in the channels which was hard to remove.

This semester, the team considered stereolithography (SLA) printing in order to achieve a better resolution for the design. SLA works by taking layers of photocurable liquid resin which are crosslinked using a UV laser [13]. Once this is complete, the product must cure, meaning it must be exposed to heat and UV light. On the UW-Madison campus, the best SLA printer available is the Viper 3D printer at the Morgridge Fab Lab (Figure 8). This printer can print layers as small as 20 microns. Robert Swader, a mechanical engineering within the Fab Lab, was consulted about using this method of 3D printing. He made a point that the materials used in SLA printing have cytotoxic effects due to the presence of methacrylate and acrylate, but very little is known about these effects [14]. He mentioned that researchers have tried curing their devices in an inert environment with some, but not measured, success. Some studies have been conducted with SLA materials. One study used DSM Watershed and found that if treated with ethanol after curing, zebrafish embryos could be cultured [15]. Another study cultured Chinese

hamster ovary cells in a SLA printed device and found nearly identical growth curves when compared with regular tissue culture materials [16]. Further research must be done on the specific materials used on the Viper 3D printer and how they can be cured and treated to ensure biocompatibility.



Figure 8. Viper 3D printer at Morgridge Fabrication Lab

Fabrication Method 2 - PDMS Photolithography

Polydimethylsiloxane, or PDMS, is an elastomeric polymer often used for fabrication of microfluidic devices. PDMS devices are fabricated using a very precise mold, and they are able to replicate features down to the nanoscale [17]. One of the most common methods to create a mold is via photolithography. This method involves the creation of a master, which is essentially the negative of the desired features within the PDMS device. Masters can be created from SU8, which is an epoxy based negative resist. This viscous material is applied to a silicon wafer and the desired thickness is attained by spinning the wafer at a particular RPM. The wafer is then treated with heat, covered with the desired template, and then crosslinked with UV light. Excess SU8 is washed away with a developing solution, leaving the master with the crosslinked features.

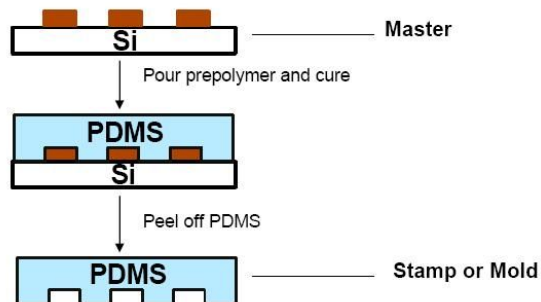


Figure 9. General process for soft photolithography.

In order to fabricate the PDMS microfluidic device, liquid PDMS and a curing agent are mixed together. The mixture is placed in a vacuum to eliminate all bubbles before pouring onto the master. This pour occurs on a hot plate and is left to heat for approximately 4 hours. Once the PDMS has fully cured, the microfluidic device is able to be peeled off of the master and is ready for use. This process is illustrated above in Figure 9.

The team considered PDMS photolithography for a variety of reasons. This method is very cost efficient and would allow us to stay within the goal of \$10 or less per use. It is also well-known that PDMS is a biocompatible material, which is an important aspect in our design. The team has worked with this fabrication method before and the materials are readily available in the BME tissue lab. However, the drawback of this fabrication method is that the device would not be able to get as thick as the team desires. In order to get to the desired thickness, an alternative type of mold would need to be used. Micromilling is most often used to create thicker molds, which is in itself a fabrication method the team is considering.

Fabrication Method 3 - Laser Cutting

Laser cutting is a process that uses a high powered precise laser to cut a design from a vector file into the material by melting, burning, or vaporizing. Laser cutting requires a very good exhaust ventilation system in order for many materials to be used due to the fumes that are created during the cutting process. The laser width directly correlates with the accuracy of the device. The laser cutter that is available to the team for fabrication is the ILS9.150D which has a 10.6u C02 laser that operates at up to 150 watts (Figure 10). The device would be fabricated using many laser cut layers glued together. Unfortunately, the laser printer available at the UW-Madison Makerspace can only be used with organic materials, so the plastics we would like to use may not be available for us to cut.



