QUANTIFYING CARDIAC MICROSTRUCTURE USING AUTOMATED TWO-PHOTON MICROSCOPY

by

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Abstract

Two-photon fluorescence microscopy is a three-dimensional imaging technology based on the nonlinear excitation of fluorophores. It has been widely used to map the 3D pattern of neuronal connections in the brain, and for reconstructing anatomical structure of organs such as skin, kidney and others. Here, we present the design of an automated imaging system using low cost, highly modular Automated Volume Imaging Machine (AVIM) and its interface with a Zeiss 7MP microscope. We apply this system to mapping murine cardiac structure.

Primary Reader: Dr. Raimond Winslow (Advisor)
Secondary Reader: Dr. Feilim Mac Gabhann, Dr. Sonia Cortassa
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Chapter 1

Introduction

1.1 Two-Photon Fluorescence Microscopy

1.1.1 Overview and Advantages

Two-photon fluorescence microscopy (TPM), invented by Denk et al in 1990 [1], is a three-dimensional (3D) imaging technology based on the nonlinear excitation of fluorophores. Traditional optical microscopy techniques, which use linear (one-photon) absorption processes for contrast generation, are limited to use near the tissue surface (less than 100 µm) for high-resolution imaging, because at greater depths strong and multiple sources of light scattering blurs the images. In comparison, nonlinear optical microscopy techniques such as TPM have special features that make them less sensitive to scattering and thus are well suited for high-resolution imaging in tissues [2].
TPM is considered a revolutionary development in biological imaging because of its four unique capabilities. First, TPM greatly reduces photodamage and allows imaging of living specimens. Second, TPM can image turbid specimens with sub-micrometer resolution down to a depth of a few hundred micrometers. Third, TPM allows high-sensitivity imaging by eliminating the contamination of the fluorescence signal by the excitation light. Fourth, TPM can initiate photochemical reactions within a sub-femtoliter volume inside cells and tissues [3].

Neuroscientists in particular have used this technology to image physiological functioning in microscopic and subcellular neural compartments [4]. In addition to the brain, specimens as diverse as lymphatic organs, kidney, heart, and skin can now be examined in detail at depths of up to one millimeter, while leaving the tissue intact [2]. Moreover, TPM is used as a tool to study the development, progression and potential treatment of pathological conditions such as tumors [5] and Alzheimer’s disease [6].

1.1.2 Brief Overview of TPM

Fluorescence is the process of photon emission by a molecule subsequent to the excitation of that molecule by absorption of a photon. It is the result of the molecule or fluorophore undergoing a three-stage
process of 1; excitation, 2; internal conversion, and 3; emission. Two-photon-excited fluorescence differs from single-photon-excited fluorescence only with regard to stage one of the three-stage fluorescence process. In two-photon excitation, a fluorophore accomplishes the transition from its ground state to an excited state by the near-simultaneous ($\sim 10^{-16}$ s) absorption of two photons. One photon excites the fluorophore to a ‘virtual’ intermediate state while the second photon further excites the fluorophore to the excited state. This is demonstrated in Figure 1.1. The two photons have approximately half the energy and double the wavelength of the photon required for a single photon excitation quantum event to occur [4].

![Jablonski Diagrams](image)

*Figure 1.1 Jablonski Diagrams showing the three-stages of excitation, internal conversion, and emission (labeled 1-3 in A) involved in the process*
of fluorescence induced by the absorption of a single photon (A) or two
photons (B), based on [4].

The probability of a two-photon absorption event occurring within a
fluorophore is extremely low. This is illustrated by the fact that although
the two-photon phenomenon was predicted by Maria Goppert-Mayer in
her doctoral dissertation in 1931 [7], the first investigations of this
phenomenon only became possible with the advent of laser sources that
provided the required high photon flux densities [4]. The
Titanium:Sapphire crystal-based laser is the typical choice for biological
laser scanning microscopy in TPM system [8]. This laser utilizes a
titanium-doped sapphire crystal as a gain medium that allows
wavelength tunability from ~700 - 1000nm.

1.2 Application to Measuring Cardiac

Microstructure

The structure of the heart is highly organized and is a key determinant of
its electrophysiological and mechanical properties. The myocytes are
organized into myofibers which run in various orientations through the
heart. In addition to the myofibers, the myocardium also consists of
nerves, blood vessels and lymphatics, embedded in ground substance,
bathed in interstitial fluid, and bonded together by a weave of collagen
A systematic description of this arrangement is necessary for complete understanding of the cardiac function.

It is now well known that the myocardial wall is a well-ordered fiber wound continuum of interconnected muscle fibers, as first noticed quantitatively by Streeter et al., 1969 [10]. It has been well documented that propagation velocities of electrical conduction are several times faster along fibers than in the transverse directions [11] making fiber organization the most important determinant of the activation sequence. Alterations in the normal myofiber architecture occur in diseases such as ischemic heart disease and ventricular hypertrophy [12]. These alterations may predispose the myocardium to abnormal electrical propagation and arrhythmia. Fiber organization also plays an important role in the generation of myocardial stress and strain and in the structural adaptation of the myocardium [13]. Therefore, a detailed quantification of the myofiber architecture is of great interest.
1.3 Dissertation Outline

1.3.1 Setting up and working of an Automated Volume Imaging Machine (AVIM)

Tissue structure including cardiac fiber orientations were first measured using histology [10]. Specialized automated histology techniques to measure the trans-mural fiber orientation gradient have been implemented using confocal microscopy [14]–[17]. However these studies have not acquired a whole mammalian heart, only chunks of tissue in the myocardium. In addition, confocal microscopy cannot image as deep into tissue as two-photon microscopy due to increased scattering of light with depth [18]. Whole mouse brain imaging using histology and TPM have been performed to trace neuronal connections [19]. A technique based on tissue cytometry and high-speed two-photon microscopy [20]–[22]. However there is no documented procedure of the technique being used for cardiac tissues to quantify cardiac structure.

In addition, by characterizing the anisotropy of water diffusion in fibrous tissue structures, Diffusion Tensor Magnetic Resonance Imaging (DTMRI) has also been used to quantify the orientation of the fibers and sheets in 3D [12], [23]–[25]. However the resolution of the acquired images obtained using DTRMI is of the order of hundreds of microns compared
to the submicron resolution (~200 nm) of a TPM system. A 21 Tesla magnet can at best produce an in plane resolution of 35 microns [26]. These systems are not commonly available and have high cost. TPM also allows external labeling by the use of fluorophores and hence any tissue and structure can be visualized by using appropriate dyes.

This dissertation outlines the set up and operation of an indigenously developed AVIM which is interfaced with a Zeiss LSM 7 MP Microscope and is used to acquire an entire small mammalian heart image volume.

1.3.2 Reconstruction, Visualization and Orientation Calculation of Cardiac Structure.

TPM acquires images in the form of ‘tiles’ and these ‘tiles’ have to be assembled in order to build an image cross-section, or ‘slice’ [27]. The slices are generated from optical stacks and mechanical sectioning (cut-step), in a process similar to that described in [14]. In addition to stitching the ‘tiles’ into ‘slices’, in-plane and out-of-plane (depth) corrections, and registration are performed on the images to improve the overall quality of the images acquired and the volume visualized. These image slices are of the order of hundreds of gigabytes and various visualization software were tested for efficient 3D volume visualization.
We review the algorithms used for reconstruction pipeline and visualization software tried in Chapter 3.

