

2 Materials and Methods

2.1 Materials

The samples used for the analysis of the myocardial microstructure were provided by the Department of Transplant Surgery, Medical University of Graz, Austria. While a porcine heart (provided by an abattoir) was used for the feasibility study performed by means of diffusion tensor imaging, all further examinations were executed using human heart samples. The different samples were derived from nine distinct patients, both female and male. The information on the patients such as age, sex, size, weight, heart wall thickness, ejection fraction, heart conditions and cardiac diseases, cause of death, and location (left ventricle (LV) vs. right ventricle (RV)) are denoted in Table 2.1. However, we were not endowed with more intrinsic information about heart no. 1, 3, and 9 by the time this thesis was accomplished. Although the 40 samples were derived from 9 different hearts, the data of only 6 patients is provided, resulting in a mean age of 54 years.

Table 2.1: Patient data from the 9 different hearts (the data of heart no. 1, 3, and 9 was not available) used, under specification of age, sex, size, weight, wall thickness, ejection fraction, heart conditions and cardiac diseases, cause of death, and location (left ventricle (LV) vs. right ventricle (RV)).

Heart no.	Age, (yrs)	Sex	Size, (cm)	Weight, (kg)	Wall thickness (LVFW/IVW), (mm)	Ejection fraction, (%)	Heart condition/ cardiac diseases	Cause of death	Location	Samples
1	-	-	-	-	-	-	-	-	LV	I
2	57	f	168	63	10/10	58 (56)	slight-moderate MR, HTN	SAH	RV	XXI
3	-	-	-	-	-	-	-	-	LV	II, V, XVI
4	57	f	167	80	11/11	84	concentric LVH, PAP borderline	aSAH	LV	VI
5	48	m	180	69	9/10	>60	sinus tachycardia, incomplete RBBB, intact LV function, minimal MR, SSS, CAD, paroxysmal	SAH	LV	III, VII, VIII
6	71	f	169	80	14/14	-	AF normofrequent after PM implant, HTN	SAH	LV	IV
7	39	m	180	91	-	-	small cardiac and mediastinal silhouette, liver transplant	ICH	LV	XVII, XVIII
8	49	m	180	109	-	-	K1 streptococcus agalactiae, hypernatremia	meningitis, SAH, sepsis	LV	XIX, XX
9	-	-	-	-	-	-	-	-	LV	IX-XV, XXII

AF...atrial fibrillation; aSAH...aneurysmal subarachnoid hemorrhage; CAD...coronary artery disease; HTN...hypertension; ICH...intracranial hemorrhage; IVW...intraventricular wall; LVFW...left ventricular frontal wall; LVH...left ventricular hypertrophy; MR...mitral regurgitation (mitral insufficiency); PAP...pulmonary artery pressure; RBBB...right bundle branch block; SAH...subarachnoid hemorrhage; SSS...sick sinus syndrome

The left ventricular wall is approximately 10–15 mm thick in human adults, however, the exact thickness of all hearts (denoted in Table 2.1) is not given. Moreover, the ejection fraction, the volumetric blood fraction that is pumped out of the ventricle with each heartbeat, of all patients is not available. While an ejection fraction (denoted in %) of more than 60 is considered “nonfailing“, values between 50 and 59 (as heart no. 2) are considered “borderline“.

2.2 Methods

This chapter describes both imaging methods used, diffusion tensor imaging and multi-photon microscopy, as well as the respective specimen preparation. Furthermore, the software used for data processing and visualization and the accordant method for the analysis of the data are introduced.