Figure 10. ILS9.150D laser cutter

Fabrication Method 4 - Micromilling

Milling is the process of using a rotating tool in a movable head to remove material from a product. It is a widely-used machining process and can be scaled down to the micro scale for use on biomedical research applications (Figure 11). It can be used by designing a 3D model on a CAD program and the translated into computer numerical controlled (CNC) tooling paths that automatically machine the piece into the material of choice. Micromilling uses end mill tooling that can reach sizes of less than 50 microns. This poses problems since machining exerts bending forces on these small bits and causes them to break at very small forces, so machining at any depth we would be limited to wider tooling or risk of frequent breakage.

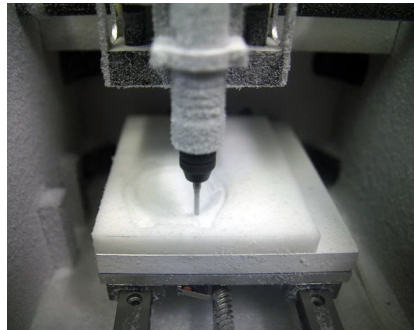


Figure 11. Micromill with small end mill.

VI. Preliminary Design Evaluation

Design Matrix

The team created a design matrix comparing the four fabrication methods with the following criteria: accuracy, materials, ease of fabrication, and cost (Table 1). Accuracy was defined as the ability of the fabrication method to produce a device with the desired geometric properties and resolution. This criteria also takes into account the ability of the device to successfully apply shear forces to the tissue. Accuracy was weighted the highest (35/100) because the device must be able to function and dissociate tissue or the device is essentially ineffective.

The materials category was defined as the compatibility of the fabrication method with certain materials of interest. Some of the fabrication methods required specific materials which were not autoclavable or may be cytotoxic. Therefore, this category was rated highly (30/100) to determine which fabrication method allows the use of the best material for this project (ideally non-cytotoxic, autoclavable, non-degradable, etc.)

Ease of fabrication was defined as the general effort that would be needed for the team to produce the prototype device (time, skill, safety, etc.). This category was rated third highest (20/100) because if the previous two criteria are not met then the device is not worth fabricating

at all. However, ease of fabrication is very important because testing should begin as soon as possible, and if it takes half of the semester to make the device, then little time is left to prove its efficacy. Also, once the team is done with producing a prototype device, the client would ideally be able to create further devices independently if given proper direction.

The final category, cost, was defined as the total cost of fabrication method, including various materials (device material, end mill bits, Makerspace fees, Morgridge Fab Lab fees, etc.). The cost of the fabrication itself is not of utmost concern of the team, therefore it was given a lower rating (15/100). Because the final device needs to cost less than \$10 per use, materials must be chosen carefully.

Table 1. Preliminary Design Matrix - Fabrication Methods. The light green shading indicates the fabrication method was the highest score for each criterion. The dark green shading indicates the highest overall score comparing the four different methods.

Criteria	3D Printing	PDMS Photolithography	Laser Cutting	Micromilling
Accuracy (35)	28 (4/5)	21 (3/5)	21 (3/5)	35 (5/5)
Materials (30)	18 (3/5)	30 (5/5)	24 (4/5)	30 (5/5)
Ease of Fabrication (20)	20 (5/5)	12 (3/5)	12 (3/5)	8 (2/5)
Cost (15)	12 (4/5)	12 (4/5)	15 (5/5)	3 (1/5)
Total (100)	78	75	72	76

Accuracy: Micromilling was rated highest because the bits can get extremely small and thus, practically any size channel could theoretically be fabricated. 3D printing was ranked next because it has a resolution of about 150 um. Laser cutting was rated 3/5 because the nozzle is cone shaped, so the channels would be wider on the top than the bottom. And finally, PDMS was also rated 3/5 because it wouldn't be easy (or possible) to make the master as thick as is needed (0.5 mm) to fit the tissue samples.

Materials: Micromilling also scored the highest in this category because nearly any material can be micromilled. PDMS scored full points in this category as well because PDMS is a known

biocompatible material that shouldn't disrupt the cell markers in any way. Laser cutting and 3D printing scored lower because materials are more limited, and in the case of 3D printing, the biocompatibility of the material has not been fully investigated.

Ease of Fabrication: 3D printing scored the highest in this category because the team is most familiar with this method and it would be the fastest. The other designs scored lower because they would take more work and time (especially micromilling).

Cost: Laser cutting scored the highest category because essentially the only cost would be the raw material. PDMS and 3D printing scored equally because they are fairly cheap. Micromilling scored the lowest because many expensive bits will be needed to produce the design.