Finally, in Chapter 4 we describe an algorithm for calculating the cardiac structure orientation from the intensity values [29] generated by the TPM detectors has been implemented and cardiac structure angle has been quantified.
Chapter 2

Automated Two-Photon Microscopy Imaging for a whole small Mammalian Heart

2.1 Introduction

An Automated Volume Imaging Machine (AVIM) was designed by members of Dr. Raimond Winslow’s lab at the Johns Hopkins University. The AVIM is interfaced with a Zeiss LSM 7MP microscope. The highlights of this design are its low cost and its modular nature, such that it can be easily interfaced with any microscope system. Briefly, it involves a 3D stage, which is used to position the sample below the microscope objective, controlling software that moves the stage to acquire images of the exposed tissue surface, a microtome used to cut away imaged tissue, and an actuator to push the sample across the microtome blade. This is depicted in Figure 2.1.
Figure 2.1 The AVIM design. The sample is placed atop the embedding cassette, (orange). The automated 3D stage (yellow) positions the sample beneath the objective. Mechanical sections are performed using the actuator, (blue) pushing the linear slide (green) across the blade (red).
Figure 2.2 Zeiss LSM 7 MP interfaced with the AVIM. The objective is connected to the microscope via an inverter and images the sample on the 3D stage.
The sample is labeled with appropriate dyes, fixed, and embedded in a paraffin block. The paraffin block containing the sample is then placed atop a 3D stage and under the microscope objective lens. ‘Z’ optical sections (‘tiles’) are collected at a particular ‘x’ and ‘y’ location, and then the stage is moved to an adjacent position and ‘z’ optical stacks are collected again. In a similar manner, the entire surface plane is imaged, to a certain depth supported by the optical sectioning capabilities of the TPM. Following this, the entire plane is sectioned at a predetermined cut depth, with the help of an actuator pushing the paraffin block containing the tissue sample across a microtome blade with the help of a linear slide. The new exposed surface is then imaged in the manner described above. The process is repeated until the entire sample has been imaged. Specifically, our ‘z’ optical stack depth was 130 microns, with optical sections collected at 10 micron intervals. The cut depth was 40 microns, and the ‘x’ and ‘y’ stage translation was 750 microns for a 10x objective lens. These numbers gave sufficient redundancies to account for any calibration and alignment errors and were determined after extensive trial and error. This entire is process is better understood with the help of Figure 2.3 and Figure 2.4. It should be noted that the portion of sample removed is less than the portion imaged (depth of optical stack is greater than the cut depth). The portion of the sample remaining after a
cut will be imaged again as a reference for image registration performed later. This is explained later in the Chapter 3 regarding reconstruction.

**Figure 2.3** Image acquisition process. Individual optical stacks are collected at each position over the surface of the sample. The sample is
sectioned with a microtome, leaving a portion of the sample imaged prior. Then the process is repeated until complete.

Figure 2.4 Image acquisition process shown from another point of view

2.2 Uniform labeling of a mouse heart

In order to have correct approximation of the cardiac structure in a rodent heart, it is important to use appropriate dyes that label the cardiac structure. Studies showcasing cardiac structure using histology sections typically use Hematoxylin & Eosin stain [30] or even Toluidine
blue [31], however they can’t be applied in our case since we are not imaging just the surface and also these are contrast dyes. This presented a unique challenge with regards to tissue preparation, since we need specific fluorescent dyes compatible with our setup.

A protocol was developed for uniform labeling of the myocardium using fluorescent markers compatible with the AVIM. The main compatibility criteria is that the dye should be able to distribute uniformly across the entire tissue. The entire protocol is listed in Appendix A. Protocol for Perfusing and Labeling a Mouse Heart with Di-4-ANEPPSBriefly, the animal would be first given a dose of sodium pentobarbital i.p. as a sedative, followed by a dose of heparin through the jugular vein. The chest would then be opened up and the heart would be excised. The aorta would be located and cardioplegic solution (High K⁺) would be retro-gradely perfused at constant flow with the help of a syringe pump. Following stabilization (~ 3 mins, and all the blood has cleared out), the heart would be perfused with the dye and then fixed using pH-balanced paraformaldehyde (PFA) solution. The heart would then be taken to pathology for embedding in a paraffin block. An unexpected difficulty encountered during development of the protocol was the non-uniform loading of the fluorescent dye, creating intensity
variations in the acquired images that lacked details in certain regions of the heart. This is demonstrated in Figure 2.5.

![Image of heart sections]

*Figure 2.5 Non-uniform signal intensity across the heart wall. A. Structural detail is lost at the epicardium due to saturation in signal. B. The endocardium loses finer detail due to lack of signal.*

Initially we tried using one of the lipophilic carbicyanine membrane dyes, DiO and Dil which have been used extensively for neuronal pathway tracing [32][33] and to label cardiac cells [34]. Unfortunately we were never able to get consistent labeling using them (Figure 2.5 A). It was also difficult to keep the dye in solution as precipitates were noticed in the coronary arteries (Figure 2.6).
Figure 2.6 DiO crystals not perfusing well and getting stuck in the cardiac-vascular structure

After the unsuccessful attempt at using DiO and DiI, we tried using Di-4-ANEPPS as reported to be used in [29] and [35] for cardiac structure. Di-4-ANEPPS is a potentiometric dye which intercalates among the lipophilic parts of biological membranes [36]. Labelling with Di-4-ANEPPS produced a more uniform imaging intensity. This along with changes such as using pH-balanced PFA as a fixative and limiting the exposure to the fixative to only 2 hours helped in reducing the non-uniform labeling. Figure 2.7 shows an image slice depicting appropriate labeling following the use of Di-4-ANEPPS and the protocol mentioned in Appendix B.
Figure 2.7 Image Slice obtained from a mouse heart and labelled with Di-4-ANEPPS and prepared using the developed protocol.

2.3 **Distortion of the heart wall**

During the perfusion, labelling, and processing of the heart, due to improper handling, such as the heart lying on the wall, the wall of the
heart can be compressed. Such a heart cannot be used to quantify the cardiac structure. An example of this is shown in the Figure 2.8

Figure 2.8 Distorted heart wall. Cardiac structure cannot be quantified for such hearts as the myocytes lose their orientation
To overcome this, we employed a couple of techniques. The first one included loading the ventricles with 5% agarose gel. This gel, when solidified, provides additional resistance to deformation during the processing and embedding of the sample. We also found that double embedding did not interfere with the signal intensity as seen in Figure 2.9. Note that artifacts in the heart wall were due to errors during the excision.
Figure 2.9 Guniea Pig heart, labelled with DiO, and the ventricles filled with Agarose gel. Agarose gel does not interfere with the signal from the
dye. **The artifacts in this image were caused during excision, the forceps used to handle the heart tore through the surface.**

Loading of the agarose was easy to achieve in a guinea pig heart due to its larger size. However, the smaller vasculature of the mouse heart created difficulties in fully loading the ventricles. As an alternative to the gel-loading protocol, we came up with a hanging protocol. During perfusion, the heart is kept hanging from the cannula while making sure that the heart does not touch the sides of the conical tube. After fixing the heart, a surgical suture is used to secure the aorta to the cannula through which the heart is ‘hung’. This provides a way to both secure the heart during transport and manipulate the heart during processing that does not involve direct contact with the heart itself. This is aptly demonstrated in the cartoon Figure 2.10.
Figure 2.10 Hanging the heart, while making sure it does not touch the side of the test tube walls during perfusion, fixing, as well as processing.

Using this ‘hanging’ protocol, we achieved satisfactory results even for a mouse heart and hence we did not use the double embedding protocol for a mouse heart.
2.4 Optical Clearing of the tissue

As mentioned earlier, cardiac tissue is particularly dense and hence we are not able to collect deep optical stacks as compared to imaging brain tissue. Normally the signal would attenuate significantly after just 80 microns into the cardiac tissue. We wanted to be able to image deeper into the tissue. This would allow us to reduce the total number of cuts required to go through the heart, and hence decrease the blunting of the microtome blade. This would decrease artifacts and improve the quality of images.

Optical clearing of tissues has been used extensively for multimodal imaging of biological specimens [30], [33], [37]. Tissue consists of particles with a higher refractive index such as collagen, elastic fibers, cells and cell compartments. In contrast, the surrounding media like the interstitial fluid and cytoplasm have a lower refractive index. This architecture makes light travel at different speeds and angles. The putative mechanism of optical clearing is refractive index matching of tissue via optical immersion [38]. This reduces scattering, which provides less attenuation and thus achieves better focusing. A very detailed account of the physical, chemical and mechanical changes due to the
use of Optical Clearing Agents (OCA) has been explained in [38], [39] and [40].

We used the protocol described in Parra et al [30] with the exception that instead of using a graded methanol series for dehydration we use a graded ethanol series. Methanol has been known to be harsh on the tissue and hence ethanol was used as described in [41]. The optical clearing agent we used is known as Murray agent or BABB (Benzyl alcohol benzyl benzoate). Figure 2.11 demonstrates the effects of clearing.

![Before Clearing](image1.png) ![After Clearing](image2.png)

*Figure 2.11 Effects of Optical Clearing. The scale lines (mm) are clearly visible under the optically cleared mouse heart*
To test the signal intensity, we imaged an optical stack of 120 microns in both a control heart and an optically cleared heart. Figure 2.12 shows the stack reconstructed for the control heart, whereas Figure 2.13 shows for an optically cleared heart.