2.2.1 Diffusion tensor imaging

Diffusion tensor imaging (DTI) is a magnetic resonance imaging method measuring the rate and direction of the diffusion of water molecules in biological tissues such as the heart. The diffusion tensor obtained from DTI can be decomposed into eigenvalues and eigenvectors of which the principle eigenvector can be used to determine the fiber direction (as diffusion is anisotropic). In case of isotropic environment, the diffusion of water is equivalent in all directions. The water contained inside and outside the myofibers cannot move freely, but rather in the direction of the fiber itself meaning that the fiber direction corresponds to the maximum direction of diffusivity. The main direction of diffusion is given by the first (and largest) eigenvector. Cleavage planes physically separate the myo- fibers which are grouped in approximately 3-4 cells thick sheets. Diffusion is assumed to be smaller perpendicular to the cleavage planes than inside the cleavage planes. Hence, the third and smallest eigenvector coincides with surface normal direction while the second eigenvector is positioned inside the sheet (Eggen *et al.* , 2012). In order to visualize the anatomic structure of, in this case, the myocardium, the path followed by the fibers has to be reconstructed using a fiber tracking method (Rohmer *et al.* , 2007).

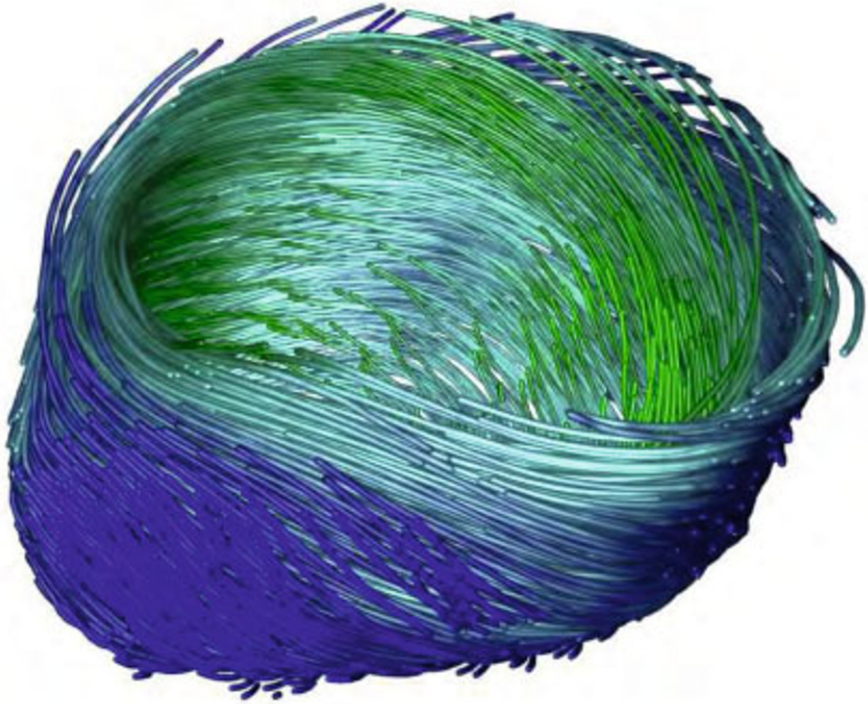


Figure 2.1: Depiction of the fiber structure of the left ventricle following DTI. Fiber tracking was performed and the clockwise to counterclockwise geometry from the epi- to the endocardium is illustrated (Rohmer *et al.*, 2006). (Original figure in colour freely available to download from our website www.springer.com)

Specimen Preparation

As DTI of microstructures is a nontrivial task, the preparation of the samples affects the resulting data. Two principal criteria for DTI are the reduction of water around the samples and the bubble-free preparation of the samples (Rohmer *et al.*, 2006). For the feasibility study a whole porcine heart, from which rectangular samples of approximately $20 \times 10 \times 1.5$ mm size were cut, was used. Immediately after cutting the sample from the heart it was

positioned into a container filled with 0.9% PBS solution. The container was put into an ultrasonic bath and sonicated for an hour to eliminate air bubbles. Spherical objects are the most suitable ones for DTI as it is essential to avoid B_0 inhomogeneities. Ideally, B_0 fields are homogeneous. In order to eliminate external field inhomogeneities, which would lead to spatial or intensity distortions, the spins have to be excited equally. The bottom of a spherical container was poured with agarose gel, the sample was positioned onto the gel and covered.

Diffusion tensor imaging and processing

The diffusion-weighted MRI data were acquired at the TU Graz MRI laboratory of the LKH Graz and the head coil on a SIEMENS MAGNETOM TrioTim 3T was used. The acquisition was performed over 10 hours of imaging time and 319 images were obtained. The following imaging parameters were used: slice thickness = 5, repetition time = 7.3, echo time = 3.41. The achieved dataset was stored as DICOM diffusion-weighted image metadata.