While all three fabrication methods are fairly close in terms of how they score, 3D printing is the best option for this semester. It scored highly in our two most important categories, accuracy and materials, and the team is most familiar with this method. 3D printing will allow the team to spend more time on testing for a proof of concept, rather than learning a new fabrication method. While it did not receive perfect scores in the two most important categories, the accuracy from 3D printing should be sufficient to make a proof of concept prototype. Secondly, it was rated 3/5 on materials because the materials that can be printed have not been thoroughly investigated for their cytotoxicity. However, the tissue sample will not be exposed to the material for very long, and cytotoxicity testing will be implemented by 3D printing a culture shaped dish with the material and culturing epithelial cells and eosinophils in order to establish if there are any detrimental effects on the cells.

Proposed Final Design

The proposed final design is based off of the design last semester, but is mirrored at the narrow channels so there can be flow alternating back and forth within the device, and has another set of narrower channels (Figure 12). This alternating flow will be accomplished by using a programmable peristaltic pump (Figure 12). One of the reasons the design didn't work last semester was due to issues with keeping the device watertight. This semester, a rubber gasket will be used, as well as a thicker acrylic cover to allow for more uniform sealing.

The peristaltic pump will be programmed and controlled via an Arduino (see Appendix C for code and circuit set up). In brief, the arduino was connected to an RJ-45 (ethernet) adaptor and plugged into the pump. Simple text commands are sent to the pump with a delay, and the pump will respond to those commands. While the pump itself has been tested for pure operation, it has not been tested with any fluid or with the device itself.

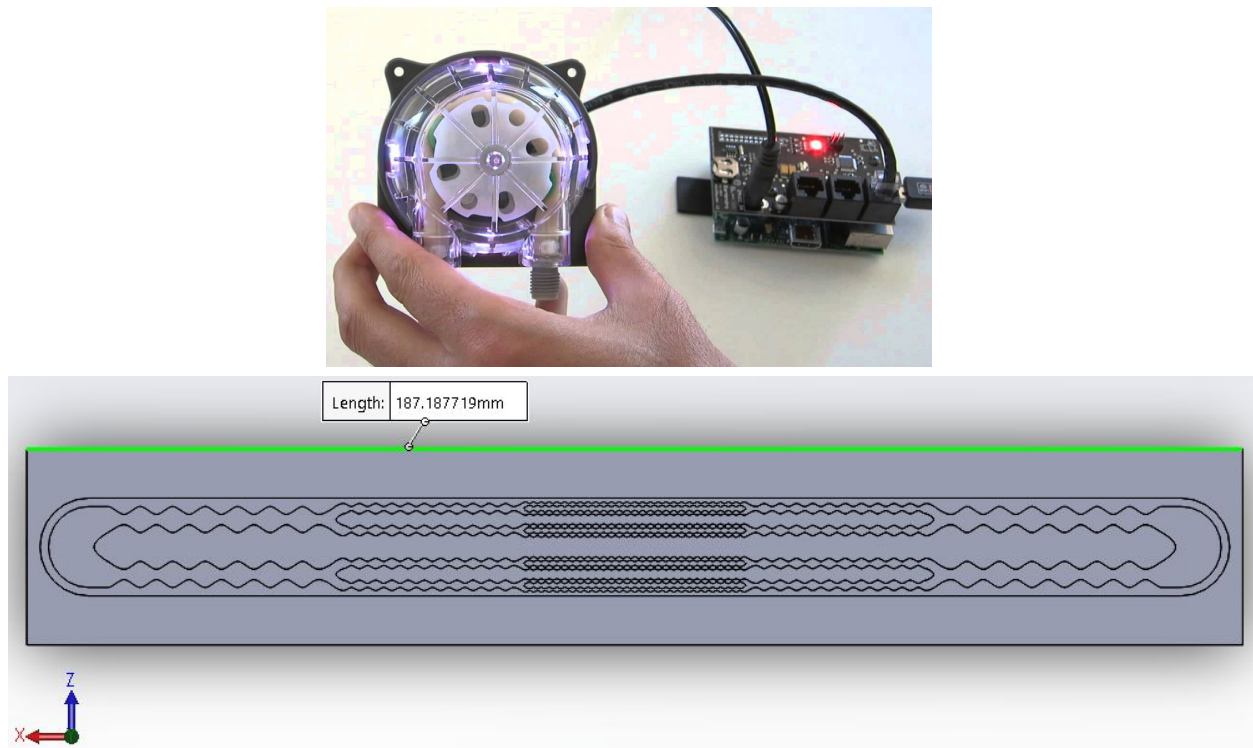


Figure 12: (Top) Programmable peristaltic pump connected to the suppliers routerboard (not used), hand for scale. (Bottom) Proposed final design with length shown. The channel minimum thicknesses from left to right are 1.6 mm, 0.64 mm, and 0.3 mm in the middle, and then mirrored about the center. The long pill shaped region surrounding the channels is an inlay for a rubber gasket.