The mean intensity at the ‘z’ depth is plotted for both the cases in Figure 2.14. It should be noted that roughly the same area was considered while calculating these values. As evident from the graph, optical clearing actually provides greater intensity values at deeper positions in the optical stack than compared to the control case.
Figure 2.12 A stack of images acquired from a control heart (which has not been optically cleared). The depth between two successive slices is 10 microns.
Figure 2.13 Stack of images from an optically cleared heart, after spending 4 days in BABB
Automated Two-Photon Microscopy Imaging for a whole small Mammalian Heart

Figure 2.14 Graph showing that optical cleared tissue has better intensity at greater depth as compared to control tissue

2.5 Striation Pattern in Images

The AVIM design is based on low cost, compact components, allowing use on virtually any laser scanning microscope. As a result we do not use microtome sectioning machines or milling machines that are commercially available to perform mechanical sectioning of the tissue. We simply use a microtome blade, and an actuator to push the sample across the blade. Due to imperfections of the blade, there appears a ‘striated’ appearance in the plane of sectioning, and this reduces the
image quality. This is evident by the striations that are noticed in Figure 2.15.

Figure 2.15 'Striated' appearance on the images. The vertical lines in the image are caused due to imperfections in the blade.
Figure 2.16 High Resolution images of the cutting edge of the blade used for mechanical sectioning. A. A new unused blade. B. the cutting edge after about 15 cuts

Figure 2.16 demonstrates the wear and tear the blade experiences while sectioning fixed cardiac tissue. The image on the right is the cutting edge of the blade after 15 cuts into the tissue. To improve the image quality, and reduce the striations we tried using a variety of microtome blades and knives. Gold/Silver/Diamond/Platinum coated disposable blades
were tried along with blades from different manufacturers such as Leica, TissueTek, C.L. Sturkey. In addition, high profile and low profile setting for microtome blades were tried. Typically there is not much difference between the two profiles, with the exception that high profile blades have a higher width and known to be sturdier. Typical width dimensions would be 8.31 mm for low profile v/s 13.98 mm for high profile. We did observe limited success with high profile setup, however calibration for the blade to ensure a flat plane of sectioning was difficult to achieve and sustain through the course of the entire heart. We are now using reusable knives by C.L. Sturkey, and have acceptable results. These knives are 185mm in length and have ‘D’ profile i.e., a wedge shaped cutting edge. Due to the length of the knife, each knife can be used for 3 mouse hearts, adjusting the blade in the AVIM mount so that a new, unused section of the blade is located at the interface with the sample. Knives are then reconditioned by the manufacturer for continued use. The use of this blade greatly reduced visible striations on the sample and increased the amount of cuts possible before replacement/adjustment is necessary, Figure 2.17.
Automated Two-Photon Microscopy Imaging for a whole small Mammalian Heart

Figure 2.17 Frontal section of an image which is striation free after using the reusable microtome knife.

2.6 Other Design Considerations

Typically, acquiring a complete mouse heart in an automated manner takes roughly 7 days of continuous imaging while using a 10x objective
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at a resolution of 1.6 microns in plane. The microscope program interface on the Zeiss 7MP, Zen 2010, has significant reliability issues over this period of time. To overcome this, we typically limit the acquisition time to <24 hours, restarting Zen, the program interface, and continuing with the acquisition. The process is still automated and the user involvement is limited to every 24 hours. The restarting also helps to periodically check the quality of images and make any necessary changes in the imaging parameters.

We wanted the AVIM to be highly modular and be easily compatible with any laser scanning microscope. To arrange for this, we use an inverter from the microscope which positions the objective on top of the sample. However, due to this, there can be a misalignment between the imaging plane and the surface of the sample. This is demonstrated by Figure 2.18. To account for this, we included adjustable legs, shown in the figure by yellow arrows at the bottom of the stage, so as to manipulate the height of the sample plane. Each leg can be adjusted individually, and hence the imaging plane can be adjusted to more closely match that of the sample surface. We would image a sample tile, and perform a calibration protocol based on trial and error to make sure the legs are
adjusted for an optimum vignette pattern before starting with imaging the whole heart.

![Image](image_url)

**Figure 2.18** Adjustable legs at the bottom of the AVIM to accommodate any height difference caused by the objective and inverter setting. The two legs of the AVIM shown in yellow here are at a different height as compared to the other two legs, which leads to the leftmost image.

The inverter can also introduce some misalignment between the axis of the automated stage in the AVIM and the imaging axis of the microscope. This is because the objective inverter can be rotated around its attachment point on the microscope. Hence, the stage travel and the image acquired by the objective is not aligned, in a manner that they are
shifted by a few microns. This manifests itself as a misalignment between adjacent tiles seen in Figure 2.19. The rotary alignment to bring the axis of imaging in line with the axis of stage movement is performed with a rotary stage mounted underneath the AVIM (yellow arrow in the figure). Before imaging any heart, we would perform a calibration protocol involving collecting a single plane of images and reconstructing them to see the offset of one tile to another. Reference images are used to guide to the correct adjustments.

Figure 2.19 Example of Rotary Misalignment in the images. This is calibrated by accommodating a rotary stage underneath the AVIM to align the imaging and the stage movement axes.
For a typical mouse heart acquisition, the optical stack depth is 130 microns, with a ‘z’ step of 10 microns, which allows errors up to 40 microns in either direction for improper cutting that may occur due to imperfections in the blade. Using a 10x objective gives us a resolution of 1.6 microns in ‘x’ and ‘y’, and a tile dimension of 850 x 850 microns. The ‘xy’ step taken to the adjacent tile is 750 microns. This allows us to have roughly 100 microns overlap between consecutive ‘tiles’ in all 4 directions. This enables us to use the information near the center of the tile rather than the periphery, which has vignette patterns and hence reduced image quality. The large value of the overlap allows for the use of any future use of tile registration algorithms, although it significantly increases imaging time.

2.7 Summary and Conclusion

Described above are the design considerations taken into account in order to label and image an entire mouse heart in an automated manner. In addition we discussed design considerations for building and optimizing an automated volume imaging machine. The same AVIM has also been used to image a guinea pig heart. To roughly estimate the timeline, it takes one day for sample preparation, 5 days if optically clearing, one day for embedding. It takes roughly one day to set up the
AVIM, and ensure calibration with the blade, the stage, and the objective, following which it takes roughly 7 days to image an entire mouse heart. This number is estimated by assuming that the images will be acquired using a 10x C apochromat objective from Zeiss. The entire process adds up to about 15-18 days for a mouse heart. Using this technique we have been able to image 6 complete mouse hearts with at least 4 being high-quality datasets.

Apart from being low cost and modular, one of the biggest advantages of this system is that we can use the same setup for not only imaging a heart but for imaging any other biological sample or tissue as long as it is embedded on a cassette in paraffin. Except for calibration and restarting, there is no user intervention necessary and it collects the images on its own. Various components are accommodated in the design to counter the effects of any possible misalignment.

Typically, we acquire hundreds of gigabytes of data for each mouse heart. The files are in the raw ‘.lsm’ file format as created by the Zen software. These files along with the parameters and coordinates files obtained from the AVIM setup is then passed through a custom reconstruction pipeline
written in python to generate slices of the tissue volume. Detailed information of this pipeline is included in Chapter 3.
Chapter 3

Reconstruction and Visualization of the Cardiac Structure

3.1 Introduction

As described in the previous chapter, we built a custom low cost modular Automated Volume Imaging Machine and used it to image an entire mouse heart. We obtain ‘stacks’ of images from the microscope. These stacks are then reconstructed by first extracting the images from the files. We then correct the images for vignetting effects, where vignetting is the reduction of the image’s intensity at the periphery as compared to the image center. These images are then stitched together based on the acquisition coordinates. Finally, corrections for cut misalignment actuator errors and corrections for image intensity for out of plane images (i.e., corrections for signal attenuation due to increasing depth) are performed. We use a python script for this purpose. The final output is a series of ‘slices’, which are based on the ‘z’ position at which they
have been acquired. The algorithms used as well as the reconstruction pipeline have been explained in the next section.

One of the things we wanted to do with these image slices was to be able to render them in 3D and hence obtain structural information from any angle and plane of viewing. After reconstructing and correcting the images for artifacts, we load the slices into a volume rendering software to visualize the data in 3D. The massive size of these datasets makes it difficult to visualize the data in its native resolution and often we need to downsample the data significantly to make use of the rendering tools available. We go into details of this in section 3.3.

