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# Optimal Tissue Clearing of Whole Mount Mouse Organs

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# OPTIMAL TISSUE CLEARING OF WHOLE MOUNT MOUSE ORGANS

by

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# ABSTRACT

## OPTIMAL TISSUE CLEARING OF WHOLE MOUNT MOUSE ORGANS

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Various tissue clearing methods have been made to optically clear tissue for high resolution imaging, but clearing whole organs is an arduous task due to limited penetration depth. To find a potential solution, 1 mm mouse organ slices were used to try four different variations of PEGASOS and BABB, organic solvent based clearing methods. Two of the variations were chosen to stain with antibodies and dyes, such as DLL4, to display vasculature. Axially swept-light sheet fluorescence microscopy was used to obtain 3D images of the kidneys. In addition, stained heart and lungs images were also acquired. Progress photos for clearing slices were observed under an inverted microscope. The outcome was that PEGASOS is the most compatible clearing medium for staining while BABB was the best for viewing autofluorescence.

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# CHAPTER 1

# INTRODUCTION

## 1.1 Tissue Clearing

Tissue clearing plays an integral role in diagnosis and treatment. It allows for the discovery and understanding of differing structures and mechanisms. Most imaging results are limited to imaging organ slices. In fact, most researchers advise to clear organ slices only up to a few millimeters due to limited penetration depth and imaging capabilities. Imaging slices only provides a limited view of the internal structure of the organ and causes aberrations in 3D reconstructions. To combat this issue, researchers have focused on developing optimal whole organ tissue clearing to get a better view of the internal structure.

One way that this is combated is by tissue clearing methods to make the organs optically transparent for imaging. There are 3 main types of tissue clearing methods: hydrophobic (solvent), hydrophilic (aqueous), and hydrogel (Ueda et al. 2020). For hydrophobic and hydrophilic, they have delipidation, decolorization, and RI matching steps. One issue with hydrophobic solvents is that they tend to shrink tissue through the clearing process. On the other hand, hydrophilic tends to swell tissue.

There are two different types of soft tissue that can be cleared: heavily colorized and non-heavily colorized (Table 1.1). Heavily colorized organs contain more pigmentation than non-heavily colorized organs which makes them more difficult to clear. Hard tissue, or bone, can be cleared as well but they take much longer. For the scope of this project, soft tissue are the organs used for testing.

Table 1.1: Heavily colorized and non-heavily colorized organs

| Heavily Colorized | Non-Heavily Colorized |
|---|---|
| Heart | Brain |
| Kidney | Lungs |
| Liver | Pancreas |
| Spleen | |
| Tongue | |

## 1.2 Immunohistochemistry Staining

Immunofluorescence is a technique that uses fluorescent-labeled antibodies to target antigens (Odell and Cook 2013). An antibody is a protein that makes B cells that activate an immune response against a target antigen (Odell and Cook 2013). Immunohistochemistry (IHC) is a type of immunostaining that uses monoclonal and polyclonal antibodies to detect a target antigen, and the main goal is to stain with minimal damage to the tissue (Duraiyan et al. 2012). The most common immunoglobulin used for IHC staining is IgG (Ramos-Vara 2005). Monoclonal antibodies are made from an animal species with a purified antigen while polyclonal are created from multiple animal species (Ramos-Vara 2005).

There are two different methods for staining: direct and indirect. Direct immunofluorescence is the use of one antibody to stain a specific structure in a sample. Indirect immunofluorescence uses two different and compatible antibodies to indirectly stain a structure in a sample. The problem with direct immunofluorescence is that it is difficult to make the stains specific. There are also dyes that can be used for staining, but they lack specificity and cause fluorescence for the whole sample. This makes it difficult to stain for specific structures.

Prior to staining, the sample must be washed in 1X PBS. Afterward, it must be placed in a blocking solution to increase the specificity of the antibody staining. For indirect immunofluorescence, it is advised to develop a blocking solution with a serum that matches the species of the secondary antibody being used. For example, if you are using a secondary antibody from a goat host species, it is best to find a blocking serum with a goat host species. In addition, you can use other blocking serums such as bovine serum albumin (BSA) or casein buffer because they can block non-specific binding. After the samples are placed in the blocking solution, they are ready to be stained. For the antibody staining process, the antibodies need to be diluted in the blocking solution previously used. If primary and secondary antibodies are used, there is traditionally a washing step in between, but researchers have found that this step might not be necessary. After the staining process is done, another washing step is performed and the tissue clearing process continues.