V. Fabrication

Materials

The final decision for the fabrication process was 3D printing using the SLA printer at the Morgridge Fab Lab. The material that will be used to print our design will be Accura 60 from 3D Systems. This is because there are only two material options available at the Fab Lab and our consult suggested the use of Accura 60 as the less cytotoxic material. We would be printing both our device and a 24 well plate for testing cell viability. The estimated cost of the device was about \$40 while the plate would probably cost about the same for a total of \$80-\$100.

Our design also incorporates the use of a silicon gasket that will be laser cut to the precise size of our 3D print. The estimated cost of the silicon is about \$5 since use of the machine will be free. Other materials will include an acrylic cover piece and set screws or clamps, all of which can be obtained for free at the UW-Madison TEAM Lab.

Methods

The final design was created using Solidworks. This design will then be sent to the Morgridge Fab Lab to be printed on their 3D Systems Viper SLA printer. It will then be cured according to their protocol. The silicon gasket will also be cut at the Fab Lab to fit the dimensions of the CAD model. Once the device is done curing it will be cured again in a nitrogen environment under UV light to remove some of the harmful effects of the photopolymer resins. A group of researchers coated their device using 99% ethanol since many of the cytotoxic effects are due to aqueous components in the material and using an organic solvent helped decrease those effects [12]. Therefore, we will be coating our product with 99% ethanol before use in testing. The silicon gasket will be placed on top of the device and sealed using an acrylic plate and set screws.

VI. Testing

After printing the proposed final design and securing the lid, the device will be tested with approximately 100,000 human epithelial cells that will be counted before and after being sent through the device. This cell number is based off of the approximate tissue size. This will allow a baseline retention rate to be set for the amount of cells that are able to pass completely through the device during a run. This test will be conducted once in our old device and three times in our new device.

At least 10 different tissue biopsy samples will be used for testing from either mouse or human lung tissue, dependent on the availability, through the device. Ideally testing will be performed with 6 frozen mouse samples, 6 fresh mouse samples, and 6 human samples which will allow samples to be run in the device as well as compare with other methods as a control. Afterwards, microscopy will be used to determine the final yield of viable cells. This final count can then be compared with the expected number of cells in the tissue and the retention rate of loose cells in the device.

The same general lab procedure that our client has been using for their tissue dissociation will be used throughout testing (see Appendix B) with modifications for specific tests. An example of the experimental set up can be seen in Figure 13. Samples will be incubated in the collagenase solution provided by Miltenyi for 30 minutes at 37°C. It will then be passed through the device using 20 ml of PBS buffer.