### 3.2 Reconstruction of ‘.lsm’ files

The ‘.lsm’ files contain image tiles acquired at a particular ‘x’ and ‘y’ location. These tiles are extracted from the ‘lsm’ files and are saved as ‘png’s. ‘PNG’ format is used as it offers lossless compression. These tiles are then processed using the following algorithms, in a pipeline as described in Figure 3.1.
Figure 3.1 Reconstruction Pipeline for images acquired from the AVIM-TPM

3.2.1 In-Plane Correction (Removing Vignettes)

Image vignetting is removed with an algorithm developed by Chow et al in [42]. Figure 3.2 In-plane image correction by Chow et al. For a given z, all images in that plane are stacked, and an average (B) and minimum (M) value is calculated for each x,y coordinate within the images. Figure 3.2 demonstrates how, for a given z coordinate, all images in that plane are virtually stacked. Then the average value and minimum value is determined for each ‘x’, ‘y’ coordinate within the image tiles. This produces an “average” image $I_B$ where the individual pixels for that image correspond to the average values at those coordinates from the image stack and a “minimum” image $I_M$ with the corresponding minimum.
values for each pixel coordinate. Both the “average” image and the “minimum” image are Gaussian filtered (standard deviation of 2, typically 10 pixels by 10 pixels) to remove any image-specific trends. It should be noted that although we do use a Gaussian filter, we do not apply to filter directly to the corrected image, but to the image that is used for correction. Next, the values stored in the filtered “average” and “minimum” images are used in Equation 1 to correct each image in the plane separately.

Figure 3.2 In-plane image correction by Chow et al. For a given z, all images in that plane are stacked, and an average (B) and minimum (M) value is calculated for each x,y coordinate within the images.
Reconstruction and Visualization of the Cardiac Structure

\[
I_O(n, [x, y]) = \frac{(I_c(n, [x, y]) - I_M([x, y]))}{I_B([x, y]) - I_M([x, y])}
\]

Equation 1 where \(I_c\) is the collected image, and \(I_O\) is the corrected image, \(I_B\) is the average image and \(I_M\) is the minimum image.

This process of calculating an “average” and “minimum” image for in-plane image correction is repeated throughout the collected volume.

3.2.2 Image Stitching – Creating Image Slices

Following in-plane image correction, individual images for a particular \(z\) value are rigidly stitched into a single image. This stitched image for a single \(z\) value is referred to as an image slice.

Figure 3.3 demonstrates the stitching process. A blank template is created large enough for the amount of pixels collected at a \(z\) value as well as the amount of offset that has to be taken into account due to the actuator-induced error. For a volume, the coordinate system is centered on the stitching template ((0,0) offset). Each image acquired at the initial \(z\) value is stitched onto the template at the coordinates recorded during its acquisition. Once all images for that \(z\) value have been stitched into the template, the resulting image is saved and the template is cleared.
This is repeated until an image slice has been output for each value of $z$ in the image volume.

![Diagram](image.png)

*Figure 3.3* Stitching individual tiles into image slices based on stage coordinates.

### 3.2.3 Image Registration

To determine the actuator-induced error between intercut volumes (the blue and the yellow portion in Figure 3.4), the portion of the sample imaged twice (before and after the cut, yellow portion in Figure 3.4) will be registered with itself. The image slice stitched at the bottom of the optical stack before a cut is registered to the corresponding image slice (same $z$ coordinate) after the cut (green portion in Figure 3.4). The two image slices are registered with a Nelder Mead Simplex algorithm adjusting the ‘x,y’ offset of the second-imaged area with respect to the
first in order to maximize the cross-correlation of the overlapping areas. The cost function that is minimized is given by:

\[ 1 - \text{Normalized Cross Correlation (NCC)}. \]

In other words we are maximizing the NCC between two images that are used for registration. If \( I_1 \) and \( I_2 \) are the subregions of the two images that are overlapped with offset \( x \) and \( y \), then the NCC of this overlap will be:

\[
\frac{\sum_x \sum_y I_1 \cdot * I_2}{\sqrt{\left(\sum_x \sum_y I_1 \cdot * I_2\right) \left(\sum_x \sum_y I_1 \cdot * I_2\right)}}
\]

\textit{Equation 2}

This NCC has a theoretical maximum of ‘1’ representing perfect overlap (the same image perfectly aligned with itself). The ‘x,y’ offsets are determined in this way for each error-inducing step (sectioning of the sample). The offset found is the error between the two intercut volumes being registered. In order to bring all of the intercut volumes into the same frame of reference, each offset is added to the preceding offset value starting at the top of the image volume and working down. Once the offsets between each intercut volume are determined, the stitched image slices are adjusted on their templates to bring the image slices into alignment.
Figure 3.4 Column representing the imaging and cutting of a single image area throughout the depth of the sample. The three cases of the top of the sample, middle of the sample, and bottom of the sample are demonstrated as to their contributions to the image reconstruction process.

3.2.4 Out of Plane Image Correction (Depth attenuation Compensation)

The signal attenuation, or dimming, with depth is a result of scattering and absorption both of the incident and reflected photons. Images acquired from deeper in the sample will be dimmer than those acquired closer to the surface. Many approaches have been developed to remove this effect from two-photon microscopy images. Because of the large depth of the optical stacks collected by the AVIM, it was necessary to
have a correction method that was robust and insensitive to the noise that is inherent in images acquired at large depths. After testing several algorithms, a mixture-modeling algorithm developed by Gopinath et al. [43] was found to work the best. The algorithm takes the output slices and labels the brightest image slice the reference image, usually the top image slice. It then assumes the image has two components in its histogram, a foreground component and a background component. The expectation-maximization algorithm is used to fit a Gaussian distribution to each of these components in the histogram of the reference image. This process is repeated for the other images in the optical stack. The non-reference images are then adjusted according to Equation 2 so that the parameters of their foreground and background components more closely resemble those of the reference image. In this equation, \( x' \) is the compensated intensity image stack (\( n \)th pixel, \( j \)th image), \( F \) is the membership probability for the background (denoted as \( 1 \)) and the foreground (denoted as \( 2 \)), \( \mu \) is the mean of the corresponding Gaussian with \( \sigma \) as its standard deviation, and the subscript \( r \) corresponds to the reference image. This process is carried out on each intercut volume collected. An example is shown in Figure 3.5

\[
(x')^j_n = F^j_2(n) \cdot \left\{ \frac{(x^j_n - \mu^j_2)}{\sigma^j_2} \sigma_r + \mu_r \right\} + F^j_1(n) \cdot x^j_n
\]

Equation 3 Gopinath et al [43].
Figure 3.5 Depth attenuation correction. The figure describes the effect of the applied algorithm. The image labelled ‘Gopinath’ shows the corrections performed on the ‘bottom image’.
3.3 Visualizing Cardiac Structure

The final output of the reconstruction pipeline is a series of PNG files, which are slices and are represented by the ‘z’ coordinate they have been acquired at. For a typical image acquisition i.e., with an in-plane resolution of 1.6 microns and a z-resolution of 10 microns, for an adult mouse heart, results in roughly 80,000 images corresponding to 600 slices, and a final dataset size of roughly 50-70 GB uncompressed. To view such a large dataset in its true uncompressed form is a challenge on its own. We tried various available open source visualization and rendering software for efficient visualization on a regular workstation, as described below. However it was not possible to hold such a large dataset in the memory of the computer.

ImageVis3D is a visualization software developed by the Scientific Computing and Imaging (SCI) institute at the University of Utah. This was the only open source software that did not load the data into the workstation’s memory but rendered the volume out of core (directly from the hard drive). ImageVis3D converts the series of slices into a volume in their `.uvf` format which is then read into the program. Although ImageVis3D can load the dataset as a whole, there was significant reduction in the image resolution and quality while rendering.
Another 3D rendering tool that has been tried for visualization was Fluorender, and is developed by the SCI institute. The application is great in terms of microscopy data visualization, especially since it supports multichannel data. It also enables the users to obtain digital cut planes as well as record rendering movies. The biggest downfall, was the need to down-sample the data significantly for rendering, since it loads the entire data into memory. Fluorender in the future, plans to add out of core rendering capabilities, following which, it should be a great tool for visualization.

