Automation of Full Organ Immunolabeling Protocol for Light Sheet Fluorescence Imaging

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***Abstract*—Visualization of the molecular and morphological complexity of organs can be achieved through advanced imaging techniques. Imaging has increased our understanding of disease, pathology, and mechanisms. Classic 2D-imaging modalities provide detailed information at a microscopic scale and while valuable, it lacks the ability to explore the spatial resolution of disease mechanisms such as the location of rare cell populations. Light sheet microscopy surpasses this limitation and allows for 3D imaging of organs. The sample processing and staining procedures can be lengthy and demanding. This project has automated these protocols in order to reduce human errors. The machine requires materials which are chemically compatible with the demanding protocols and can withstand mechanical stress for prolonged periods. The protocols also include elements of heating as well as cooling, while keeping a constant pressure and flow into the organs. A user interface has been designed to facilitate interaction with the machine with the goal to further develop and improve said protocols. The machine has successfully run a full protocol using coloured water. While this was without temperature control, it shows that this prototype works.**

I. Introduction

ANIMAL models are important in studying disease [1]. There is a vast number of reasons for examining parts of, or whole animals. Visualization of morphologies and specific molecules in these animal models provide important insights into disease mechanisms and phenotypes. 2D and 3D imaging techniques have a wide range of applications and require different approaches for sample processing. Histology, for example, is based on getting a two-dimensional view of a section generated by slicing the organ thinly [2]. It does however fail to show the complex three-dimensional structures. Certain hypotheses require the study of sparse structures within organs, and when slicing of the organ is performed it can be hard to find the structure and the examination may alter the structure. One way of reducing these limitations is to image the intact organ. This can be achieved by several microscopy methodologies such as confocal microscopy, MRI, CT, PET, and light sheet microscopy. The advantage of using light sheet microscopy is that it shines a sheet of light through the tissue, activating fluorophores. This enables the possibility of capturing light from the fluorophores perpendicular to the light sheet, causing the image to capture a slice of the organ. The light sheet can then be moved. This results in images of slices throughout the organ which can be projected in 3 dimensions to visualize the tissue being imaged. Using light sheets minimizes the time which the fluorophores are exposed to light and thereby reduces the photobleaching of the tissue. [3]

To achieve high quality images, the samples need a vast amount of preparation which makes them transparent. This process is called tissue clearing and requires time and manual labor which could lead to human error. Reducing this would improve the general quality of samples. This project aims at automating protocols for tissue clearing and immunolabeling, to produce samples for light sheet fluorescent imaging.

A. Tissue clearing and immunolabeling

Tissue clearing is the process of removing all undesired parts of the organ from an imaging perspective. These include all things that are not fixated cells or proteins and removal of these results in a transparent tissue. The protocols for tissue clearing can be either hydrophobic or hydrophilic. The tissue is naturally hydrophilic and the hydrophobic thus requires pre-treatment before the adding of hydrophobic chemicals [4]. An example of a hydrophobic method is immunolabeling enabled three-dimensional imaging of solvent-cleared organs (iDISCO [5]). iDISCO uses dichloromethane (DCM) to clear the tissue. DCM is hydrophobic, meaning that the environment it is used in also needs to be hydrophobic. This is achieved using methanol and phosphate buffer saline. After DCM is used the samples also go through a peroxide treatment using hydrogen peroxide. Lastly, the samples are rehydrated. After tissue clearing the organs are immunolabeled, which is the process of blocking antibodies from binding to undesired surfaces and then adding antibodies which allows them to bind to the desired proteins.

inFLATION (infusion of Flourescently Labelled Antibodies in Tissue of Intact OrgaNs) is an adaption of the iDISCO+ protocol which has been developed by the research team where this project is situated. The difference between inFLATION and iDISCO+ is that the chemicals are circulated through the organ instead of incubating the organ in them. This results in a more homogenous distribution of the chemicals and antibodies in the organ and therefore a better clearing and staining. Multiple variations of the protocol exist [6] such as iDISCO, iDISCO+, vDISCO, uDISCO, wildDISCO and 3DISCO [7]. They share a similar approach but have slight differences in the chemicals used, timings, and which tissue the protocol is adapted for. For example, the wildDISCO protocol is developed for whole mice while the rest are specified to organs.

There are other available methods for tissue clearing and staining. One of these is called CLARITY [8], which utilizes

Submitted June 9, 2024

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Swedish title: Automatisering av antikroppsfärgning av helt organ för light sheet flourescence imaging

electrophoresis in hydrogels. The method consists of embedding the organs in hydrogels and then applying an electric field over the hydrogel. This results in the undesired parts being dragged out of the organ and into the hydrogel. A drawback of CLARITY is that the electric current induces heat in the tissue which could potentially denature a number of proteins. Additionally it drags all the undesired parts in the same direction causing one side to be inferiorly cleared which impacts the result. One big advantage of using this method however is it being automated. This is desirable seeing as the process is 2-8 weeks long, depending on the organ, and has a multitude of steps.