One limitation to fluorescence staining is that the successfulness of the staining depends on the concentration of the antibody and, in the case of indirect immunofluorescence, the compatibility of the primary and secondary antibodies (Odell and Cook 2013). The best way to obtain optimal staining is to test different concentrations and see which has the best signal-to-background ratio (Odell and Cook 2013). As a result, testing of different antibody concentrations and blocking solutions is needed to get an optimal imaging result.

### 1.3 Light-Sheet Fluorescence Microscopy

Light-Sheet Fluorescence Microscopy (LSFM) uses a plane of light to create optical sections to acquire 2D images of a sample. This custom technique is non-invasive and makes it less challenging to obtain 3D reconstructions (Santi 2011). Like confocal

microscopy, this technique is beneficial when trying to view deep into tissue samples. In addition, there is minimal photobleaching and phototoxicity (Santi 2011). It allows for sub-cellular resolution of samples with no limitation of speed or excitation (Adams et al. 2015). The basic setup for LSFM is a path for excitation of the sample and the path for imaging (the camera) (Stelzer 2014). These paths are perpendicular to each other. The limitation of LSFM is that the resolution is limited by numerical aperture (NA) and field-of-view (Teranikar et al. 2022). There are many variations of LSFM created to combat these issues, but the focus for this project is the use of Axially Sweeping Light Sheet Fluorescence Microscopy (AS-LSFM).

AS-LSFM uses the same setup as a basic LSFM with an illumination path (laser) and detection path (camera) that are perpendicular to each other. The axially sweeping aspect is a motor moving the laser axially through the sample which allows for the field-of-view to be changed without compromising resolution (Teranikar et al. 2022). This allows for better imaging of larger tissue samples and whole organs. There are still limitation to this technique, but it is a variation of LSFM that certainly improves the resolution compared to conventional microscope techniques. One of the limitations is that the sample needs to be imaged from multiple sides to get the most accurate reconstruction of the sample (Kim et al. 2021).

# CHAPTER 2

# METHODOLOGY

## 2.1 Clearing Process

The mouse organs to be cleared were received in confocal tubes submerged in a Phosphate Buffered Saline (PBS) solution. The clearing process has four main steps: fixation, decolorization, delipidation, and RI (Refractive Index) matching. Dehydration is another step of tissue clearing, but, due to limited time, it was not explored during this project.

Fixation is used to maintain the original integrity of the organ outside of the body. Paraformaldehyde (PFA) was used as the tissue fixative. PFA fixes the tissue by binding to amine groups within the tissue. The degree of fixation is determined by the concentration of PFA used (Richardson et al. 2021). In this case, a concentration of 4% was used. Decolorization is for pigmentation removal. The pigment must be removed to prevent scattering and increase transparency. 25% Quadrol was used for pigment removal (Table 2.1). In addition to this, heavily colorized organs were submerged in 5% ammonium to remove heme. Delipidation is the removal of lipids. Lipid removal decreases light scattering and allows for improved fluorescent staining (Richardson et al. 2021). Delipidation was done via submersion in a series of Tert-Butanol dilutions (30%, 50%, and 70%). RI matching is for matching the refractive indices of the organ and the clearing solution. This makes sure light is refracted when passing through the sample of interest. BABB (Benzyl Alcohol/Benzyl Benzoate) was made by mixing 1 part benzyl alcohol with

2 parts benzyl benzoate. PEGASOS (polyethylene glycol (PEG)-associated solvent system) was created by mixing 75% benzyl benzoate, 25% PEGMMA500, and 3% Quadrol.

Table 2.1: Required chemicals for each tissue clearing step

| Process | Required Solution |
|---|---|
| Fixation | 1X PBS, 4% PFA |
| Decolorization | 25% Quadrol, Ammonium (heavily colorized organs only) |
| Delipidation | Tert-Butanol & PEGASOS |
| Tissue Clearing | PEGASOS, BABB, PEGBABB, or PEGatone |

As for the time duration, the whole tissue clearing process took approximately 5 days for non-heavily colorized and 7 days for heavily colorized. The fixation process took about 1 day to complete on an orbital shaker at 4°C at ~15 rpm (Table 2.2). For the rest of the tissue clearing process, the samples were shaken in a rotator at 37°C at ~100 rpm. The only difference between clearing heavily colorized and non-heavily colorized organs is that the decolorization process is 2 days longer for heavily colorized organs in order to properly remove pigmentation from them.

