3D MICROSTRUCUTURAL CHARACTERIZATION OF

HEART VALVE LEAFLETS

by

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ABSTRACT

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Valvular heart disease is common in the world today and understanding the three-dimensional microstructures between the various heart valves is very important for developing effective treatments. For this study, four heart valves were retrieved from the heart of a pig as they closely mimic the human's heart. The purpose of this study was to obtain the three-dimensional microstructures of the mitral, pulmonary, aortic, and tricuspid valve after tissue clearing and imaging with a light-sheet microscope. The tissue clearing process is essential to the success of this project as it entails procedures that render the samples transparent for in-depth imaging of the heart valves using the light sheet microscope, which is a technique that allows one to view the three-dimensional imaging of a structure. Results retrieved from these comparisons set the foundation for researchers or medical personnel to study the patterns of the valves for better diagnosis, treatment, and prevention of valvular heart diseases.

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CHAPTER 1

INTRODUCTION

1.1 Background

Valvular heart disease is a condition in which there is a complication with one or more of the heart valves including but not limited to: mitral, aortic, pulmonary, and tricuspid. Examples of this disease include valvular stenosis, valvular regurgitation, and valvular atresia. Abnormalities in the structure of the heart can alter the function of the heart leading to valvular heart diseases. The heart has four chambers: the right ventricle, right atria, left ventricle and left atria. Between these chambers are where the valves which are made up of ECM are situated. The valves open and close, regulating the flow of blood into and away from the heart by preventing the back flow of blood. The sound of the heartbeat is the sound of the opening and closing of the heart valves (Dornbush & Turnquest, 2022). The mitral and tricuspid are atrioventricular valves, while the pulmonary and aortic are semilunar valves. The atrioventricular valves have leaflets while the semilunar valves have cusps. Having two leaflets, the mitral valve separates the left atrium and the left ventricle; with three leaflets, the tricuspid valve separates the right atrium and the right ventricle. The pulmonary valve separates the right ventricle and the pulmonary artery whereas the aortic valve separates the left ventricle and the aorta (Hinton & Yutzey, 2011).

Understanding the three-dimensional microstructures of heart valves is critical for the development and advancement of heart valve disease treatment. In-depth imaging of

the heart valves to understand the structure has been conducted over the years utilizing various techniques such as electron microscopy, confocal microscopy, etc. However, to better image the structural properties of the heart valves, a 3D imaging technique is needed that generates a high resolution with less complications, hence the use of the light sheet microscopy for the course of this study (Schoppmeyer, 2018). The 3D microstructural data obtained from this study will help researchers and medical professionals to better understand the mechanical behavior of heart valves.

1.1.1.1 Significance

The increased mortality rate from heart valve diseases may be caused by a structural or functional defect in the valves. To acquire knowledge on how the structure influences the function and remodeling of heart valves under changing stress and diseases, a thorough understanding of the heart valve microstructure is needed. This study reveals the 3D microstructures of the tricuspid, mitral, pulmonary, and aortic valves by using the light sheet microscopy technique, which can serve as a solid foundation for heart valve biomechanics and computational modeling.

1.2 Tissue Clearing

The comprehension of the structure of the heart valves better promotes an impact on its functionality. The biological relationship between structure and function is the reason a three-dimensional analysis is needed, hence tissue clearing procedure (Richardson et al., 2021). Tissue clearing is a procedure that reduces the opacity of biological samples to enable in-depth imaging without damaging the structure of the samples. There are three types of tissue clearing techniques, such as the organic solvent-based (hydrophobic),

aqueous, and hydrogel-embedding approach (Tian et al., 2020). In this experiment, the organic solvent-based (hydrophobic) technique was used for tissue clearing of the valves.

In this study, we performed tissue clearing on the samples to make them transparent by using various chemical solutions. The protein and lipids that form cells and tissue have a high refractive index, and for light to pass through the biological samples with reduced light scattering, the refractive index must be reduced and equalized. Tissue clearing procedure equalizes the refractive index, causing a more transparent sample (Fasoli & Florindo, 2022). This procedure also saves time by not undergoing tissue sectioning techniques which introduce artifacts.