3.3.1 FIJI/ImageJ

ImageJ (http://imagej.nih.gov/ij/) is a Java based image processing tool developed by the NIH [44] and is widely used by the biomedical imaging community. It is designed with an open architecture that provides extensibility via Java plugins and recordable macros. FIJI (Fiji Is Just ImageJ) is an image processing package [45] which is based on ImageJ. It is basically a distribution of ImageJ with Java, Java 3D and a lot of plugins organized into a coherent menu structure. Due to the inherent plugins available, FIJI was the preferred image processing software used by us for image and volume manipulation.
Speckles are random ‘dots’ in the background of the image slice that are introduced to the image due to the presence of dust on the sample. The first step is to de-speckle the slices that are generated, performed using a simple median filter. This reduces background noise and helps reduce. Following filtering, the slices are then downsampled to roughly 30% in ‘x’ and ‘y’ using bilinear interpolation. This is done to reduce the size of the slices and the resulting image volume, so that they can be loaded into memory and rendered in 3D.

Figure 3.6 shows a montage of a few slices made using ImageJ. We see great structure and organization of the cardiac myocytes in the heart, in the form of sheets. These images validate the entire process of acquisition and reconstruction, since we see such beautiful and highly detailed structure.
3.3.2 3D – Volume

The downsampled slices are then loaded onto FIJI, and we use the plugin ‘3D Viewer’ to visualize the data. The data properties are specified so as to get correct x:y:z ratio. A screenshot of the volume rendered is shown in Figure 3.7. ‘3D Viewer’ plugin has the ability to record 360 degree rotating view of the volume rendered and this allows us to create movies of the volume rotating.
Figure 3.7 Snapshot of 3D rendered Volume using the '3D Viewer' plugin in FIJI
Another plugin that is extensively used is the ‘Volume Viewer’ plugin. This plugin has the capability of generating digital sections and this allows us to display any plane of virtual sectioning. An example of the digital sectioning performed by the plugin is shown in Figure 3.9. This heart was actually sectioned with the plane of sectioning perpendicular to the long axis of the heart during imaging. So the slices were acquired starting from the base, and going down through to the apex. The slices were rendered using ‘Volume Viewer’ plugin and the snapshot in Figure 3.8 was acquired. This demonstrates the ability to collect snapshots and digital cut planes at almost any angle using the plugin. The only downfall is the downsampling of data. Figure 3.9 is another cutplane generated on the same heart. Both these figure show the best visualization of sheets in the mouse heart.

Figure 3.10 depicts great visualization of cardiac myocytes into sheets. A small tissue near the septum was cropped from the slices, and rendered in a 3D volume. The image shown here is a screen shot from the volume, clearly showing the best sheet structure visualized.
Figure 3.8 Snapshot of digital sectioning creating cut planes with the help of 'Volume Viewer' Plugin in FIJI. We are at the endocardium, opening right into the LV.
Figure 3.9 Cut plane generated after rendering the volume in 3D, using FIJI. This shows distinct arrangement of cardiac myocytes into sheets. We are viewing the mid cardium, towards the end of the LV.
Figure 3.10 A snapshot of a tissue block, cropped from the slices from the septum area. This cropped region was then rendered into a volume and we
can see clear organization of cardiac structure into sheets. This is the best sheet visualization we’ve seen.

Figure 3.11 Snap shot of cardiac sheet structure obtained after loading the slices into FIJI.

Figure 3.11 shows that we are seeing great structure in the heart. The snap shot was generated after cropping a part of transmural wall from the images which were acquired in a transverse plane (i.e., the plane of imaging was perpendicular to the long axis of the heart), and rendering them. We see clear sheet pattern from a transmural point of view. It
appears as the sheets start at the base of the heart, plummet down vertically from there, and then change orientation by almost 90 degrees to radiate through the heart.

3.4 Summary and Discussion

The images acquired with the AVIM require significant post-processing before they can be rendered into a 3D image volume. The reconstruction pipeline is used to compensate for many shortcomings of the acquired images. We have described above the algorithms used by us to correct for imaging artifacts, which are caused due to the way the images are acquired using our setup.

While all the algorithms discussed above are ‘off-the-shelf’, we did evaluate other algorithms before deciding to use the ones mentioned in this dissertation. Further detail on the algorithms evaluated can be found in the dissertation of Robert Kazmierski entitled “Metabolic heterogeneity in ischemia reperfusion injury: The inside story”. Going forward, it is likely that further improvements can be made to the reconstruction pipeline, upgrading the quality of the output image volume.
Visualizing such large datasets presents in itself a unique problem. Given the imaging resolution, the sheer size of the datasets makes 3D rendering on regular workstation onerous. We demonstrated the use of ImageJ and FIJI as an effective tool for managing these datasets. FIJI in particular, by using Java 3D, has been effective for rendering with the constraint of downsampling the data. Open source softwares for rendering aren’t capable of handling such large datasets well. We even tried using the free trial version of the commercially available software “Amira” but it did not yield results better than FIJI in terms of visualization. Other commercially available software packages, could provide improved rendering.

Such rich datasets present a unique challenge to the visualization community and by collecting data at such high resolution having a high volume size should motivate the community to develop rendering softwares. With regards to this, we are talking to developers at the SCI institute at the University of Utah, Salt Lake City, Utah.
Chapter 4

Quantifying Cardiac Structure

4.1 Introduction

Histology has traditionally been the standard method for mapping cardiac structure organization in rodents [10], [46]. More recently, Diffusor Tensor Magnetic Resonance Imaging (DTMRI) has also been used to measure the fiber orientation at high spatial resolution. Cardiac fibers are also known to be organized in sheets with surface orientation varying throughout the ventricles [47]. It has been postulated that the primary eigenvector at each voxel in these DTMRI dataset gives the fiber orientation, with the secondary eigenvector giving the sheet orientation and the tertiary eigenvector giving the sheet normal direction [12], [48], [49]. After collecting such high-resolution data, we wanted to be able to identify and quantify cardiac structure. DTMRI and Histology data suggest that the fibers in the myocardium follow a counter clockwise rotation as we move through the wall from the epicardium to the endocardium[50]–[52]. However, sheet orientation has yet not been well documented. There have been data suggesting that the sheet orientation
undergoes a clockwise rotation i.e., from a positive angle in the epicardium to a negative angle in the endocardium [12], [47], [53]. As a frame of reference, the angles lying in the upper right quadrant would classify as positive angles, and the angles lying the lower right quadrant would be negative angles. Figure 4.3 explains this nomenclature.

We wanted to measure the fiber inclination angles of the cardiac sheets as they extend through the ventricular wall. The images acquired and tools developed in earlier chapters provide the context that these structures can be visualized. We use the algorithm proposed by Karlon et al [49] to measure the orientation of these structures in an automated fashion, a technique which has been used on confocal microscopy images to quantify the cardiac structure [29].

4.2 Method

The method used by Karlon et al.[49], was adopted from Chaudhuri et al. [54]. In brief, the algorithm determines the local orientation based on formation of a pixel-by-pixel gradient vector. The steps are as follows:

- A mask is generated to amplify the gradient information in the image of interest I,

\[
h_x(i, j) = \frac{2i}{\sigma^2} \exp \left( -\frac{i^2 + j^2}{\sigma^2} \right)
\]
Quantifying Cardiac Structure

\[ h_y(i,j) = \frac{2j}{\sigma^2} \exp\left[ -\frac{i^2 + j^2}{\sigma^2} \right] \]

*Equation 4*

where \( h_x \) is the x-directed mask, \( h_y \) is the y-directed mask, \( i \) is the x position, \( j \) is the y position and \( \sigma \) is the area of influence of the mask. The mask size is given by \( s \) such that \(-s \leq i, j \leq s\). Mask size is determined such that the minimum value of the mask, except along the lines defined by \((0, j)\) and \((i, 0)\) was at least 0.01. Hence the mask size is constrained by the following equation:

\[ s \approx \sigma \sqrt{-\log(0.005) - 2 \log \sigma} \]

*Equation 5*

The mask is then convolved with the image \( I \):

\[ G_x = (h_x * I), \quad G_y = (h_y * I) \]

*Equation 6*

Pixel-by-pixel gradient magnitude and directions are computed from these components:

\[ G = \sqrt{G_x^2 + G_y^2}, \quad \varphi = \tan^{-1}\left( \frac{G_y}{G_x} \right) \]

*Equation 7*
The local direction of orientation is perpendicular to the intensity gradient $\varphi$

$$\varphi = \varphi + \frac{\pi}{2}$$

*Equation 8*

- The dominant “local” direction is then calculated in small image subregions $W$ of size $m \times m$ using an accumulator scheme. This is done by defining an array $A^W$ to represent possible orientations from $0^\circ$ to $179^\circ$.