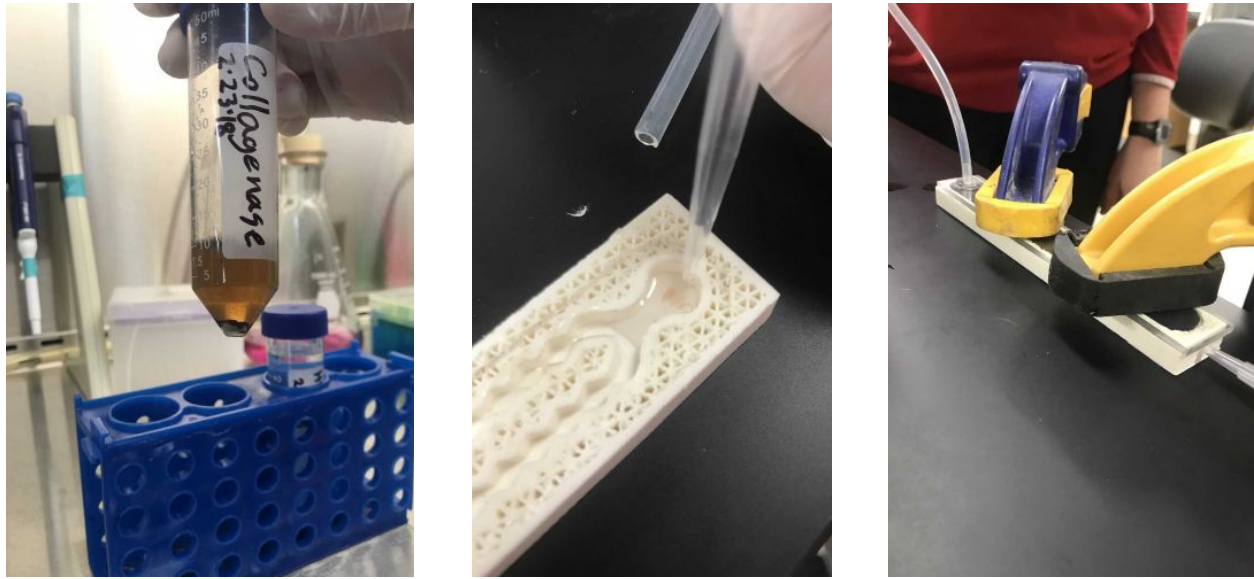


Figure 13. The basic testing steps for running a tissue through the dissociation device. **(Left)** Placing the tissue in collagenase and incubating **(Middle)**, placing the tissue in the device **(Right)**, clamping on the gasket and using the peristaltic pump to push fluid through the system

The first round of testing will use two frozen mouse samples. One of the samples will be exposed to sonication during the collagenase incubation step and then run through the device. This technique will then be compared with just running the tissue through the device. The sonication process has been used in other studies to loosen cells from the ECM to obtain better dissociated tissue samples [18]. For this device it will be used as a part of the process so cells can more easily be removed from the tissue in the device. The amount of time and the intensity of the sonication will be determined based on three rounds of preliminary testing with this process measuring cell counts and cell viability after each round. If the sonication process proves to improve the cell yield rate then it will be incorporated into the final procedure.

To judge the success of our device we will be using the Miltenyi gentleMACS as a control using three different samples to get an average value of cell yield. This will be compared to the average cell yield obtained with the microfluidic device using a paired t-test to determine significance. Significance will be assessed as $p < 0.05$. Success will first be evaluated as yielding more cells than the Miltenyi gentleMACS but will ultimately be successful if the device can yield the 10,000 viable cells as defined in the PDS.

Another set of tests will be to assess the biocompatibility of the 3D printed material. A sample 24 well plate will be 3D printed using the same material as our device. 9 wells will be cured using the nitrogen atmosphere and coated with ethanol while 9 wells will be cured only using the Fab Lab method. This will be compared with 9 well of cells cultured on regular tissue culture polystyrene. For each condition cells will be counted and viability assessed at 3 different time points. These time points will be 10 minutes, 1 hour, and 24 hours. If cell viability is

maintained at all three time points we can assume that the any cytotoxic effects due to the material of our device will not affect the viability of the lung tissue for dissociation.

VII. Discussion of Future Work

The results of testing in the previous semester and preliminary testing this semester revealed that more work needs to be done to make the device as effective at tissue dissociation as possible. One of the main problems that the team has faced both this semester and last semester was leakage of fluid out of the device. The team has brainstormed several possible improvements/solutions to confront this problem including: utilizing a water-tight seal with a rubber gasket, combining a top and bottom device components in a “lego-like” tight seal, or fabricating the entire device as one component.

The water-tight seal idea stems from the rubber gasket used in the preliminary testing. Even with significant pressure applied to the lid and gasket, leakage was still apparent. Some possible ways that the team could improve this method: fabricate a lid that minimally deforms under a load, fabricate and apply a rubber gasket that is perfectly fitted to the device, and research other possible methods of sealing the device.

The “lego-like” tight seal idea would incorporate two equally-sized components with protruding pieces to allow the pieces to be combined. If enough pressure could be applied to the components, and if the two components are fabricated accurately with minimal warping, then this design idea could prove functional.

Finally, fabricating the device as one single component would very quickly simplify and localize the leakage to only the input and output. However, if the device is a single component, then it is impossible to visualize what is occurring within the device. It also prevents the removal of tissue from within the device. This may pose a significant problem for determining whether a piece of tissue is stuck within the device, and may make the cleaning/autoclaving of the device more difficult.

There are a few other areas of future work that the team is hoping to address this semester including: identifying the ideal channel length and size through research, testing, and in silico modeling, and determining an effective protocol for making sure we can accurately count the individual live cells obtained as a result of the dissociation protocol.