1.2 Light Sheet Microscopy

For this study, the light sheet microscopy technique was used to image four different heart valves from a pig because it closely mimics the human heart. With the valves having two to three leaflets or cusps, one from each valve was sampled. Light sheet microscopy is a technique that allows for the 3D imaging of samples by using a thin sheet of laser light to view a sample from all angles. The microscope is placed orthogonal to the light sheet which illuminates the sample in a single plane, and because it is a fluorescence microscope, it gathers fluorescence signals from the light sheet and images the sample by using a camera. There are several other types of microscopy techniques for imaging, such as spinning disc microscopy, scanning electron microscopy, and transmission electron microscopy. As opposed to the light sheet microscopy, these techniques do not have the ability to perform three-dimensional imaging. Other techniques include confocal microscopy, two-photon microscopy, electron microscopy, etc. These techniques allow for 3D imaging but increase the risk for phototoxicity and photobleaching (Santi, 2011).

Unlike these techniques, the light sheet microscopy is advantageous because it allows for high-speed imaging with high resolution, reduces background noise, and reduces the risk of phototoxicity and photobleaching by illuminating the sample one region at a time, which reduces the laser power that causes damage to the sample (Schoppmeyer, 2018).

CHAPTER 2

LITERATURE REVIEW

Similar research like this has been carried out over the years, but none have accurately depicted the 3D microstructure of all the four heart valves. Gumpangseth et al., (2020) investigated the microstructure of the four heart valves in relation to age as it is important for treatment development of valvular disease. This was carried out by immunostaining with picrosirius red for the evaluation of elastin and collagen fibers. This experiment made use of heart valves from donors between the ages of 20-90 years of age, and it was seen that collagen and elastin increased with older aged heart valves

Carruthers et al. (2012) investigated the use of a 2D imaging technique to image one of the heart valves and examine the structure to determine how the structure affects valvular diseases. Using a multiphoton microscope, the mitral valve was imaged to explore the microstructure and uncover the fundamental causes of valve illnesses. This study does not provide sufficient information to determine how the structure of valves affect valvular diseases because it only images the mitral valve leaflet instead of all the heart valves. Also, the use of multi-photon microscopy, which is a 2D imaging technique, makes it challenging for the heart valves because the valves are 3D structures that cannot be accurately imaged by the multi-photon 2D imaging system. In general, using 2D imaging procedures also causes low resolution that restricts the ability to construct models for the valves (Ayoub 2018).

To image the structures of the heart valves, various imaging techniques have been used such as computed tomography, magnetic resonance imaging, and echocardiography. Multiple procedures have been used to compensate for each technique's shortcomings as they each cannot work independently. These approaches, however, are time-consuming and difficult, which is why for this study the light sheet microscopy technique was implemented for the 3D imaging. The advantages of using the light sheet microscopy procedure includes deep tissue penetration, high-spatiotemporal resolution, minimal photobleaching, and low phototoxicity. Overall, light sheet microscopy promotes better analysis and understanding of the valvular microenvironment (Ding et al., 2018).

CHAPTER 3

METHODOLOGY

3.1 Dissection

The heart of the pig was dissected to obtain all four heart valves by using a scalpel. Then the heart valves were submerged into Phosphate buffered saline (PBS) in four different tubes labeled accordingly. The PBS was used to preserve the valves before experiments began. The samples were stored in the fridge while submerged in PBS for about 3 weeks, and the PBS solvent was changed weekly to keep it fresh. After 3 weeks, tissue clearing procedure that entails fixation, dehydration and depigmentation, pre-clearing, and clearing was performed on the valves.

3.2 Fixation

Fixation is an initial stage in studying pathology for preserving tissues and preventing autolysis and degradation. Two types of fixative include denaturing fixatives and crosslinking fixatives. Fixation can be performed with chemicals like glutaraldehyde, methacarn, or formaldehyde (Howat & Wilson, 2011). Formaldehyde, a crosslinking fixative, was used in this study to preserve the valves for a successful experiment. The PBS was removed from the valves after three weeks, and formaldehyde was added.