Table 2.2: Time duration for entire tissue clearing process without IHC staining

| Process | Time Duration | Temperature & Speed |
|---|---|---|
| Fixation | ~1 day | 4°C at ~15 rpm (shaker) |
| Decolorization | 2 days (non-heavily colorized)<br>4 days (heavily colorized) | 37°C at ~100 rpm (rotator) |
| Delipidation | 1 day | 37°C at ~100 rpm (rotator) |
| Tissue Clearing (RI matching) | 1 day | 37°C at ~100 rpm (rotator) |

PEGASOS and BABB are popular hydrophobic clearing solutions. Since they are well-known for their optical transparency abilities, different combinations of the two were

used for testing. The four different variations tested were PEGASOS, BABB, PEGBABB (a mix of PEGASOS and BABB), and PEGatone (a mix of PEGASOS and pure acetone) (Figure 2.1). These different variations were tested on various 2 mm organ slices. Slices were utilized to ensure quick testing before performing any experiments on whole organs. A custom ABS organ slicer was created in SolidWorks (Figure 2.2). Due to lack of time, the organ slicer was unable to be used, so the slices were cut with a blade.

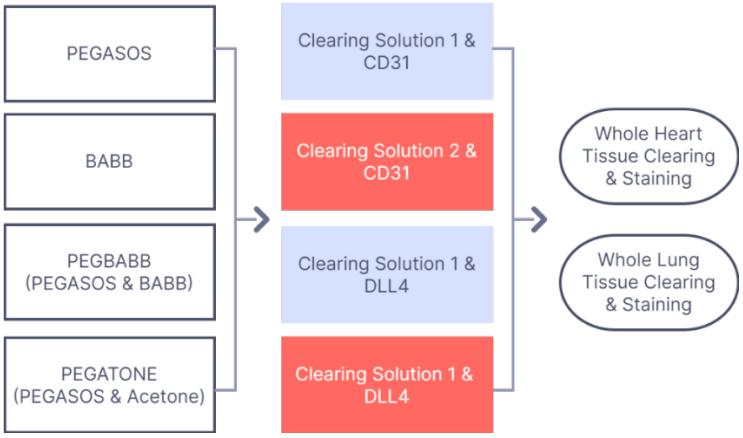



Figure 2.1: Timeline of experiment

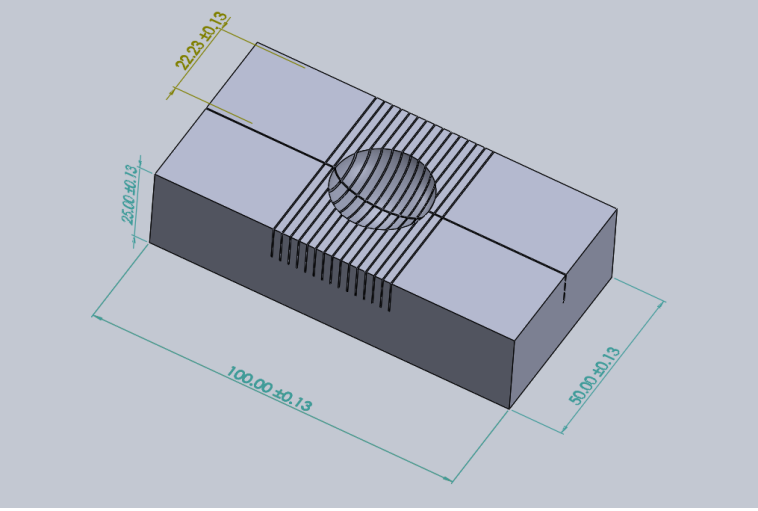



Figure 2.2: SolidWorks file of custom organ slicer

## 2.2 Antibody Staining

After the four different clearing variations were tested on slices, they were compared and the best two were chosen for antibody staining with cluster of differentiation 31 (CD31) and Delta-like ligand 4 (DLL4), respectively. The two best clearing methods were PEGASOS and BABB. Thus, these two clearing mediums were both tested with CD31 and DLL4, respectively. The clearing and staining were again tested with organ slices. Standard IHC protocols were followed for all staining experiments.

Prior to this experiment, direct immunofluorescence was used with Fluorescein isothiocyanate (FITC) but this did not display vasculature as well. Thus, indirect immunofluorescence was used instead. Examination under an inverted microscope showed that CD31 had some signs of successful staining. On the other hand, DLL4 was unable to be seen under the inverted microscope. Thus, CD31 was chosen as the antibody to use for the whole heart and lung staining (Figure 2.1).

There are four main steps to the staining process: pre-washing, blocking, staining, and post-washing (Figure 2.3), all of which was performed after the decolorization process. First, The organs were washed in 1X PBS and 0.3% Triton-X 100 (PBS-T). This was done for 30 minutes at room temperature. The blocking solution was created with casein buffer, 10% dimethyl sulfoxide (DMSO), and 1 mL of the PBS-T previously used. The blocking process took 1 day of incubation at 4°C.