To improve the efficiency of the iDISCO+ protocol, or more specifically, the inFLATION protocol, it needs to be automated. The protocol has 21 different steps with multiple sub steps and normally takes 2 weeks with ideal conditions, depending on the organ. Automation would additionally reduce the human errors associated with prolonged processes. The inFLATION protocol includes parts where the temperature and flow of the chemicals need to be controlled over a long period of time. There are many aspects that pose challenges to automation. It requires a system which removes the risk of pumping air bubbles into the organs, as this would cause uneven staining. An interface of the machine is required both for operator input and monitoring of the process. Since the automation should preferably be able to process more than one organ at a time, it needs to treat every organ equally regardless of where in the machine it is placed. The organs and the liquid pumped through them also need to be separated to prevent cross-contamination. Finally, the materials used need to be chemically compatible with the protocol.

B. Approach

For the automation of tissue clearing and immunolabeling protocols to be possible, there are some requirements which need to be met. The machine needs to be able to mix precise amounts, maintain a stable temperature, withstand the chemical conditions and the tubing needs to withstand the stress exerted by the pumps for prolonged periods. To meet these requirements, electrical components are required.

C. Explanation of electrical components

The specifications require some electrical solutions. Peltier elements have been used for their space efficient heating and cooling abilities. [9]

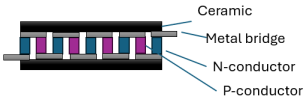

Fig. 1: Graphic explanation of Peltier element

This is a component with alternating n and p conductors, that when exerted to a current moves heat from one side to the other. (Figure 1). When the energy from the hot side is removed, the cold side can reach freezing temperatures. If the current changes direction, the sides are altered, meaning that the hot side becomes the cold side.

Peristaltic pumps have been used for the circulation due to their precise flow control. A peristaltic pump exerts pressure on a tube and pushes it forward. The pressure is exerted by cylinders which rotates along a part of the tube, thereby pushing the content forward. (Figure 2)

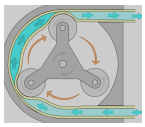

Fig. 2: Schematic drawing of a peristaltic pump [10]

Solenoid valves were used due to their ability to control flow through tubes. A solenoid valve consists of a housing and a membrane which can be moved using a magnetic coil, the coil is activated via an electric signal. The membrane is spring loaded and can be either normally open, normally closed, or bistable. The difference is where the spring is fitted, the bistable variant has a permanent magnet in the top which can hold the membrane and therefore does not need a constant signal to be open. The membrane is used to cover a hole in the housing that the fluid goes through, and thus the valve only works in one direction.

The relay's ability to alter large currents using a small input necessitated the use of them when controlling valves and Peltier elements. A relay consists of three gates, and a switch. The gates are named common, normally open, and normally closed where the common is connected to normally closed until the switch gets a signal. When this happens, a coil is activated and moves the connecting cord from normally closed to normally open. This makes a characteristic clicking sound that most people recognize from the indicator in cars.

This project aims at automatizing the inFLATION protocol. Here we show the proposed machine to be equipped with suitable materials, explain how different parts of a machine does the tissue clearing and immunolabeling. It will also discuss some of the choices that were made along the way, why it is needed and what will be the next steps to bring this vision into reality.

II. Method

A. Testing of tubes

Materials were tested to see if they would withstand the chemical and mechanical stresses they would be exposed to.

Fig. 3: Illustration of tube in chemicals, made in Biorender

For the sake of confidentiality, the two tested tubes will be referred to as tube A and tube B. First, the two different kinds of tubes were cut into pieces (8, 13 and 18mm) using a scissor and each piece manually weighed. The different lengths were used to separate the tubes seeing as they were placed in the same falcon tube. Using the handheld microscope, DinoLight (AM4815ZTL), the tubes' inner diameter, outer diameter and length was measured. This was done with both type A and type B tubing. The pieces were placed in falcon tubes containing

either 66% dichloromethane (DCM) or in 100% methanol to monitor possible reactions. When examining the tubes they were tapped against the falcon tube to release any remaining liquid. They were then measured using the DinoLight, weighted, and finally put back in the tube. This was done once a day. The DinoLight sets a magnification rate on the images based on the distance to the object. In this case when the object is not laying on the same level as the background, the magnification can sometimes be wrong. To negate these changes, the ratio between the inner and outer diameter was measured, due to the ability to use the same picture and therefore the same magnification.