The team has identified channel lengths and sizes for our device fairly arbitrarily. The team is currently discussing what the smallest channel width should be. One argument is that the smallest channel should be as small as we can precisely 3D-print, because these small channels would only allow the smallest cells/pieces of tissue to pass. However, since the large pieces of tissue would then be unable to pass through these small channels, the tissue could become easily stuck. If the larger pieces of tissue become stuck then the shear forces upon that tissue from the moving fluid are minimized. Therefore, a second argument is to have the smallest channels

slightly larger than the smallest possible tissue size. This would allow for sufficient shear stresses on the large pieces of tissue while also promoting the separation of the tissue into smaller pieces. The team will finalize these dimensions in the next week prior to the fabrication of our final device.

Another future goal to work on either this semester or in future semesters is to create and test a protocol for counting the live cells obtained from the device. The team did some basic cell viability testing with Trypan Blue last semester, however very few cells were obtained so the counting was done by manually counting cells on individual slides. The team hopes to obtain enough individual cells to stain with Trypan Blue and utilize a digital cell counter to obtain larger sample sizes and more precise results.

Another note on future work: the team has not been able to find sufficient literature on the cytotoxicity of the 3D-printed materials which may be used to fabricate the device. For the current dissociation protocol the tissue is intended to be in contact with the device for a maximum of 10-15 minutes. Since this is such a brief time, the effects of the material on the cells is expected to be minimal. In the future, more work should be done to analyze the effects of the material and perhaps improve the biocompatibility of the material with a surface coating or inert atmosphere curing after fabrication.

Finally, the team hopes to identify a suitable company for professional fabrication of the device so there is more control over material choice.

VIII. Conclusions

The use of tissue biopsies is an important aspect in the field of asthma research. Tissue dissociation is used to analyze, via flow cytometry, the cellular makeup of the tissues. The client, Dr. Sameer Mathur, supplied the team with the task to create a device that would allow his lab to dissociate tissues of 1-2 mm³ instead of the standard 3-4 mm³. After analysis of design specifications the team was able to develop a microfluidic design to fit the criteria and choose the method of 3D printing as the most suitable means of fabrication. The microfluidic device utilizes a set of diminishing channels as well as pressurized air to force tissue samples that are in solution through channels with smooth constrictions. The team believes that this device will allow the client to achieve his 10,000 viable cell count based on results seen in a similar study. The team will move forward with the fabrication of the microfluidic design and will start testing and optimizing the process to meet the client's specifications. If this device can help dissociate lung tissue, it will greatly improve the research results obtained from this asthma study. Hopefully the outcomes will help our client better understand the asthmatic response and use that knowledge to help individuals all over the world breathe better.

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X. Appendix

Appendix A: Product Design Specifications

Microscale Tissue Biopsy Dissociation Device Product Design Specifications 2018/3/7

Raven Brenneke, Nathan Richman, Lauren Ross, Thomas Guerin, Chrissy Kujawa

Function: To dissociate cells from small (1-2 mm³) lung biopsy samples. The design must produce a measurable amount of viable cells for flow cytometry (approximately 10,000 white blood cells).

Client Requirements:

- Dissociate cells from lung biopsy samples retrieved from asthma patients during the duration of the asthma research study.
- Must be able to recover cells with minimal disruption to surface markers, so that the cells can be analyzed via flow cytometry.

Design Requirements:

1. Physical and Operational Characteristics

a. Performance Requirements: The device should successfully dissociate tissue samples to obtain at least 10,000 cells, ideally 10,000 white blood cells. The device will be used daily by lab technicians using sterile techniques to load tissue and unload cells.

b. Safety: The device must be sterile and protect the lab tech from possible contamination due to the use of human tissue samples. The device should also be able to withstand spills and drops without shattering or breaking into sharp shards.

c. Accuracy and Reliability: The device must yield at least 10,000 cells from the sample of tissue. It should completely dissociate the tissue samples without disrupting cell markers and not resulting in cell lysis.

d. *Life in Service*: Life in service will depend on whether or not the device is reusable. If it is reusable it needs to last enough runs so that the cost per use is less than \$10. If non-reusable, it would only need to last for a single tissue dissociation.

e. *Shelf Life*: While not in use the device should have a shelf-life of at least 5 years in case the client's study ends and starts up later.

f. *Operating Environment*: The device will be used in a laboratory setting. During use, the device will be exposed to various enzyme-containing solutions including collagenase G, sterilization agents, and possibly high temperatures and pressures present in an autoclave (if device is reusable, it should withstand temperatures of -20 to 130 °C).