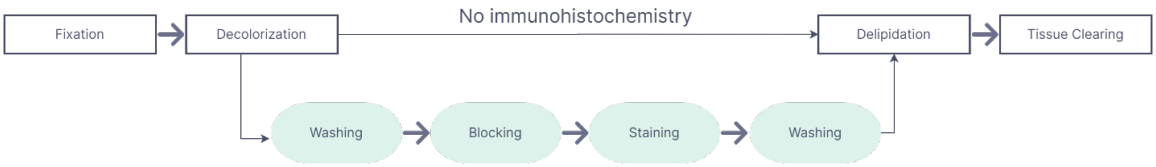



Figure 2.3: Flowchart of tissue clearing process including IHC staining steps

The primary antibodies used were CD31 and DLL4. For indirect immunofluorescence, CD31 and DLL4 were used together with Goat Anti-Rat IgG and Goat Anti-Rabbit IgG, respectively (Table 2.3). The concentration required for CD31 and DLL4 was 1:50 (ratio of antibody to blocking solution). For Goat Anti-Rat IgG, the concentration was 1:200. For staining, the organs were placed on the orbital shaker at approximately 15 rpm at 4°C (Table 2.4). Since the antibodies are light-sensitive, the conical tubes with the organs were covered in aluminum foil to prevent heavy light exposure. Moreover, the organs were continuously covered during the remainder of the tissue clearing process until they were ready for imaging. The entire staining process took approximately 7 days.

Table 2.3: Chemicals for IHC staining

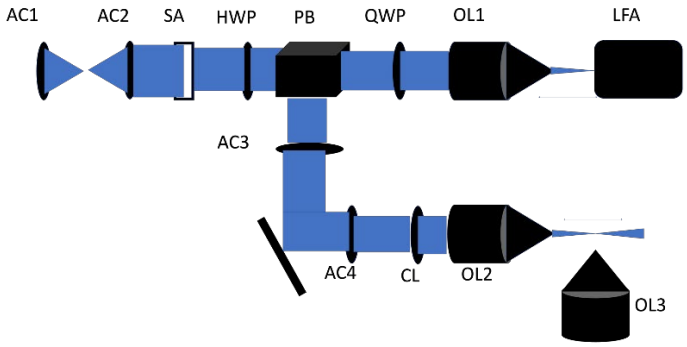
| Process | Required Solution |
|---|---|
| Washing | 1X PBS, Triton-X 100 |
| Blocking | 1X Casein buffer, 10% DMSO, PBS-T (PBS & Triton X-100) |
| Primary Antibody Staining | CD31, DLL4 |
| Secondary Antibody Staining | Goat Anti-Rat IgG (H+L), Highly Cross-Adsorbed (for CD31); Goat Anti-Rabbit IgG (H+L) (for DLL4) |

Table 2.4: Time duration for IHC staining

| Process | Time Duration | Temperature & Speed |
|---|---|---|
| Washing | 30 minutes | 37°C |
| Blocking | 2 days | 4°C |
| Primary Antibody Staining | 1 day | 4°C at ~15 rpm |
| Secondary Antibody Staining | 1 day | 4°C at ~15 rpm |
| Washing | 1 day | 37°C |

## 2.3 Imaging Setup

The imaging setup was a custom axially swept light-sheet fluorescent microscope (Figure 2.4). In order to get a high resolution to view the organs, a distinctive frequency was input into a motor for each organ. This way, each organ was able to be imaged at the best resolution possible. The samples imaged were held in a cuvette and immersed in the clearing medium used (i.e., PEGASOS or BABB) (Figure 2.5). The RI of PEGASOS is 1.543 (Jing et al. 2018) while BABB is 1.559 (Becker et al. 2012). To hold the sample up to the laser, a sample holder was created in SolidWorks as well, and a glass slide was used to put the organs in place for imaging (Figure 2.5).




AC1, AC2: Achromatic lens doublet
SA: Slit aperture
LFA: Linear focus actuator
OL1: Objective lens
HWP: Half wave plate
PB: Polarizing beam splitter
QWP: Quarter wave plate
CL: Coil actuator
OL2: Illumination objective lens
OL3: Detection objective lens