To further test the suitability of tube A, a piece was cut of it and placed in methanol for 2 weeks. The methanol was then tested using an FTIR sensor to see if it contained anything else. An FTIR sensor measures how much of the infrared spectrum is absorbed in the bonds of the molecules and provides a spectrum which can be seen as a fingerprint for the molecule.

The valves were chosen to be chemically resistant to DCM, methanol and hydrogen peroxide. This was however not tested since there was no safe way to pump the chemicals through them.

B. Examination of mechanical stress resistance

The tubes' response to mechanical stress was also examined. They were put in a peristaltic pump, three tubes next to each other. To stop the tubes from being fed into the tube, stops were designed, 3D-printed and glued to the tubes. The ring on the right side in figure 4 was designed to have the same diameter as the tube. The cuboid on the left of figure 4 was designed to be larger than the cover of the tubes on the pumps. They were designed to clamp onto the tube and have one part which reached out to log into the cover of the tubing in the pump, see the outer grey area in figure 2. Chemical compatibility charts [11] suggested that superglue (ethylcyanoacrylate) should be appropriate with the used tubing. The glue was applied to the tube, then the stop was slided into place and rotated to ensure an even spread of the glue. Finally, a drop of glue was applied to each edge to fill air pockets. The tubes were then measured using the DinoLight. The outer diameter was measured both at the end of the tube and at a mark where the tubes would be in the pump. They were set to pump water in a loop. Each day, the tubes were emptied of water by pumping air through them, and the same diameters as before were measured. After the tubes had been tested for 2 weeks, two of them were put into a pump with two unused tubes. They all pumped water into different falcon tubes to see if the wear would cause the tubes to create air bubbles or pump less efficiently. This was also done with unused tubes in all positions in the pump (MasterFlex Ismatec 78018-20) to make sure the machine was not pumping unequally. To ensure the result, multiple speeds were used and the pumps were calibrated for the tubes used. Additionally, tests were performed to evaluate if the pumped amount changed due to temperature. This was done using water at 4°C, 22°C and 37°C. During the test, two tubes were used to examine both the individual amount and the combined to see if the pump was affected by a higher load.

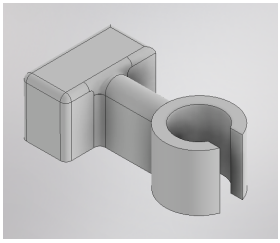

Fig. 4: CAD model of stop, made in AutoDesk Inventor

C. Examination of circulation using manual valves

A setup of the machine was designed and drawn according to the specifications mentioned in I-A. It was then tested to see if it would work. It was done using water and manual three-way valves with luer locks. The pumps were turned on and each of the steps in the protocol were tested. (Figure 5). The setup was designed to take fluid from beakers, via anti siphone valves and two manifolds(1), to the first pump. Anti siphone valves restrict the flow to one direction using a membrane which lets fluid through a hole in one direction and restricts it in the other. This means that all the liquid that is being pumped into the machine will mix after the first manifold and then separate equally into every organ after the second manifold. From the first pump to a drip stand which removes air bubbles (2), and on to the second pump. From the second pump it went to a manifold which could direct the fluid to either waste (3) or to the organ (4). Lastly, the fluid went back to the manifold before the first pump to be circulated (5). The drawing (figure 5) shows one channel but the setup was tested on four parallel channels.

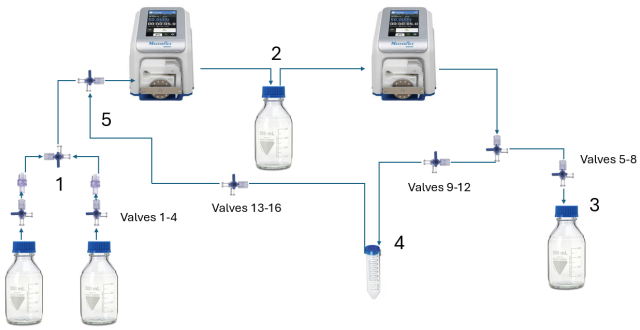

Fig. 5: Drawing of the setup showing one channel

D. Controlling the pumps

The pumps were controlled via analog input. Jumper wires were soldered to a DB25, which is a connector for ports with 25 in-going pins, according to the pump's manual. When pin 17 and 19 are connected the pump will start, and when the connection is broken, the pump will stop. The amount of current that is being applied between pin 2 and 3 will control the pump's speed. To control the speed, the input current needed to be in the interval 0-20mA. The pumps were fed with power from one channel. That channel was connected to two different channels which both were connected to a power source of 5V via a relay.

To manage to run the pump at 2 different speeds, a relay was connected which switched between circuits (figure 6). The circuits had different resistance, one had 4.7kΩ and the other 1kΩ. The 1kΩ was connected to the normally closed connection of the relay seeing as it was the one which would be used most frequently.