For each subimage:

$$A^W(\theta) = \sum_{i,j} G(i,j) \frac{\exp[2 \cos[2(\theta - \varphi_{ij})]]}{\exp(2)}$$

*where $0^\circ \leq \theta < 180^\circ$*

*Equation 9*

The dominant direction is the largest accumulator bin value. The angles were then converted to range from $-89^\circ$ to $+90^\circ$. In effect the accumulator scheme comes from Von Mises distribution, which is analogous to a normal distribution in circular statistics.

The direction is such that the x axis is angle $0^\circ$, any positive angle will be in the first (upper right) quadrant and any negative angle will be in the fourth (lower right) quadrant. Thus, clockwise rotation of angles would be
going from positive to negative values and other way around for counter clockwise rotation. We used the same parameters as described by Karlon with \( s = 6 \) and \( \sigma = 4 \). The image subregion size \( m \) varied and was generally constrained by the computing resources and the application for the algorithm.

### 4.3 Results

#### 4.3.1 Validation

The first step was to validate the algorithm and its implementation. We selected random texture images to test the Karlon implementation (Figure 4.1). In addition, we also generated images with known orientations of \(+45^\circ\), \(-45^\circ\), \(+90^\circ\) and \(0^\circ\) lines in them to be able to validate the algorithm (not shown).
Figure 4.1 A. An example texture image used to validate the implementation of the algorithm. B. The histogram of the angles computed for the same image clearly showing the two predominant angles.
To further ensure correct implementation of the algorithm, we used the ‘Directionality’ plugin (http://fiji.sc/wiki/index.php/Directionality) in FIJI under the ‘Analyze’ section. It is able to compute the direction associated with the image using two techniques. The first technique involves finding the local gradient orientation. The gradient of the image is calculated using a 5x5 Sobel filter, and is used to derive the local gradient orientation. This is similar to the algorithm described by Karlon. The second technique involves calculating the Fourier component of the image and analyzing it. The algorithm chops the image into square pieces, and computes their Fourier power spectra. For a square image, structures with a preferred orientation generate a periodic pattern at +90° orientation in the Fourier transform of the image, compared to the direction of the objects in the input image. The result of using this plugin is shown Figure 4.2.
Figure 4.2 Results from the 'Directionality' plugin in FIJI for the same image in Figure 4.1.A.

The discrepancies between the exact values from the Karlon implementation and the Directionality plugin are a result of the bin size of the accumulator array as well as the size of the subimage selected to calculate the angle values from.

4.3.2 Structural Inclination Angles for Mouse Ventricular Wall

The next thing was to calculate the transmural structural inclination angles, i.e. the arrangement of cardiac myocytes into fibers and sheets, for a ventricular wall using the images generated by the AVIM and the TPM. The frame of reference is best demonstrated in Figure 4.3 by
Streeter et al., [10]. The angle $\alpha$ is the angle that we are calculating with the images acquired.

Figure 4.3 Diagrammatic representation of cardiac structural inclination angle based on Streeter et al. [10].

With regards to this, we used cropped sections of the images generated, to select a region of the LV free wall at the base and the apex of the heart, in which the images were actually sagittal sections of the heart. The area was roughly 800 microns by 800 microns. An example image is shown in Figure 4.4.
Figure 4.4  A. Cropped region in the base of the heart considered for calculating structure inclination angle. B. Histogram of the angle values for the image shown in A.

Doing this for all the images as we go through the wall, we notice a counter-clockwise rotation of the angles. The epicardium (Figure 4.5 A) has largely negative angles, and as we move through the wall into the mid-myocardium, the angle seems to be moving towards positive values, and as we reach the endocardium (Figure 4.5 B), the angle is largely positive, as seen in Figure 4.6. Cross referencing the angular information obtained from our analysis with angles available in the literature, we can validate our measurements. The information contained in these image cross section is rich and we can quantify cardiac fiber inclination angle.
Figure 4.5 A. Epicardial surface having a negative angle. B Endocardial surface depicting a positive angle. Reference image for the viewer for orientation.
We did similar analysis for not only the base, but also for mid-ventricular region of the heart, Figure 4.7. The area selected here, is slightly below the one used in Figure 4.6.
Figure 4.7 Distribution of Cardiac Inclination angle across the LV Free wall, at the base of the heart. The region used here is slightly below the images used to generate Figure 4.6 (i.e., in the mid-ventricular region)

We also notice that as we get towards the endocardium and especially when we hit the trabeculae carneae/papillary muscles, there is a distinct bimodal behavior where the angle fluctuates between a higher positive and negative value (Figure not shown). We see that cardiac structure is significantly arching, as we move through the wall. In fact this behavior can be seen even in a single image (Figure 4.8). The lower portion of the
image would have a high positive angle, while the upper portion of the image would have a high negative angle.

Figure 4.9 shows another aspect of the bimodal behavior. Image A shows that the 800x800 micron region, would predominantly have a large negative angular orientation of -84.76°, whereas image B would have a predominantly positive angular orientation of 87.17°. The two portions are separated by just 40 microns in space in ‘z’.

![Cropped Slice](image)

*Figure 4.8 Cropped Slice roughly 800 x 800 microns in dimension, demonstrating the significant arching of the cardiac sheet structure. This is the clearest visualization of sheet we have ever seen.*
Figure 4.9 A. An image, which has an average ‘negative’ value, with a value of -84.76°. B. An image, separated by just 40 microns in space from ‘A’, having a ‘positive’ angle value of 87.17°. This clearly depicts the fluctuating nature of cardiac sheet.

Finally, if we look closely at images for a heart where the slices are sectioned in a transverse plane (i.e., the plane of imaging is perpendicular to the long axis of the heart) we see the circular arrangement of the sheets in the mid myocardium. They run almost horizontal to the plane of imaging (Figure 4.10). These images strongly demonstrate that the slices and images generated by the AVIM and TPM could also be dealt with as a vision problem. There could be different ways with which these images could be analyzed and further help us
understand the intricate arrangement of the cardiac myocytes. Future work could include more detailed exploration of the collected heart volumes to investigate sheet angle for conduction studies, interstitial space segmentation for bidomain modeling and more.

Figure 4.10 Slice depicting the transverse nature of the sheet arrangement, in the mid myocardium. The red arrow points to the specific regions.
4.4 **Summary and Discussion**

We have described the implementation of an algorithm that calculates the local orientation based on the gradient in the image intensity. This method is more direct than the DTMRI data since it calculates inclination angle based on the visualized structure of the heart. The fact that we are able to get angle values from our images also validates our imaging technique and the AVIM setup, and demonstrates one of the applications of this instrument. Another application is the use of the setup to capture metabolic sinks and transmural heterogeneity in the myocardium as demonstrated in the dissertation of Robert Kazmierski at the Johns Hopkins University.

The structural information obtained from these images by visualization, and from the angular values computed from the images is highly detailed. They provide a unique perspective in terms of visualization. This is the clearest visualization of cardiac structure that we have, to the best of our knowledge. We wished to briefly demonstrate one of the many possible applications of the AVIM. We mapped murine cardiac structure based on the intensity gradient in the images. Most certainly more work can be done on the images acquired to better quantifying cardiac inclination angles by implementing tools such as meshing, fiber tracking.
and other computer vision techniques to greatly expand on the data collected from the AVIM.

The fiber inclination angles computed from the images obtained, agrees with histological evidence, as well as data from DTMRI. This allows us to validate what our acquisition, reconstruction and analysis technique. We observe a distinct counter-clock wise rotation of fiber inclination angle as we move from the epicardium to endocardium. We can use this technique to various knock-out models and now we are in a unique position to have great structural visualization as well as be able to quantify and perform different analysis on the images obtained. The angular information obtained using this algorithm is based on the intensity gradient. Although this is a good technique and the most applicable based on the nature of the images obtained, further work can be performed on the collected data regarding the arrangement of myocytes in the heart.

The bimodal behavior near the endocardium depicts the arching nature of the cardiac myocytes as they combine to form sheets. This agrees with the previous assumption that the sheets run transversely, along the direction of the long axis of the heart, in the endocardium. However, there is a significant curvature associated with these sheets. Imaging
more hearts, especially those embedded in a manner that enables the plane of imaging to be sagittal plane/frontal plane would permit us to look more into this finding.