g. *Ergonomics*: The device must be simple for lab technicians to control. This includes being able to easily load a sample into the microfluidic device and unload the output from it.

h. *Size*: The device should be capable of dissociating a tissue sample size of 1-2 mm³. The device should be able to fit on a lab bench, but otherwise, the size of the device is not of huge concern as long as it is able to perform the task successfully.

i. *Weight*: The weight of the device is currently not applicable to the design criteria given by the client's wishes. The microfluidic device is small enough that weight will not be a factor in its utility.

j. *Materials*: The material for the device must be cheap enough to obtain the goal of the cost per run being less than \$10. The materials used will depend on the final fabrication method chosen. The material will need to not induce any inflammatory reaction with the cells. The current material used is PLA and ABS.

k. *Aesthetics, Appearance, and Finish*: The device must be simple and not confusing to use. The specific aesthetics and appearance of the final product is not of large concern as long as the device functions properly.

2. Production Characteristics

a. *Quantity*: The client initially requested one device to be manufactured for use, but an additional device may be requested later on.

b. *Target Product Cost*: The budget for this project is \$300 dollars. The cost of fabrication of the device will be determined at later time depending on the type of material, volume of material, and fabrication technique selected. The existing device is non-reusable and costs roughly \$10 per

cap with the tubes accompanying the device costing \$6 per tube¹. The target cost of the microfluidic device is \$5-\$10 per use.

3. Miscellaneous

a. *Standards and Specifications*: This is a custom device being used in a research setting; there are no international or national standards to abide by.

b. *Customer*: The client would prefer to have a removable lid on the device in order to remove potentially valuable tissue samples if the device does not run correctly.

c. *Patient-related concerns*: Patients will not be using this device; it will be used in a research setting. There is no storage of patient data incorporated in this device and the devices should be sterile with every use.

d. *Competition*: A current device for tissue dissociation is made by Miltenyi that includes a tube cap with an attached grinding component that is compatible with a machine, gentleMACS™, that initiates the grinding of the tissue. This device is currently used by the client, but since their tissue sample size is very small it is unable to be properly dissociated by the gentleMACS [3].

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Appendix B. Testing Protocols

Microfluidic Device Protocol- from last semester

- The tissue sample was about 1.5 inches in diameter (this sample was VERY large compared to what they usually get for this study)
- Took 4 smaller biopsies with a biopsy tool
- Two samples were placed in a 50 ml conical tube containing only 1x PBS solution
- Two samples were placed in a 50 ml conical tube containing 1x PBS, 20x Buffer S, Enzyme A, Enzyme D
20x Buffer S, Enzyme A, Enzyme D are all from the dissociation kit that came with the GentleMACS device
- Samples were then placed in the incubator for 30 minutes on a gentle rocking device
- Samples were then sucked from conical tube using tubing connected to peristaltic pump
- The tubing from the peristaltic pump was connected to an adapting connector that feeds into another tube
- The tube coming from the adapter is connected to the adapter on the device
- The dissociation process is finished when all material and fluids are collected in the retrieving well on the far end of the device.
- It is optional to run the solution through the device multiple times for further dissociation
- The solution was then transferred to a conical tube.
- Conical tubes were vortexed for 5 seconds
- Solution was filtered using a 50 micron filter
- Tubes were centrifuged at room temp, 1300 rpm for 10 minutes
- Supernatant was taken off, and cell solution at the bottom of the tube was re-suspended in PBS and loaded into cytospin device
- Centripetal force from that device forced cells onto glass slide
- Slide was stained with HEMA 3

Original protocol for the Miltenyi gentleMACS device

- The tissue sample was about 1.5 inches in diameter (this sample was VERY large compared to what they usually get for this study)
- Took 4 smaller biopsies with a biopsy tool
- Two samples were placed in a 50 ml conical tube containing only 1x PBS solution
- Two samples were placed in a 50 ml conical tube containing 1x PBS, 20x Buffer S, Enzyme A, Enzyme D
 - 20x Buffer S, Enzyme A, Enzyme D are all from the dissociation kit that came with the GentleMACS device
- Samples were then placed in the incubator for 30 minutes on a gentle rocking device
- Conical tubes were vortexed for 5 seconds
- Solution was filtered using a 50 micron filter
- Tubes were centrifuged at room temp, 1300 rpm for 10 minutes
- Supernatant was taken off, and cell solution at the bottom of the tube was resuspended in PBS and loaded into cytospin device
- Centripetal force from that device forced cells onto glass slide
- Slide was stained with HEMA 3