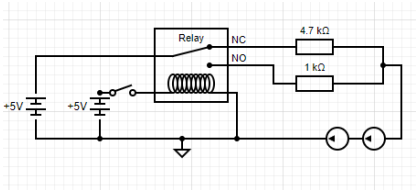

Fig. 6: Electrical circuit of a relay connected to two pumps

E. Raspberry Pi

To control the relays a Raspberry Pi (RPi) was used. An RPi is a circuit board with a Linux operating system. It can write programs in any language, but Python is preinstalled. The RPi has USB, USBc for power, aux, micro-HDMI, and ethernet ports as well as specific ports for a camera and a touch screen. It also has GPIO pins that can be used as power sources (3.3V or 5V), digital and analog signals. It is controlled by connecting a computer mouse and keyboard. To view the coding it also needs a screen. This can be connected either via HDMI, or remotely with SSH (Secure Shell). SSH is a tool where a TCP port is opened on the RPi and can be connected to via a computer. From the computer the RPi's terminal can be opened, from which the terminal programs can be written or started. It can also run other commands, such as opening the camera or taking a picture. In addition it is possible to use Raspberry Pi Connect which is a web-program that allows the user to enter the entirety of the RPi remotely using the RPi's desktop.

F. Valves and relays

The valves were numbered according to when in the protocol they were to be used. They were connected to different relays to be controlled by the Raspberry Pi. Valve 1-4 was connected to the relay matching the valve's number. Since they were controlling one liquid each, they needed to be controlled separately. Valve 5-8 was connected to relay 5 at the normally open gate and would direct the liquid into waste. Valve 9-12 were also connected to relay 5 but at the normally closed gate and would direct the liquid into the organs. Valve 13-16 were connected to relay 6 and opened to let the liquid back to pump 1 and into circulation. Valves 5-16 were soldered together in pairs to reduce the number of wires being used. The valves were then connected to a breadboard, where those using the same relay were connected to the same channel which connected to said relay. (Figure 5). Since the valves require 24V they could not use the Raspberry Pi as power source. Instead, they were using a Powerbox 3100 with controllable current and voltage. The Powerbox was connected to the breadboard and supplied all the valves from there.

G. Human Machine Interface

A graphical user interface (figure 7) was designed to make it easier to operate the machine. The interface has 6 buttons: New step, start, stop, enable camera, disable camera, and take picture. The new step button opens a new window where the user enters information regarding which fluid or fluids they want for the step. They can specify percentage of each chemical and the complete amount, as well as for how long it should be circulating and at what temperature they want the step. The window contains a button which gathers the information from the window and closes it, allowing the user to add more steps. When the user has added all desired steps, they can use the start button to start their protocol. If they then press the enable camera button, a camera will take pictures every half an hour that the user can view from anywhere with an internet connection. This can later be disabled by the disable camera button. They can also take a picture when they desire by using the take picture button. If the program needs to be stopped, they can use the stop button, and then resume the program by pressing the start button. The stop button was made red while the rest were made orange, to help the user find the button quickly.

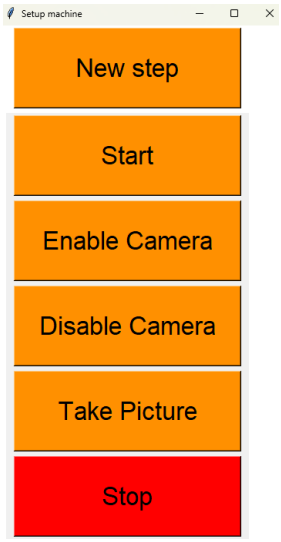

Fig. 7: Graphical User Interface

H. Proof of concept using an electronic setup

The electronic setup was the last step to prove the automation could work. All the valves and pumps were connected to their relays. The four liquids were water with food colouring to make sure the correct chemical was being pumped. Underneath all the valves were paper napkins to see if any liquid was leaking. A camera and a touch screen were connected to the Raspberry Pi. The screen was used to start the software and present the stop button. Instead of using the touch screen, the Raspberry Pi could be controlled via SSH.

I. Testing efficiency of the mixing

After the setup had been run and tested, it was used to test if the protocol for mixing was working. It was programmed to mix two different colors of water (first yellow + blue, then red + blue) in different percentages (20%, 40%, 60%, or 80%). The same percentages from the same beakers of water were then pipetted by hand. The mixtures were pipetted into a 96 well plate and studied in a spectrophotometer (EPOCH). The spectrophotometer studied the absorbance in the different wells and could thereby study how much of each color had been added (Figure 15). It was pipetted by hand three times and machine pumped twice (Figure 14). The ratio between the values at the peaks of the absorbance was then calculated and compared between hand mixed and the ones mixed by the machine.