There is anisotropy in the data that is generated because the ‘x’ and ‘y’ resolution is 1.6 microns whereas the ‘z’ resolution is 10 microns. This anisotropy makes it difficult to obtain perfect information from the out-of-plane images by the use of digital sectioning. We can always acquire images with a better ‘z’ resolution and obtain a better representation of the heart, but this also means increasing the image acquisition time equivalently. As an example, imaging at a resolution of 1.6 microns in ‘z’ would mean an image acquisition time of roughly 70 days. It is a tradeoff between shorter imaging time and better resolution in ‘z’. The balance between these two competing needs is determined by the ultimate application for the resulting dataset. The acquisition parameters used in this study were adequate to measure inclination angle throughout the ventricular wall. Other applications may require high resolution in ‘z’ over a smaller region of the heart.
References


[35] A. Matiukas, B. Mitrea, M. Qin, and A. Pertsov, “Near-infrared voltage-sensitive fluorescent dyes optimized for optical mapping in


Appendices

Appendix A

Protocol for Perfusing and Labeling a Mouse Heart with Di-4-ANEPPS

Materials

- Di-4-ANEPPS
- DMSO
- NaOH
- 100 mL Cardioplegic (High K+ Solution)
- 24 inch tubing with luer lock connectors
- 3 x 25 mL syringes
- 4 x 50 mL Tubes
- Aortic cannula for mouse heart
- 12 inches suture thread
- Petri dish
- Syringe pump calibrated to 5 mL/min flow rate
- Paraformaldehyde salt
- 100 mL PBS
Methods

2 hours before mouse heart perfusion

- Prepare fresh 4% pH Balanced paraformaldehyde (PFA) solution.
- Measure 2 grams paraformaldehyde salt and combine in 100 mL erlenmeyer flask with 50 mL PBS
- Place solution on a heating pad in chemical hood, set to 60 C, and stir with stir bar.
- Stir on heated pad until solution is clear.
- Use NaOH to Balance the pH of PFA to 7.4
- Remove from heat pad and place in 50 mL tube.
- Prepare 1 mL Di-4-ANEPPS stock solution
  - Dissolve 1.0 milligrams Di-4-ANEPPS in 1 mL DMSO
- Prepare 100 mL Cardioplegia Solution
- If syringe pump is available, determine setting for 3 mL/min with 25 mL syringe
- Collect the rest of the materials needed and bring them to Kreiger Hall for mouse heart perfusion

Immediately before excising heart

- Spray Cardioplegia solution and Di-4-ANEPPS stock solution with ethanol cleaning solvent and place in 37 C bath.
• Cut 3 two inch segments of suture thread and pre-tie into loose overhand knots.

• Place the petri dish under stereoscope.

• Connect tubing and mouse cannula and mount cannula so that the mouse heart can be attached while submerged in the petri dish.

• When Cardioplegia Solution is at 37 C, draw out a 30 mL aliquot into the petri dish.

• Place pre-tied knots into the petri dish and “hang” them on the cannula. This is done so that they can be pulled down onto the aorta later.

• Load 20 mL Cardioplegia Solution into first syringe, and use this syringe to prime the tubing and cannula.

• Load 18 mL Cardioplegia Solution and 1 mL Di-4-ANEPPS stock solution into the second syringe.

• Load 25 mL of pH Balanced PFA in another syringe with its own tubing.

• Connect first syringe loaded with normal Cardioplegia Solution to the tubing and cannula and place in syringe pump (if available).

• Fill 50 mL tube with PBS and label for pathology.

**Perfusing the Heart**
Appendices

- Anesthetize mouse per standing protocol.
- Open mouse chest cavity and rapidly excise heart.
- Place heart in petri dish, and identify aorta using stereoscope.
- Using forceps place the mouse aorta over cannula and use sutures to tie in place.
- Test heart mounting by turning on syringe pump or manually ejecting solution from syringe. Heart should begin to noticeably clear of blood.
- Adjust mounting and retest if necessary.
- After this, carefully move the setup from the petri dish to a 50 mL tube. “Hang” the heart from the cannula and the edge of the 50mL tube should be able to support the tubing from which the cannula and the heart is attached.
- Switch to second syringe containing Cardioplegia Solution and Di-4-ANEPPS. Perfuse heart with entire contents of syringe as close to 3 mL/min flow rate as possible. Repeat this for 30 mins. When you run out of the solution in the syringe, pipette out the solution and fill it back up in the syringe, and continue the process.
- If first syringe has remaining Cardioplegia Solution, switch to the first syringe and perfuse remaining solution through mouse heart to wash out excess dye.
- Detach the cannula from the tubing, and attach it to one which contains the syringe filled with pH balanced PFA.
• Also move the setup to a clean 50 mL tube, with the heart again hanging with the help of tubing and the edge of the 50mL tube.

• Perfuse the heart at 3 mL/min with the pH balanced PFA.

• Remove heart from the cannula and place the heart in pH balanced paraformaldehyde for 2 hours.

• Rinse the heart once with PBS.

• Place heart in 50 mL tube filled with PBS and labeled for pathology. Wrap tube in aluminum foil to protect from light. Specify “non-formalin” in the form at processing. This is done to limit the hearts exposure to PFA from not more than 2 hours.

• Embed the heart sagittally/transversely/frontally based on desired orientation.
Appendix B

Cardioplegia (High K\(^+\), No Ca\(^{++}\)) Solution

<table>
<thead>
<tr>
<th></th>
<th>Stock/M.W.</th>
<th>mM for</th>
<th>1000</th>
<th>10x (1000mL)</th>
<th>mL</th>
<th>Total Cl</th>
<th>Total K</th>
</tr>
</thead>
<tbody>
<tr>
<td>K-Glutamate</td>
<td>185.2 g/mole</td>
<td>120</td>
<td>22.224</td>
<td>222.24 g</td>
<td>27</td>
<td>145</td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>74.55 g/mole</td>
<td>25</td>
<td>1.86375</td>
<td>18.6375 g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgCl(_2)</td>
<td>1 M</td>
<td>1</td>
<td>1</td>
<td>10 ml</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEPES</td>
<td>238.3 g/mole</td>
<td>10</td>
<td>2.383</td>
<td>23.83 g</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EGTA</td>
<td>380.4 g/mole</td>
<td>1</td>
<td>0.3804</td>
<td>3.804 g</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

pH w KOH 0.1 M

7.4 K-Glutamate KCL MgCl\(_2\) HEPES EGTA
Appendix C

Protocol for Optical Clearing of a Mouse heart

- After fixing using pH-Balanced PFA, wash the heart twice in room temperature PBS by pouring the PFA out of the glass vial and replacing it with PBS. Swirl the heart in PBS solution before pouring out and replacing with PBS for a second washing.

- Dehydrate the heart at room temperature by a graded series of ethanol incubations (once in 50%, 70%, 95%, and twice in 100%) at 1 hr per incubation to extract the water from the fixed tissue. For each incubation, pour out the previous ethanol solution from the glass vial and replace it with the subsequent solution until the heart is completely submerged.

- After the second incubation in 100% ethanol, pour out the solution and replace with equal parts ethanol and the clearing solution containing benzyl alcohol and benzyl benzoate (1:2 vol:vol ratio). After 4 hr of incubation, decant this solution and replace with a 100% solution of benzyl alcohol and benzyl benzoate (1:2 vol:vol ratio). The refractive index of the clearing solution is n = 1.54.

- Once in BABB, the heart will become noticeably transparent, within 4 - 5 hr. For best clearing results, leave the heart to clear
overnight/at least 12 hours at room temperature while shielded from bright light.