Appendix C: Programmable Peristaltic Pump

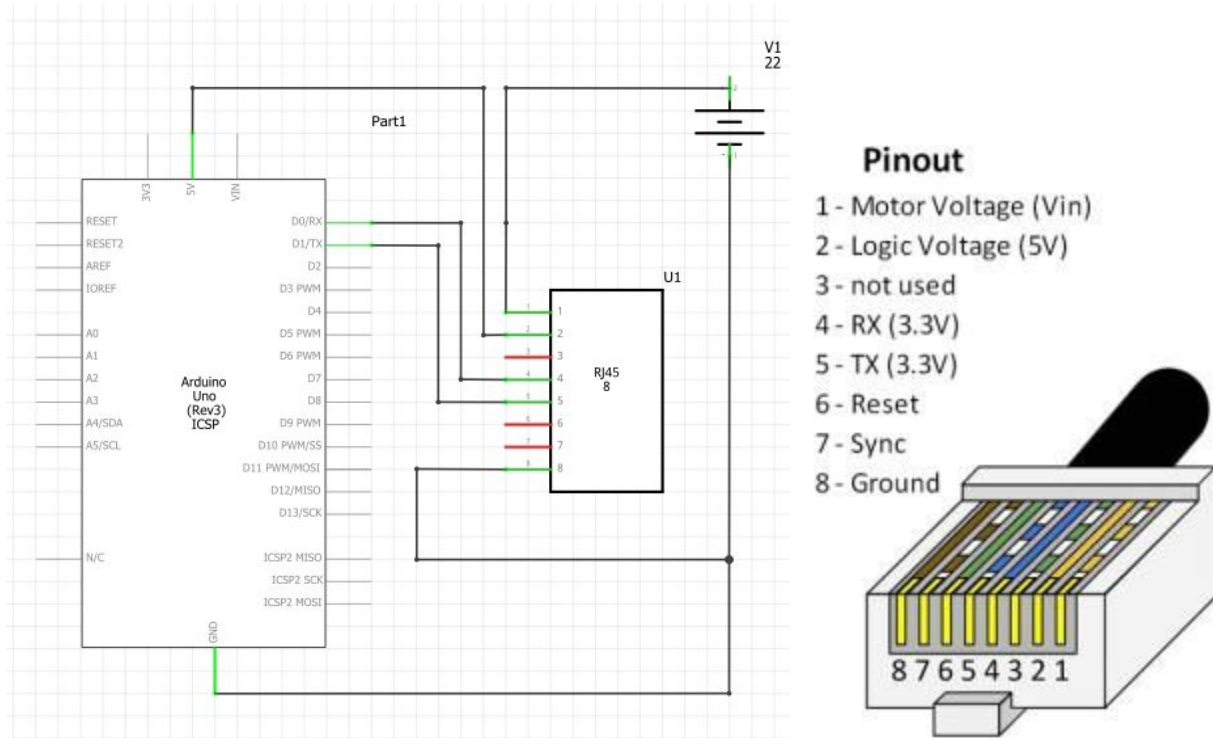


Figure 1: Circuit diagram for Arduino connected to pump. Arduino is connected to the RJ-45 adaptor, the RX and TX pins are connected to their respective pin on the Arduino, the logic voltage is connected, as well as the external motor voltage (22V), all is then grounded to that voltage source.

Initial arduino code to control pump:

```
#include <SoftwareSerial.h>
```

```
SoftwareSerial mySerial(0,1); //RX, TX
```

```
void setup() {
  pinMode(0,INPUT); //Set input and output modes
  pinMode(1,OUTPUT);
  mySerial.begin(9600);
  delay(100); //Delay after setting up serial
}
```

```
void loop() {
```

```
  mySerial.println("!!!"); //Send start signal to pump (from datasheet)
  delay(1000); //Todo: find a way to only send start signal once
  mySerial.write("speed 100 1\n\r"); //Send signal to start, delay, then tell pump forward
```

```
delay(50); //Might need to switch order around, i.e. forward first
mySerial.write("forward\n\r"); //then speed
delay(50);
mySerial.write("timedisp 1000\n\r"); //Tell it to run for 4 seconds, then tell it to run
delay(4000); //backward for 4 seconds
mySerial.write("backward\n\r");
delay(50);
mySerial.write("timedisp 1000\n\r");
delay(4000);
}
```