J. Testing prototypes for temperature control

A temperature-controlled box was built for the parts of the protocol that could not be done at room temperature. This was done using a Styrofoam box, Peltier elements, fans, heatsinks, aluminium cube, and a powerbox. (Figure 8). The Peltier element was placed with the cold side against the aluminium cube and the warm against the heatsink. It was screwed in place. A hole was cut in the Styrofoam box where the aluminium cube could go through the box's wall and the heat sink would prevent any leaks. Then a large fan was placed on top of the heatsink to remove the heat. To test the efficiency of the setup, a beaker with 40 ml of water was placed in the box and the temperature of it was measured every 15 minutes.

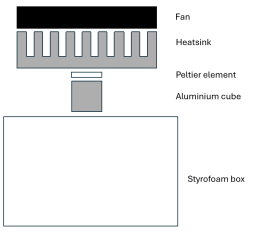

Fig. 8: Schematics of temperature prototype

Using materials which easily transfers heat is important when building an efficient system. During the styrofoam-test described above, air was often used as the medium. However, by replacing the air with a medium with higher heat transfer coefficient the system would both cool and heat quicker. A tube stand of aluminum was designed that could be cooled or heated using Peltier elements and heatsinks. This idea was however only tested in theory by cooling an aluminium cube with a Peltier element, measuring the cooling and then extrapolating an expected value for the larger part.

III. Result

A. Tests of tubing materials

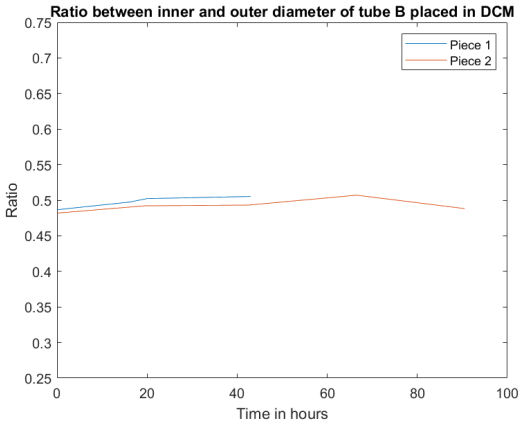

Fig. 9: Tube B in DCM

The tubes' weight and ratio between outer and inner diameter was measured when put in chemicals, to observe if they were dissolving or absorbing the liquid. The ratio between outer and inner diameter of tube B shows trends towards increasing which is shown in figure 9. "Piece 1", "Piece 2", "Piece 3" refers to the different pieces of the same tube that was submerged in the chemicals. Additionally, the weight of tube A was unchanged when submerged in methanol which can be seen in figure10. There is no graph for how tube A behaved in DCM since it swelled to twice its weight in 24 hours and was deemed inadequate enough to entail further evaluation.Chemical compatibility charts state that tube A has excellent compatibility with methanol [11]. Evaluation of potential tube change from mechanical strain was measured by comparing the diameter of the tubing in the pump with tubing at the end, which had not been exposed to any strain. The mechanical strain did not have any noticeable effect on tube A either which is shown in figure 11.

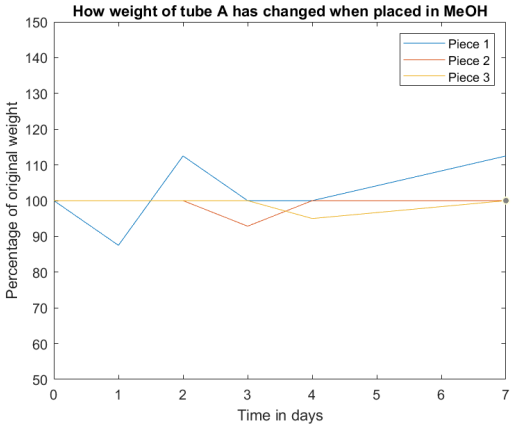

Fig. 10: Weight changes of tube A in methanol

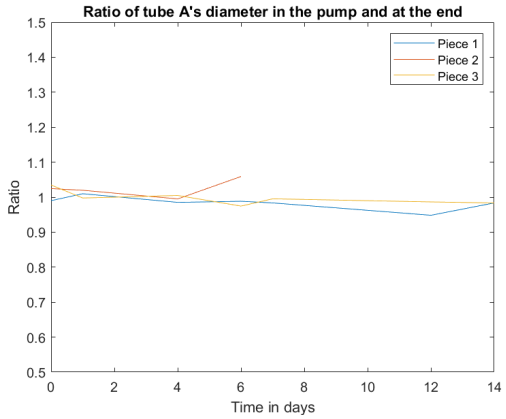

Fig. 11: Ratio of tube A's diameter in the pump and at the end

The red line stops abruptly since the tube stop failed. It should also be noted that after the protocol had been run through once, using only water and food coloring, the tubes were not leaking or creating air bubbles, and was used during further testing with the machine. The temperature tests of the tubing indicated changes of 1 ml/minute if the temperature differed between 4°C and 37°C. The pumps also reduced the pumped amount with 10% when the load was doubled. The FTIR test (figure 12) shows high similarities with the infrared spectrum for methanol. However, at 3400 cm^{-1} there is a peak not found in methanol.