- Proceed to pathology for embedding in paraffin. Be sure to specify no xylene protocol, directly embedded in paraffin.
Appendix D

Code for Karlon Intensity Gradient Angle Calculation

% This program reads the image and calculates the fiber orientation
% based on Karlon, W., Hsu, P., & Li, S. (1999). Measurement of
% orientation and distribution of cellular alignment and cytoskeletal
% organization. Annals of Biomedical ..., 27, 712–720. Retrieved from
% http://link.springer.com/article/10.1114/1.226

function karlonWithPlotting(inputimage)

% Load, convert and display image
image1 = imread(inputimage);

if size(image1,3) == 3
    image1 = rgb2gray(image1);
end

fig1 = figure('Visible','off');
RA = imref2d(size(image1)); % to get the image axis in regular frame of reference
imshow(image1,RA);
axis off;
hold on;

image1 = double(image1);

% Define mask for gradient formation
s = 6; %mask size
sig = 4; %area of influence
i = -s:s;
j = -s:s;

hx = zeros(size(i,2),size(j,2)); %initialize
hy = zeros(size(i,2),size(j,2)); %initialize

%calculate the mask for gradient formation
for index_i=1:size(i,2)
    for index_j=1:size(j,2)
        hx(index_j,index_i) = 2*i(index_i)/sig^2*exp(-
(i(index_i)^2+j(index_j)^2)/sig^2);
    end
end
\[ hy(index_j,index_i) = \frac{2 \cdot j(index_j)}{\sigma^2} \exp\left(\frac{-i(index_i)^2 + j(index_j)^2}{\sigma^2}\right); \]

\]

\]

\]

% Convolve masks with image

Gx = conv2(image1,hx,'same');
Gy = conv2(image1,hy,'same');

G = (Gx.^2+Gy.^2).^0.5;
phi = atan(Gy./Gx)+pi/2;

% Accumulator scheme for angles from 0 to 179 deg
theta = 0:0.1:3.13;
m = 50; %sub image size

%accumulator array -- predefine
A = zeros(floor(size(G,1)/m),floor(size(G,2)/m),size(theta,2));
angle = zeros (floor(size(G,1)/m),floor(size(G,2)/m));
for \( x = 2:\text{floor(size}(G,1)/m) \) \% index defining start of subvolume on full image in \( x \) dimension

for \( y = 2:\text{floor(size}(G,2)/m) \) \% index defining start of subvolume on full image in \( y \) dimension

for \( k = 1:\text{size}(\theta,2) \) \% index for different \( \theta \) values

for \( i = 1:m \) \% index for image subregion \( x \) dimension

for \( j = 1:m \) \% index for image subregion \( y \) dimension

\[ A(x,y,k) = G((x-1)*m+i,(y-1)*m+j)* \exp(2*\cos(2*(\theta(k)...
\phi((x-1)*m+i,(y-1)*m+j)))/\exp(2) + A(x,y,k); \]

end

end

end

end

end

\% Determine most common angle seen in 'A' distribution

if isnan(max(A(x,y,:)))

angle(x,y) = 0;

else

angle(x,y) = 0.1*find(A(x,y,:) == max(A(x,y,:)));

end
% Convert the angular range

if angle(x,y) > pi/2
    angle(x,y) = angle(x,y)-pi;
end

angle(x,y) = -angle(x,y);

% Plot the Fibers on the image

if angle(x,y) ~= 0
    center = [(x-1)*m (y-1)*m];
    liney = [ center(1)-m/2*(cos(angle(x,y)+pi/2)) center(1)
               center(1)+m/2*(cos(angle(x,y)+pi/2)) ];
    linex = [ center(2)-m/2*(sin(angle(x,y)+pi/2)) center(2)
               center(2)+m/2*(sin(angle(x,y)+pi/2)) ];
    plot(linex,liney,'r');
end

end

end

% Save image with plotted fibers on them

inputimage = inputimage(1:end-4);
plotImageName = strcat('\Directory\',inputimage);

saveas(fig1,plotImageName,'png');

% Convert angle to degree, display histogram
angle = angle*180/pi;

% Plot and Save the Histogram
idx = angle(:) ~= 0;

fig2 = figure('Visible','off');

hist(angle(idx),[-89:90]);

histName = strcat('\Directory\',inputimage);
saveas(fig2,histName,'png');

% Save the mat file
matName = strcat('\Directory\',inputimage,'.mat');
save(matName,'angle','m');
end
Curriculum Vitae

Aagam Shailesh Shah
3400 N. Charles Street, Hackerman Hall 318, Baltimore, MD 21218; 410-340-9937; 
aagamshah@outlook.com

EDUCATION
● Masters of Science and Engineering, Biomedical Engineering GPA: 3.97/4.0
  Feb 2012 - May 2014
  Johns Hopkins University, Baltimore, MD.

● Bachelors of Engineering in Biomedical Engineering Percentage Marks: 75.15% (Topper)
  Aug 2007 - May 2011
  University Of Mumbai, India.

● Senior Secondary School, HSC Board Percentage Marks: 83.33%, PCM: 90.66%
  2006 - 2007
  Jai Hind College, Mumbai, India.

● Secondary School, SSC Board Percentage Marks: 88.40%
  2004 - 2005

MASTERS' THESIS
Quantifying Cardiac Micro-Structure using Automated 2 Photon Microscopy, Johns Hopkins University
Feb 2012-May 2014
- Developed histology protocols to label a rodent heart uniformly across various metabolic conditions.
- Developed and implemented automated sectioning protocol to get through whole heart using Labview.
- Assisted in developing and implementing an automated imaging setup to acquire high resolution whole heart microscopy images using a Zeiss 7MP (multi photon) Microscope.
- Assisted in developing algorithms for automated image volume reconstruction using Python.
- Set up collaboration with SCI institute, Utah regarding developing visualization and rendering tools for such large volume data.
- Developing algorithms to calculate fiber orientation and sheet structure of a whole rodent heart using MATLAB and ImageJ.
- Aim to achieve high resolution complete heart models under different metabolic conditions and other knock out varieties.

PUBLICATIONS

URL: http://ieeexplore.ieee.org/stamp/stamp.jsp?tp=&arnumber=6178980&isnumber=6178945
SKILLS

● Languages: C, C++, JAVA, Python, 8085 8086 8087 8051 Assembly Language (familiarity).
● Software Packages: MATLAB, NEURON, AutoCAD, PSPICE, LabVIEW.
● Operating Systems: Linux, Windows, Mac OS.

ACADEMIC PROJECTS AND PRESENTATIONS

● Probabilistic Graphical Models, Johns Hopkins University, Baltimore
  Sept 2013 – Dec 2013
  ○ Working on a presentation of probabilistic graphical models, especially their application in the field of cancer genomics and cell signaling networks.
  ○ In depth review of ways in which Bayesian Networks are applied as machine learning technique to learn and identify pathways for phenotypic traits, molecular markers.

● Implementation of statistical machine learning techniques, Johns Hopkins University, Baltimore
  Sept 2012 - Dec 2012
  ○ Designed workflow and applied machine learning algorithms dealing with high resolution SNP array data of the BRAC1 Breast cancer gene.
  ○ Applied various classifiers to test their accuracy across a variety of input conditions using MATLAB.

● Biodegradable Hydrogels for Cell and Drug Delivery, Johns Hopkins University, Baltimore
  Feb 2012 - May 2012
  ○ Presented a term paper on Biodegradable hydrogels with in depth review of its application to cell and drug delivery.
  ○ Studied the mechanism behind biodegradable hydrogels, the various types and their application in the field of cell and drug delivery.

● Computational Investigation of effect of UP state on EPSP in striatal MSN, University of Mumbai
  June 2010 - May 2011
  ○ Researched on striatal neurons having direct implications towards research in possible cure of Schizophrenia and drug addiction using Neuron Suite.
  ○ Modified preexisting compartmental model for striatal MSN to understand the kinetics of EPSP with respect to the resting membrane potential of the Neuron.
  ○ Sodium and Potassium channel were studied to correlate the kinetics with the EPSP width.

● Automatic Plant Irrigator, University of Mumbai
  Dec 2009 - May 2010
  ○ Designed an automatic plant irrigator to make life simpler for farmers in the rural setting.
  ○ Developed the design using microcontrollers and embedded systems, implemented using PSPICE.
TEACHING EXPERIENCE

• **Systems Bioengineering II Lab**  
  Spring 2014  
  o Involved in guiding more than 120 students in a lab identifying human systems response to stimulus. This included modelling human responses as systems responses.

• **Freshmen Modeling and Simulation**  
  Fall 2013  
  o Involved in guiding 6 undergraduate students in a Biomedical Modeling and Simulation lab, modeling the dynamics of the arm and the human circulatory system.  
  o Guided the team through a foam core ping pong ball transportation device competition, as well as an independent project for modeling human learning based on audio and visual cues.

• **Systems Bioengineering II Lab**  
  Spring 2013  
  o Involved in guiding more than 120 students in a lab modeling the response of human auditory system, along with the effects of cochlear implants.

• **Systems Bioengineering I Lab**  
  Fall 2012  
  o Involved in guiding more than 120 students in a lab studying the effects of extracellular chemicals on membrane potential and action potential using frog heart.

• **Signals & Systems**  
  Spring 2012  
  o Involved in grading assignments, tests and exams of more than 120 students in a course covering representation of signals and linear time-invariant systems and Fourier analysis.