3.3 Dehydration and Depigmentation

In initiating this process, the PBS in the samples was disposed and then 20% methanol and 80% deionized (DI) water solution was made. The solution made was added to four 5ml tubes that contained the four valves. The tubes were then wrapped with foil and

left on a laboratory shaker for an hour. After an hour 40% methanol and 60% DI water solution was made and then exchanged with the initial solution in the valves. The new solution was transferred to the 5ml tubes, and the tubes were wrapped in foil and left on a shaker for an hour. One hour went by and a new solution: 60%of methanol and 40% of DI water was prepared, then the solution in the valves' tubes were changed with this new one. This process was repeated for 80% methanol and 20% DI water, 100% methanol, 100% methanol again, and lastly a new solution of 100% methanol. On the final round, the solution was in the valves on a laboratory shaker for much longer (Table 3.1).

Buffer	Time duration	Temp
20ml Ch3+ 80ml DI H20	1h	RT
40ml Ch3+ 60ml DI H20	1h	RT
60ml Ch3+ 40ml DI H20	1h	RT
80ml Ch3+ 20ml DI H20	1h	RT
100ml Ch3	1h	RT
100ml Ch3	1h	RT
100ml Ch3	1 week	4℃

Table 3.1: Time duration and Temperature for dehydration & depigmentation

Solution y was prepared and used to replace the initial solution in the 5ml tubes of the heart valves after one week (Table 3.2). These valves in the tubes were submerged in solution y for 2 days, wrapped in foil and on a shaker. After 2 days, the solution was changed with 100% methanol. After 30 minutes, this process was repeated 2 more times with a one-week duration for the last solution change. After a week, solution z was prepared, and these heart valves were submerged in this solution for two days (Table 3.2).

After two days, 80% of methanol and 20% of DI water solution was made, and the solutions from the tube were exchanged with this new solution and placed on the shaker, wrapped in foil. The same process was repeated for 60% of methanol and 40% of DI water solution, 40% methanol and 60% DI water solution, 20% of methanol and 80% of DI water solution after one hour each. The initial solution in the tubes was with 1x PBS and left on the shaker for one hour. A new solution, solution x, was prepared, and the initial solution in the valves' tubes were replaced with it. The valves with solution x remained on the laboratory shaker for an hour. This process was repeated with a fresh batch of solution x, but this time around, it was left on the shaker for 5 days. (Table 3.2).

Solutions	Content	Temperature
Solution y	66ml DCM + 33ml Ch3	RT
Solution z	3ml h2o2 + 15ml Ch3	RT
Solution x	900ml DI water + 100ml of PBS+2ml of Triton X-100	RT

Table 3.2: Preparation of solutions

3.4 Preclearing

After 5 days, the samples were precleared by preparing embedded solution with 1% agarose in 1x PBS. Using 10x PBS, 1x PBS was made by mixing 5ml of 10x PBS to 45 ml of DI water. One percent agarose was made by adding 0.5 grams of agarose to 50ml of the 1xPBS. Furthermore, the agarose was dissolved by heating up in a microwave two times. First it was heated for 30 seconds, and it was then heated for an extra 15 seconds to ensure the agarose solution was completely clear. The agarose solution was heated and left to cool to 40°C. The heart valve leaflets were placed on different weighing dishes with the

agarose poured over them. After 40 seconds, the texture of the agarose started to change with the valves submerged in it. When it was jelly-like, the part of the heart valves in the agarose was dissected within the 5mm to 1cm size range.

3.5 Clearing

The dissected jelly-like agarose valves were placed in four new 5ml tubes. The clearing process began by mixing 20 ml methanol and 80 ml DI water and added to the 5ml tubes and left in a foil on a shaker for one hour. The same was done but the solution was replaced with a 40 ml methanol and 60 ml DI water solution. After an hour, the solution was replaced with a 60 ml methanol and 40 ml DI water solution, and the same was done with an 80 ml methanol and 20 ml DI water solution. After an hour, the solution was replaced with 100ml of methanol. An hour later, a fresh batch of 100ml methanol was replaced with the initial methanol and the tubes were left overnight on a shaker. The next day we replaced the solution with a new solution of 66 ml of dichloromethane and 22 ml of methanol. This solution was left in the tubes for a week. After a week, the solution was replaced with the dibasic ester, and it was left for exactly one week then the light sheet microscopy technique was performed to image the samples.