Figure 2.4: Schematic for custom AS-LSFM setup

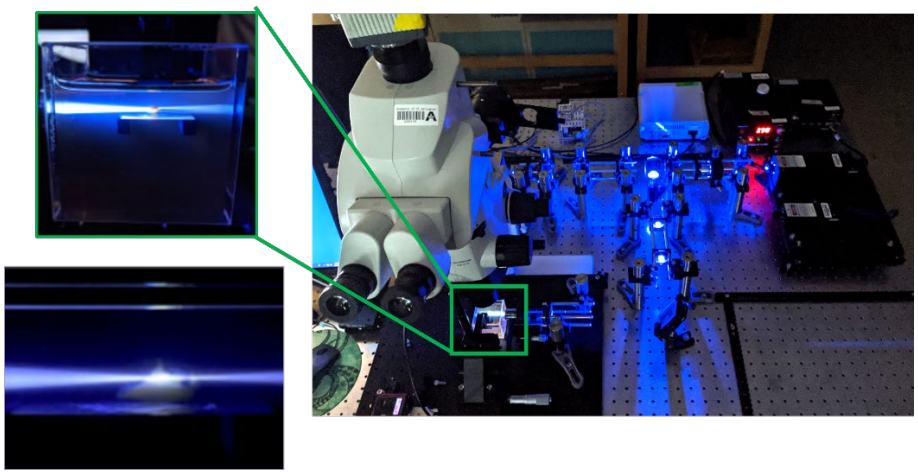


Figure 2.5: Image of microscope setup with organ in cuvette holder (left side pictures)

After the entire sample was scanned, the image stack was taken into ImageJ, an image processing application (Figure 2.6), and a 3D reconstruction was generated. There are features such as “Subtract Background” and “Variational Stationary Noise Remover (VSNR).” The VSNR plugin was used to remove stripes from 2D and 3D images. This was done by inputting numerical values for $\sigma_x$, $\sigma_y$, and $\Theta$ to create a shape that matched the thickness and direction of the stripes in the image.

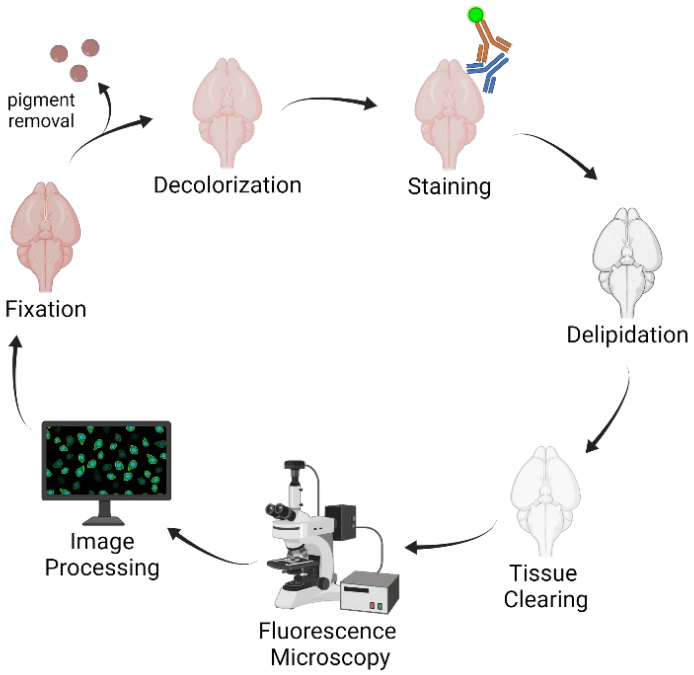



Figure 2.6: Overall tissue clearing and imaging process, created with BioRender.com

# CHAPTER 3

# RESULTS

This project showed that PEGASOS is the best clearing medium for maintaining autofluorescence while BABB is the best clearing medium for optical transparency. Fluorescent beads were used to determine the resolution of the microscope. The calculations for the axial and lateral resolution both showed to be 2 μm, respectively (Figure 3.1).