B. Tests of cooling the setup

The styrofoam box took 3 hours to cool 40ml of water from 20°C to 5°C. When using a fan to circulate the air even more,

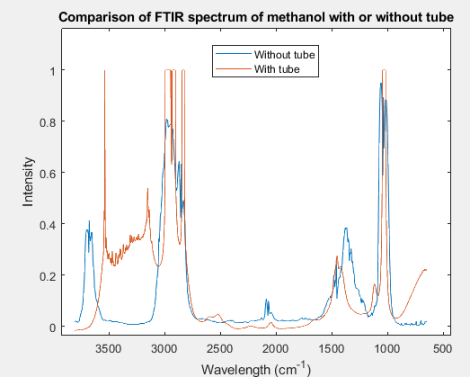

Fig. 12: FTIR spectrum of methanol after holding tube A for two weeks compared to FTIR spectrum of pure methanol [12]

it turned out that the fan produced heat and the temperature never got below 11°C. This was not deemed sufficient.

The other version that was tried was to create an aluminum tube holder. Since there was not enough time to test this, it was only calculated. The small aluminium box was cooled to 3.1°C in 15 minutes. When extrapolated, this showed that the larger tube stand would require 19 minutes to reach a temperature of 3.1°C assuming the room temperature was 22°C and the use of 2 Peltier elements.

C. Outcome of the setups of the machine

We found that liquid can come from only one tube in the case that two tubes are connected to the same manifold and are pressure driven from the third entrance. This problem can be solved by opening and shutting the valves leading up to the manifold to achieve mixing. This reduces the degree of mixing in the tubes which can be resolved by the drip stand.

When the pumps had been connected via analog input and the two different resistors they could pump at two different speeds, 1 ml/min or 4.2 ml/min. The slow speed is wanted for pumping liquids through the organs to allow for maximum diffusion without damaging the tissue with too much pressure. The high speed is used for mixing the chemicals and directing them to waste. The higher speed is desired to minimize the time demanded by these steps.

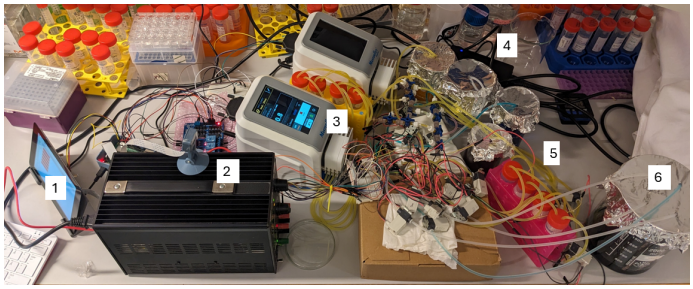

Fig. 13: Image of the full setup

During the testing of the electrical setup, the chosen manifold proved to let air through the fittings and was replaced by connecting 3 way manifolds with luer locks to keep it hermetically sealed. It was also found that the push-fittings on the chosen valves had a tendency of leaking if the tubing

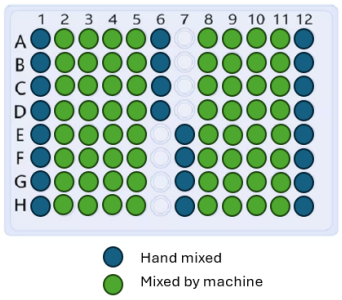

Fig. 14: Schematic of the well

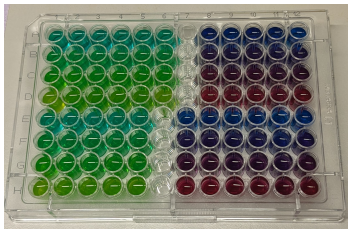

Fig. 15: 96 well plate used in spectrophotometer

was not absolutely straight when fastened. After the tubing had been properly attached, the valves performed as planned. They did not overheat, leak or malfunction in any other way. The setup managed to go through the entire protocol once, using water instead of chemicals, without any air bubbles, differences in amounts between the channels, nor any leaks. Figure 13 shows a picture of the setup. To the left (1) there's the touch screen and a keyboard to edit the code. The black box (2) is the powerbox with the surveillance camera on top and the blue relay board behind it. The red lids between the pumps (3) belong to the drip stand, we pump liquid in from (4), the tubes for organs are found at (5) and the beaker at (6) is waste. Note, no organs were used.