3.6 3D Slicer Software

We obtained the images from the light sheet microscope as a tiff file then imported them to the 3D slicer software. The 3D slicer software is used to analyze the three-dimensional images of the human body, mainly for medical applications such as disease diagnosis, treatment, computational simulation, etc. (Fedorov et al., 2012). The volume rendering feature on the software was used to display region of interest and using the crop volume feature, the valve was to range between 60mm to 90mm. The segment editor

feature enabled the use of thresholding, scissor tool, and 3D model build. The thresholding feature was effective in separating the heart valves from its surrounding tissue. The 3D model build feature was used to build the segmented valve on the 3D workspace. Next, the scissor tool was used in the 3D workspace to cut out the part that was not needed. Lastly, the segmentation feature was used to save the file as an .stl file for 3D printing of the 3D model created.

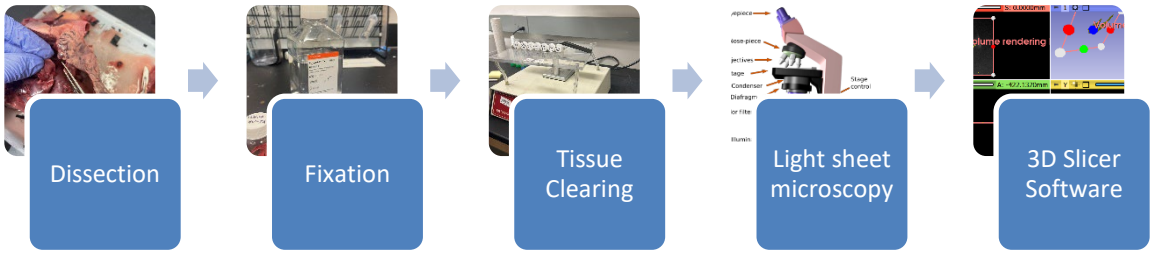

Figure 3.1: Schematics of the methodology process

CHAPTER 4

RESULTS

The tissue clearing process achieved great results in rending the valves transparent for in-depth imaging.

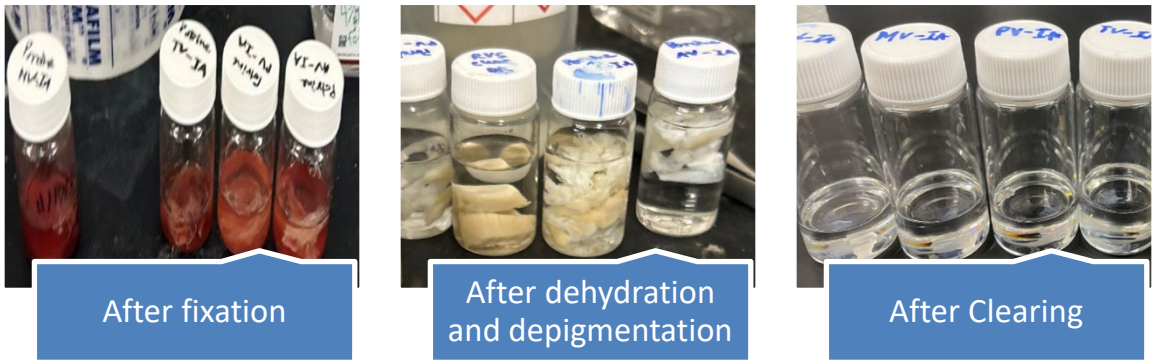

Figure 4.1: The visual result of the valves after each procedure and their transition from opacity to transparency.

After tissue clearing, the heart valves were imaged under a light sheet microscope to develop the three-dimensional image of the valves. The images were obtained with a magnification ratio ranging from 2x-3.2x. Then the images achieved from this technique were uploaded to 3D slicer software to create 3D models. The three-dimensional model of a structure appears bigger than its actual size because of the level of detailing it projects. After building the model, it was analyzed quantitatively by measuring the surface area and volume in millimeters.