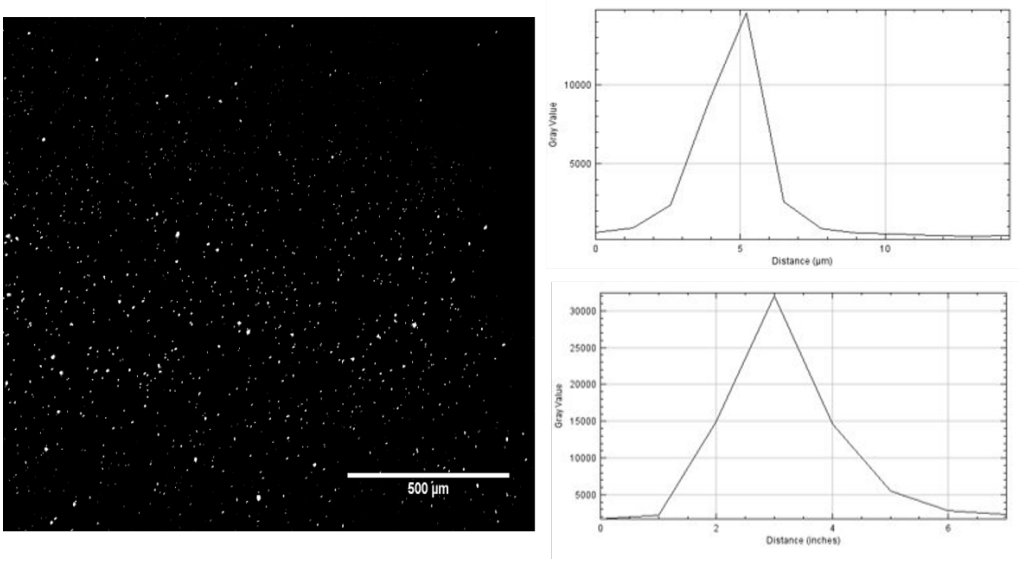



Figure 3.1: Fluorescent beads (left) used for calculating axial (bottom right) and lateral resolutions (top right)

As for the tissue clearing process, progress photos were taken for the steps. A mouse heart was cleared in PEGASOS and progress photos for post-fixation, post-decolorization, post-delipidation, and post-clearing were taken. (Figure 3.2).

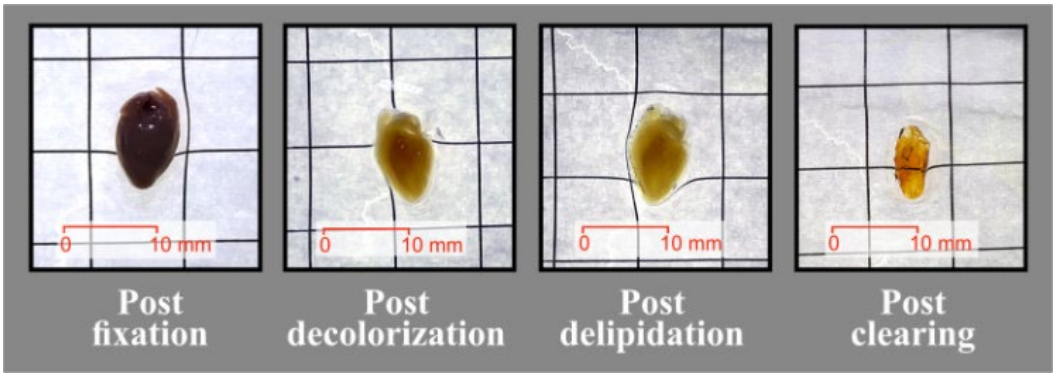



Figure 3.2: PEGASOS cleared heart progress photos

The BABB kidney was the most optically clear and a 3D reconstruction was obtained without and with axially sweeping (Figure 3.3). As for the PEGASOS heart, it was not fully cleared. Only the top section of the heart was clear enough for imaging (Figure 3.4). Since there was no axially sweeping for this image acquisition, the VSNR plugin from ImageJ was used to remove majority of the stripes from the image.

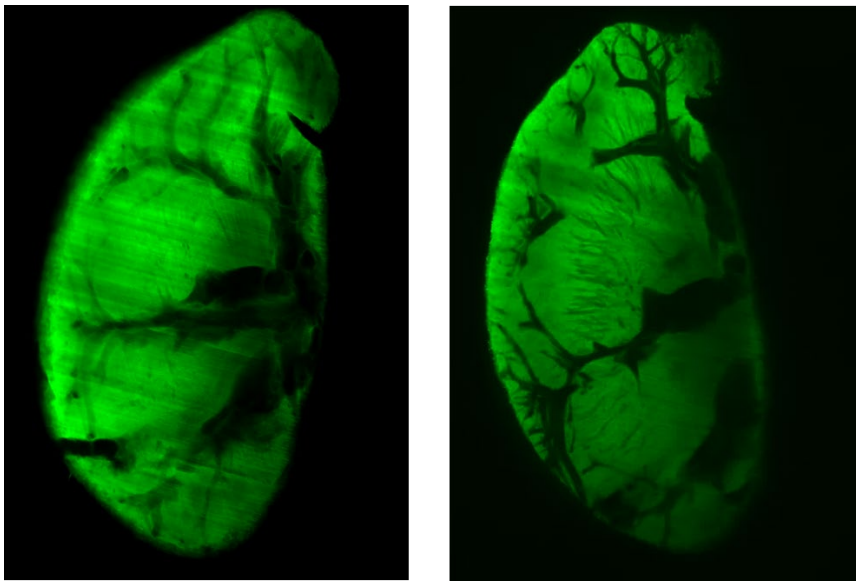


Figure 3.3: 3D reconstruction of BABB kidney without (left) and with axially sweeping (right)