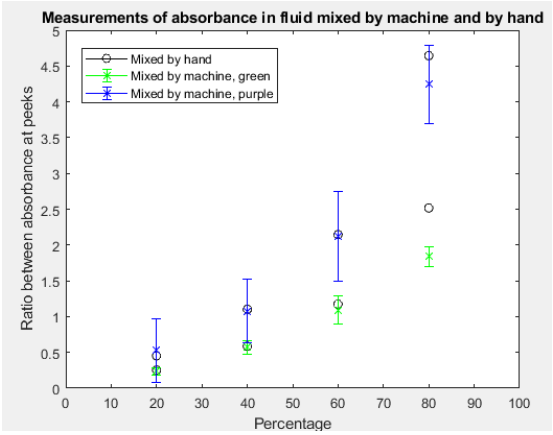

Fig. 16: Comparison of ratio between peaks in spectrophotometer at different percentages

The machine was also tested to see how well it mixed specific percentages from its different containers. The results are shown in figure 16. The values are ratios from the two highest peaks found in the spectrophotometer, 630 nm were used for both colours, additionally 460 nm was used for green, and 520 nm for purple. (Figure 15). The x is the mean value from 8 different mixes of each colour, the lines show standard deviation and the circles represent mean values from three mixtures made by hand. The standard deviations have been divided by the percentage to normalize the values seeing as the deviation should be larger with a larger ratio. The green lines represent the green fluid, and the blue lines represent the purple fluid. We observed little to no difference between handmixing and automated mixing in mistures between 20% and 60%. However, we found a larger difference at 80% in

the green mixture.

IV. Discussion

A. Tube selection

When deciding which tube to use we have done several different tests, observing how they perform under mechanical strain and when exposed to different chemicals. The greatest challenge is to withstand DCM due to it being a highly reactive compound. We have found a tube material that is compatible. However, due to the rigid behaviour, the material is not suited for use in a peristaltic pump. When we were making the decision of which tube to use, we chose tube A. It swelled to double its weight when exposed to DCM but behaved well in methanol. It is also very flexible and our mechanical tests showed no difference between a brand new tube and one that had been in use for two weeks. The tubes would be changed between every run of the machine to avoid cross contamination. Since this is a first prototype, we find it acceptable that the DCM will have to be handled manually, which makes this tube selection possible for now.

The FTIR that was done to evaluate if tube A were leaking any substances when submerged in methanol for 2 weeks was deemed inconclusive. Even though the FTIR-spectrum shows higher values around 3400 cm^{-1} than the spectrum for methanol (figure 12), this is not from our tube. The spectrum coincides with that of ethyl cinnamate, a compound that had been used earlier in the machine.

B. Machine evaluation

While the machine has not yet been tested with the actual chemicals, or seen how the organs would look after a treatment from the machine, we are quite confident that it is not too far away. Many of the vital steps have been tested and prototyped successfully. The mixing of different fluids was deemed sufficient, even though the result at 80% when mixing the green was off. The reason for this was that one of the tubes which entered the manifold at the same point as the yellow was empty. This reduced the amount of yellow fluid and thus affected the outcome. Seeing as this was not the case when mixing purple and that the remaining results were within one standard deviation, the mixing was deemed sufficient. More tests need to be done when there are no air in the system, to confirm this conclusion. If this phenomena is repeated, a better manifold will be required.

C. Use of automating the protocol

The reasoning for automating this protocol is the multitude of steps during a prolonged period. When these steps are performed manually, a greater risk of human errors exists than when it is done by a machine. This reduces the need for iterated samples due to unsuccessful clearing or staining and thereby makes it cheaper. The reason for choosing this protocol over other is that it is faster and has a more even result within the organ. It also permits a wider range of examinations than other available solutions. Due to the difference in organ density, the clearing time will vary. Therefore it is deemed necessary to have a user interface where the time and amounts of chemicals can be altered. This also allows testing of different types of animal organs seeing as they have different sizes. Among the limitations when it comes to size is the amount of fluid needed and the ability to cool the samples. The multitude of similar protocols makes the machine more useful seeing as it can operate all of them. This type of clearing does require further development due to the lack of standardization for different organs. Having a machine to be able to develop the appropriate protocols for different organs would prove helpful seeing as it could remove prolonged exposures due to work hour limitations. This could potentially reduce the required time of protocols. It does however require larger amounts of chemicals seeing as the tubing needs to be filled as well which slightly increases the cost. The automation of the protocol reduces the number of steps that require manual handling from 21 to two steps. This results in a reduction of active work time from 4 days to one hour.