Structure	Volume (mm^3)	Surface area (mm^2)
Mitral valve	66145	32784
Pulmonary valve	6193	12663
Tricuspid valve	42460	28052
Aortic valve	5356	11028

Table 4.1 Quantitative analysis

The images for the aortic and tricuspid valves were the largest and so were the sizes of their 3D models. Their models were thick and layered unlike the mitral and pulmonary valve, which were thin. The 3D slicer software, initially, could not produce a 3D model for these images without cropping them. To ensure fairness, the valves were cropped within a width range of 60mm to 80mm and a length range of 80mm to 90mm.

The model of the mitral valve appears thick and layered and spongey. The image from the light sheet microscope was thresholding on 3D slicer to highlight only the valve and exclude any surrounding tissue (Figure 4.2a).

a b

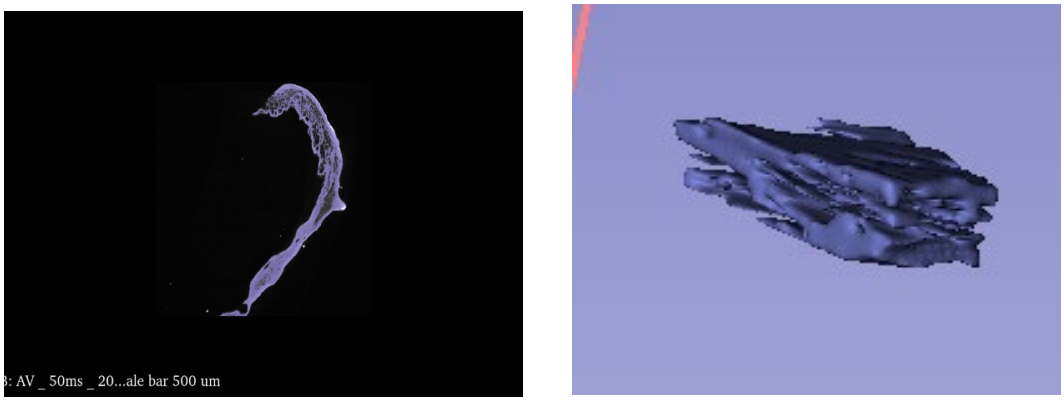

Figure 4.2: a.) Thresholded image of the mitral valve. b.) The 3D model of the mitral valve on 3D slicer software.

The aortic valve on the 3D workspace of the 3D slicer software shows the thinness of the valve; it was without layers and the surface was smooth. The model has rough edges because of an imperfect cropping.

a b

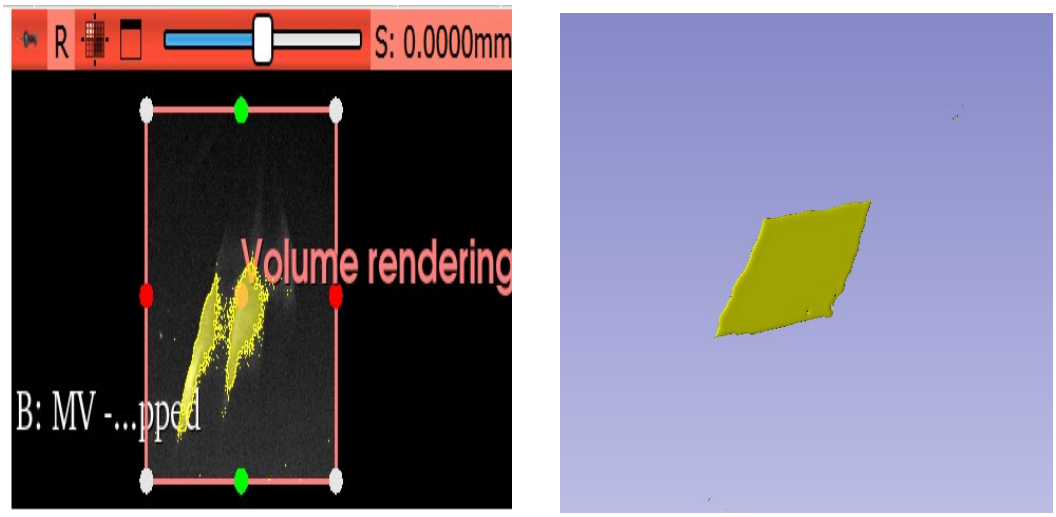

Figure 4.3: a) The cropped image of the aortic valve before it was three-dimensionally plotted. b) The 3D model of the aortic valve