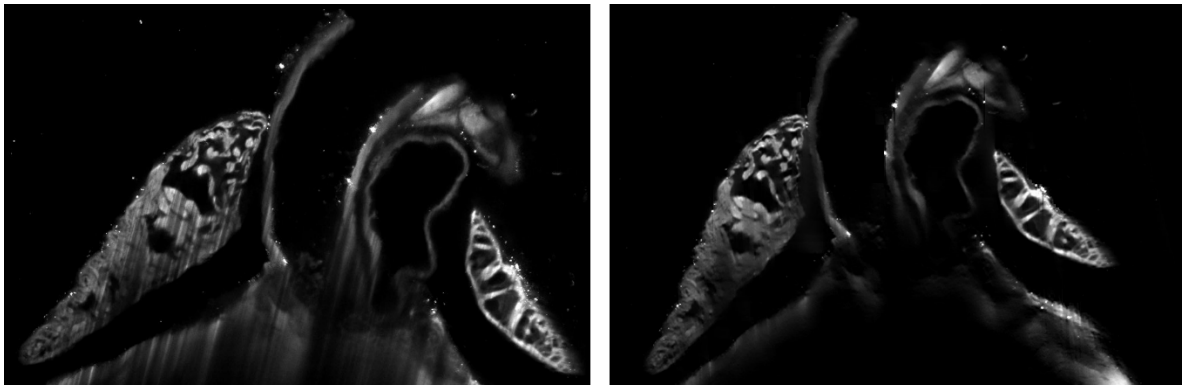


Figure 3.4: 2D image of PEGASOS heart without axially sweeping before (left) and after (right) VSNR processing

The PEGASOS heart was stained with CD31, but the image acquisition showed no signs of staining. In addition to the BABB kidney and PEGASOS heart, a PEGASOS lung was imaged as well (Figure 3.5). Again, there were no signs of endothelial cell staining with CD31.

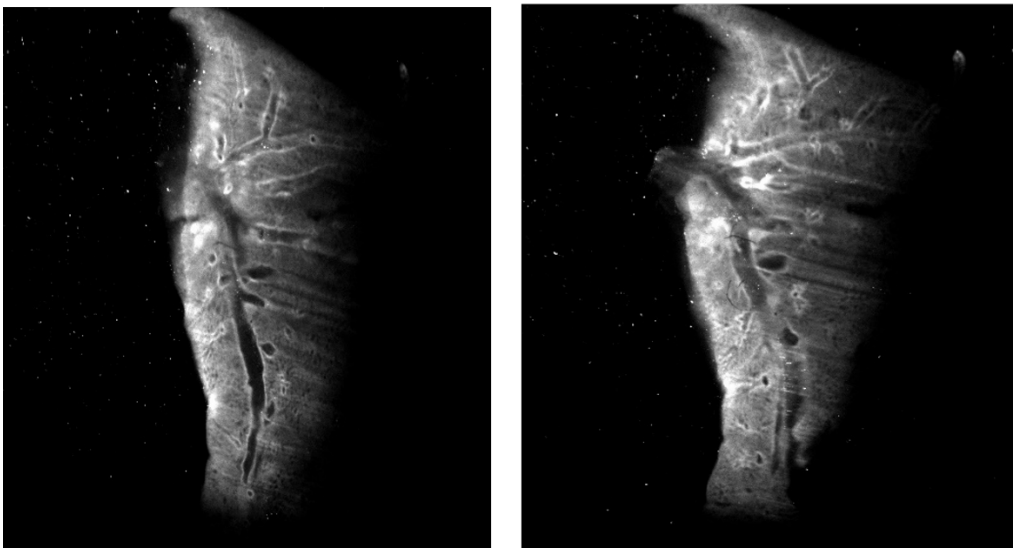


Figure 3.5: 2D slices of PEGASOS lungs with axially sweeping

# CHAPTER 4

# DISCUSSION & FUTURE WORK

This project showed that PEGASOS is the best clearing medium for maintaining autofluorescence while BABB is the best clearing medium for optical transparency. Although PEGASOS is good for maintaining the natural luminescence of an organ, it did not clear the organ as well as BABB. For example, the PEGASOS heart that was imaged was not as transparent as it could be, so the image acquisition was limited to a small section of the heart. For BABB, the main issue is that it is well known for quenching green fluorescence protein (GFP) which is the emission color for our camera (Becker et al. 2012).

Overall, the findings from this experiment will be helpful for the future testing to be done. Optimization for all areas (tissue clearing, staining, and LSFM imaging) need to continue, but substantial progress has been made in all areas. Additional quantifications need to be measured to determine the best clearing solution as well.

Due to lack of time, we were unable to measure the shrinkage ratio of all the organs cleared. For the continuation of this project, more time should be spent on comparing the shrinkage displayed between PEG1 and BABB for better comparison. Most clearing mediums are known to either shrink or swell organs. Since PEG1 and BABB are organic solvent based clearing methods, they shrink organs. There could be a possibility of exploring other types of clearing methods such as aqueous based ones since they swell organs. Since they increase the size of the organs after clearing, they are generally easier to image when compared to shrunken organs.