The reason for using these types of protocols over other is that it gives a better result. The hydrogel-based clearings have a risk of denaturing the proteins and can thereby give false results. It also results in an uneven clearing due to the electrophorese part of it dragging the residues to one side. This creates one side with poor clearing and can therefore cause loss of data on that side.

D. Interface

There is a graphical user interface designed for the machine. This allows the user to make their own steps where they define which solution or mix of solutions is supposed to be added, how much and for how long they should be circulated. This gives the operator the possibility to make changes to the protocol and adapt it to their circumstances. It also holds the ability to activate a camera which uploads pictures to google drive and gives the operator the ability to monitor the status and progress. When this button is activated, it takes a new picture every half an hour. If the user would like to start the machine or see if the program on the Raspberry Pi is still running, it is possible to log into the Raspberry Pi using SSH. This allows the user to start the program from anywhere with an internet connection, but the setup of the machine still needs to be done in place. The SSH option is only available if the Raspberry Pi is connected to Wi-Fi which does not hold limitations for SSH. It is also important to note that if this machine were used for research, the images would not be uploaded to Google Drive as this does not comply with GDPR. Instead, the images would be saved locally on the Raspberry Pi and hold the ability to be fetched via SSH.

E. Ethics

This project has had a vast demand on the materials. Despite wanting to create a sustainable machine we have had to prioritize it being chemically and mechanically stable. There are however other areas where we've been able to think environmentally. Our heatsinks and the aluminium cube used to transport cold is residuals from companies who would otherwise dispose of it. The programming is also done to

reduce the electricity used by connecting the valves to the port where they would most seldom receive power.

Another aspect of ethics closely related to this project is animal testing. This machine will be used on organs from sacrificed mice and rats, which would still have happened even without this project. However, one of the goals is to reduce human error and through that reduce the number of samples which can not be used for science. With the final steps of the protocol, we are able to preserve the tissue cleared organs for many years to come. A future ambition is adding the functionality of reverse clearing paraffin embedded organs, enabling reuse. This project will lead to fewer animals being sacrificed for science, while still being able to research medicine.

F. Future developments

The next step is temperature control. This has been calculated but not prototyped yet. The first step of the next iteration is to order an aluminum cube according to our sketch and test.

A great advancement for the machine would be to use Fourier Transform Infrared Spectroscopy (FTIR) to see when each chemical stops interacting with the tissue. This would optimize the circulation times and make it easier to operate the machine seeing as there would be a reduction in steps. In addition it would improve the quality of the samples since no unwanted compound would be left in the organ. Currently an FTIR sensor is costly and thus not included in the proof of concept. A FTIR sensor measures the spectrum of light that goes through an object and can thereby see which frequencies have been absorbed. They change depending on the amount of residues from the organ.

Air bubbles in the organs lead to uneven staining. At this point air bubbles are disposed of via the drip stand, and even though that has worked so far it might not always. If something were to go wrong with the pump, or a container would run dry, there is risk of pumping air from an empty drip stand. To get around this, air bubble sensors would be needed. It could be an infrared sensor that notices the difference in diffraction index between air and liquid, or a capacitive sensor. This could then activate a valve to waste and pump the air bubble there instead of into the organs. Another sensor that could be needed is a pressure sensor that can notice if something starts leaking or if a tube is broken. This would then stop the entire program, stop the pumps and hopefully prevent chemicals from spilling. A message could be sent to the user to alert them that something has gone wrong.

The machine also needs some form of casing, to avoid it looking as the setup shown in figure 13. The housing will need to contain a part which can heat or cool the samples along with the drip stands. This would also remove the mixing of electronics and fluids and thereby reduce the risk of using it. It would reduce the amount of space the machine takes up and make it easier to move. This seeing as it could consist of one layer of electronics and have all the fluid handling on top of it. Most parts of the fluid handling needs to be open since the tubes need to be replaced between different samples.

V. Conclusions

The automation is a viable option to perform the protocol manually. It reduces the amount of steps drastically and thereby makes it easier for the operator and reduces human error. It does however require larger amounts of the chemicals seeing as the tubing needs to be filled for a good result. It has been able to run a full protocol using water with food dyes.

The use of this automation results in a more easily adapted protocol and therefore allows optimization for each organ.

VI. Acknowledgement

We would like to thank all the people at LBR for their help in the lab. We would like to send our greatest gratitude to Hani Alsafadi for his help with the project and allowing us to try our way forward. We would like to send another special gratitude to Darcy Wagner for funding the project and discussing possible solutions to our problems in the project. We would also like to thank Niclas Palm for his help with the electrical problems we have had on the way.

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