The model obtained for the pulmonary valve was similar to that of the mitral. After thresholding the image from the light sheet, three parts were highlighted and created on the 3D workspace, but after cropping, a little piece of the thresholded portion was obtained.

a b

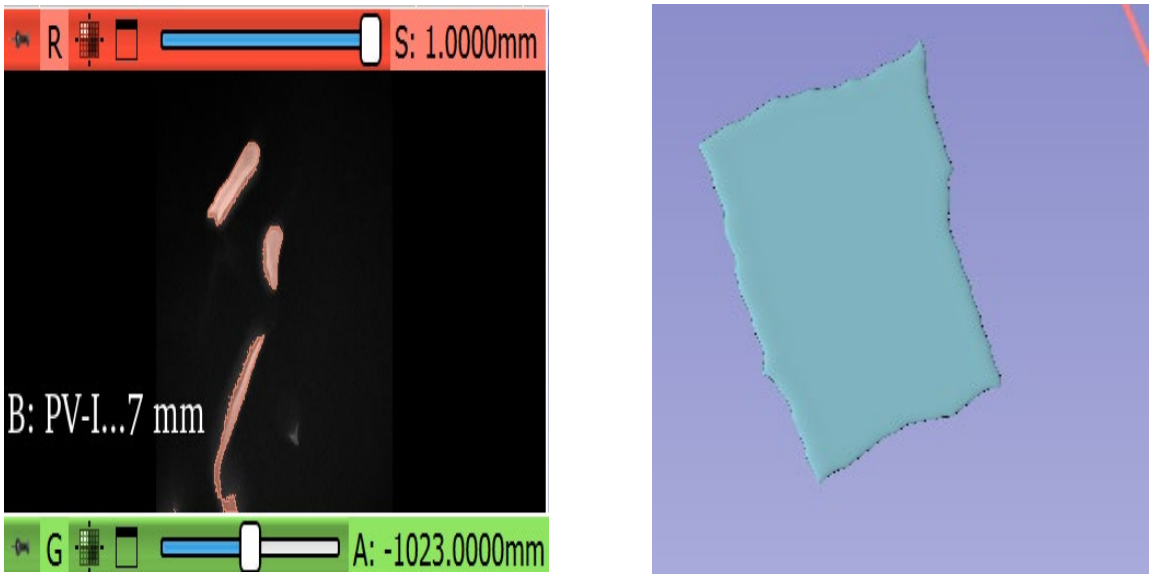

Figure 4.4: a.) The image of the pulmonary valve obtained from the microscope. b.) A section of the image in 3D

The 3D model of the tricuspid valve resembled the aortic valve. Though it seemed to have more layers, but it appeared less thick in comparison to the 3D model of the aortic valve. The tricuspid valve's image obtained from the light sheet microscope appeared the biggest on 3D slicer.