When I initially started this project, I considered finding a better alternative for benzyl benzoate. I initially proposed to change it to an acetone-based chemical. This one would not be corrosive to plastic so it will be easier to image without melting the sample holder. More research on this is needed to be done before this change is implemented. Pure acetone was tested in one of the different clearing techniques (PEGatone) but there was no significant improvement in clearing. It is possible that another chemical to try would be benzyl acetone. It has a similar chemical structure to benzyl benzoate but, again, it is not as corrosive.

Although time did not permit, utilizing the organ slicer that was created would be helpful for testing samples as well. Without the organ slicer, the 2 mm slices used for testing were not accurate and the edges were rough and tearing. In addition, it will make it faster to cut and test the slices. It will also limit the potential aberrations created from cutting the organs with more precise and clean cuts.

Work should also be more focused on improving the protocol for PEG1. The RI matching for PEG1 did not perform as well as BABB, but the drawback to using BABB is that it is known for quenching green fluorescence protein (GFP) and shrinking organs. It is possible that BABB has a stronger tendency to quench fluorescence due to a higher percentage of benzyl benzoate in it. Both PEG1 and BABB contain benzyl benzoate, but PEG1 only has about half of the amount of benzyl benzoate that BABB has. Another reason to replace benzyl benzoate is that it is corrosive to plastic. Throughout this project, imaging with BABB medium caused our sample holder to melt a great deal of times.

Another area that needs further enhancement is the immunohistochemistry staining. All the standard protocols were followed but the staining was difficult to view

during imaging. As previously mentioned, it is possible this is due to the fact that the excitation for the antibodies matched the excitation for the autofluorescence. In order for a differentiation or contrast to be seen, a dual-excitation setup might have to be used. For example, a fluorescent microscope set up with a blue laser hitting the sample on one side and a red laser hitting the sample on the other side. The creation of this will need to be precise because the frequency of each motor will have to match each other for a high-resolution image. Nonetheless, the staining protocol still has room for improvement, and the staining will continue to be done through indirect immunofluorescence.

As for the decolorization process, the use of Quadrol was good at removing the pigmentation from organs. The only difficulty was removing the pigmentation from the heavily colorized organs such as the heart and liver. Another decolorization protocol to test would be the combination of CHAPS/NMDEA and Quadrol. CHAPS/NMDEA could be used for the first day to increase the permeability of the organs and make them more susceptible to depigmentation, then Quadrol could be used in the remaining days to finish the process. This will be more cost-effective in comparison to only using CHAPS/NMDEA for decolorization because CHAPS is an expensive chemical to buy. It would also be interesting to see how the combination of CHAPS/NMDEA and Quadrol could improve the decolorization process.

Lastly, in the standard tissue clearing process, there is an additional dehydration step that takes place. In this project, this step was not utilized so it is possible that the results of the clearing process could be improved with the implementation of the dehydration process. This will follow right after the delipidation and prior to clearing and will add approximately 1 day to the clearing process.

There are numerous benefits to imaging. It allows for a better understanding of the structures and how the body works and flows. The resolution for trying to achieve optical transparency and high resolution is because cellular structures need micrometer-scale resolution to be properly viewed (Zhao 2020). Physical sectioning allows for better penetration but disrupts the natural structure of the organ. For a more accurate depiction, whole organ imaging and staining is the leading solution. It is a difficult feat, but innovative researchers are working to bridge the gap between theory and application.

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## BIOGRAPHICAL INFORMATION

Toluwani Ijaseun will graduate in May 2022 with an Honors Bachelor of Science in Biomedical Engineering with a concentration in Tissue Engineering. She has worked on various projects, such as neural network training for automatic segmentation of the heart, tissue clearing protocols, immunohistochemistry staining, and fluorescence microscopy imaging. She is interested in cardiovascular research and hopes to pursue a career path in that field. Additional projects that she has worked on include an American Sign Language Translation device for her Senior Design Project. She, and the other members of her team, worked on incorporating fingernail components to the device to increase the accuracy. In addition, she also improved the marketability of the device by incorporating an LCD screen for visual output of the predicted letters.

She currently plans to pursue an MD/Ph.D. in cardiovascular medicine. Prior to that, she would like to work in the biomedical industry and learn more about medical devices and product design. She would also like to work on more clinical research as well. If time permits, she will take more classes in Medical Humanities. She enjoys the subject matter and believes it is the best way to learn how to better serve patients and improve the patient-provider relationship, as well as the overall healthcare experience.