a b

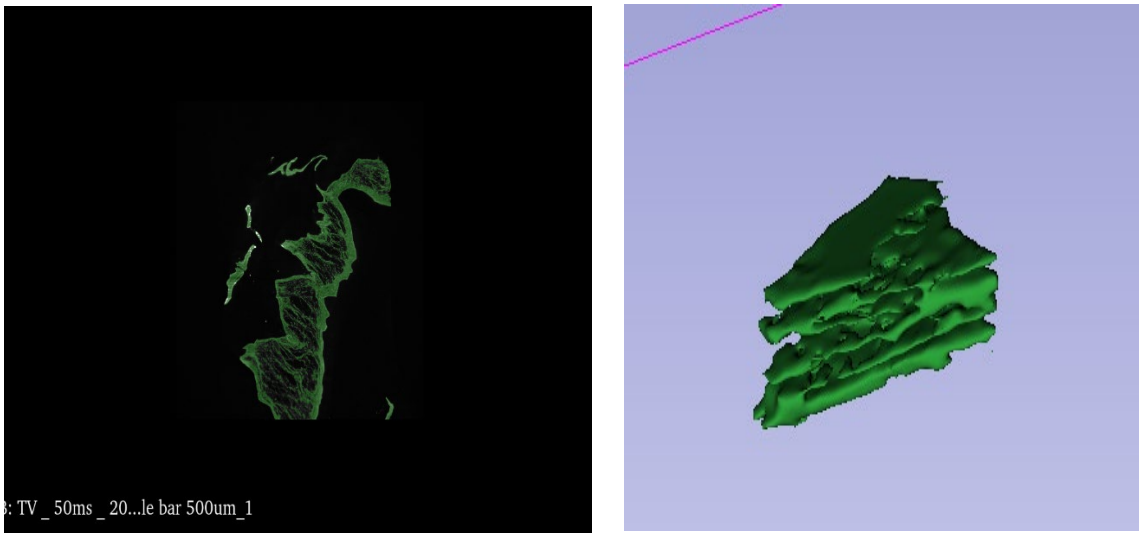

Figure 4.5: a) Tricuspid's valve image from the microscope. b.) The generated 3D model of the tricuspid valve.

CHAPTER 5

DISCUSSION AND CONCLUSION

The heart valves differ in terms of surface area, volume, and thickness. Though all heart valves have similar functions, their structure varies as they all have unique leaflets and cusps that influence their susceptibility to diseases. Typically, the thickness of heart valves is usually less than 1mm. The atrioventricular valves are thicker than the semilunar valves, and the valves on the left are somewhat thicker than the valves on the right (Hinton & Yutzey, 2011). The mitral valve, an atrioventricular valve, is said to be the thickest valve, followed closely by the tricuspid valves, which is also an atrioventricular valve (Misfield & Sievers, 2007). In this study, we see that the results obtained do not vary from previous research findings. The mitral valve was indeed the thickest, and the tricuspid was second thickest, due to their similarities as they are both located between the atrium and ventricles. From the quantitative analysis of the 3D models, the mitral valve has the greatest surface area and volume compared to the other heart valves, closely followed by the tricuspid valve. The tricuspid valve has a smaller surface area than the mitral valve because its orifice is larger than the mitral valve's orifice (Misfield & Sievers, 2007). It is already known that both valves are thicker than the aortic and pulmonary valve because they are atrioventricular valves, but it could also be because they both have thick leaflets that are attached to chordae tendineae, which connect the valves to the papillary muscles (Rajiah et al., 2019).

Research shows that the thinnest valve is the aortic followed by the second thinnest, the pulmonary valve. (Brazile et al., 2019). This study proves the same as previous research findings. The information obtained from the quantitative analysis of this study shows that the aortic valve has the smallest volume and surface area, followed closely by the pulmonary valve. Similarities occur because they are both semilunar valves and they are both present between the ventricle and two major arteries. Also, the semilunar valves have thin cusps and lack the chordae tendineae, which the atrioventricular valves have, and this could be a reason for the small surface area and volume.

Overall, the results match research findings, but better 3D images could have been obtained. For future experiments, it would be best if every region of each valve was imaged for better analysis and to obtain more information that will be useful to medical personnels creating treatments for valvular heart diseases.

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BIOGRAPHICAL INFORMATION

Ifejola Adebo joined the Honors college in her freshman year, Fall 2019. She will graduate in May 2023 with an Honors Bachelor of Science in Biomedical Engineering with a concentration in Tissue Engineering.

Ifejola participated in the UTA Smart Hospital internship in Spring 2022 where she designed and constructed a simulated thermometer for simulators. She has also worked on modifying an air bubble sensor for ECMO. She was a part of the department of biomedical engineering's first clinical immersion program, where she was a student observer at Cook Children's Hospital.

Ifejola plans to pursue a master's in biomedical engineering in Fall 2023. In the future, she sees herself working in the biomedical engineering field.