Data Processing and Visualization

BioimageSuite and 3Dslicer, which are both open source software, were used to visualize and process the data.

BioimageSuite BioimageSuite is an integrated image analysis software suite developed at Yale University supported from the National Institutes of Health (NIH) and the National Institute of Biomedical Imaging and Bioengineering (NIBIB), having its main focus on neuro- and cardiac imaging and analysis. The data obtained from DTI had to be converted into NIFTI format before loading it into the program as BioImageSuite can only process data in ANALYZE or NIFTI format. This was realized using MRICron. In order to get reasonable results, the diffusion-weighted images were preprocessed using an image filter. Therefore, after loading the images into the program they were filtered by a special diffusion filter termed *Anisotropic Diffusion* aiming to reduce noise while preserving significant parts of the image content. The creation of a mask of the region of interest is another prerequisite

before building the diffusion tensor. The Diffusion tensor itself was built with the *Diffusion Tool* of BioImageSuite. BioImageSuite is not capable of recognizing the gradient directions of the dcm2nii conversion files (MRIcron), and hence, they were added manually by means of a MATLAB script as the gradient directions are stored in the DICOM header of each file. From the previous computed diffusion tensor image the diffusion tensor can be further analyzed in order to display characteristics such as the eigenvalues and -vectors, the fractional anisotropy, the directionality, and fiber and sheet angle among others.

3Dslicer 3D slicer is a software package for visualization and image analysis developed by several institutions including the Massachusetts Institute of Technology, the Surgical Planing Laboratory and the National Institute of Health (NIH). The DICOM data obtained from DTI had to be converted to a NRRD format using a NRRD converter before loading it into Slicer for the analysis. The data was preprocessed, first by using a noise reducing filter on the diffusion-weighted imaging dataset, secondly by creating a mask. After preprocessing a diffusion tensor image (coloured by orientation mode) was estimated from the diffusion-weighted image. The images can also be coloured according to their fractional anisotropy. Fractional anisotropy describes the degree of anisotropic diffusion which is zero (dark) for fully isotropic materials and one (bright) for fully anisotropic materials (diffusion along one main axis). DTI tractography, the virtual reconstruction of the trajectory of water molecules along fibers, was performed. However, 3DSlicer is not endowed with a tool to determine the fiber or sheet angle.

2.2.2 Multi-photon microscopy

Multi-photon microscopy (MPM) based on the excitation of fluorescent probes is a standard imaging method (Williams *et al.* , 2005). However, second harmonic generation (SHG) microscopy, first demonstrated in crystalline quartz in 1962 (Campagnola *et al.* , 2002), has been emerging as a powerful imaging tool for biological and biomedical applications, especially for collagen rich tissues such as cornea, tendons and arteries (Cicchi *et al.* , 2013). In contrast to fluorescence microscopy, SHG is a label-free method

that does not have the side-effects of photobleaching or toxicity, but still provides high-resolution, high-contrast, and three-dimensional studies of tissues (Campagnola *et al.*, 2002). In contrast to two-photon fluorescence, in second-harmonic imaging, no energy is lost (Cox & Kable, 2006) and the scattering of the illuminated light is less due to the relatively long excitation wavelengths used, allowing deeper penetration depths than would be possible with other microscopy techniques. Moreover, SHG is produced in a small volume, because the intensity of the second harmonic signal depends on the square of the intensity of the incident illumination and thus, on $1/r^4$ (with r being the distance from the focal point of the illumination). The potentially increased spatial resolution results from the nonlinear relation between illumination and SHG intensity (Kumfer, 2009).

SHG microscopy is adapted from the nonlinear optical effect known as second harmonic generation or frequency doubling. When two photons, arising from a laser source, interact with a nonlinear material like collagen, a photon exhibiting half the wavelength and therefore twice the energy and twice the frequency of the incident light will be generated. The Jablonski diagram of SHG is demonstrated in Fig. 2.2.

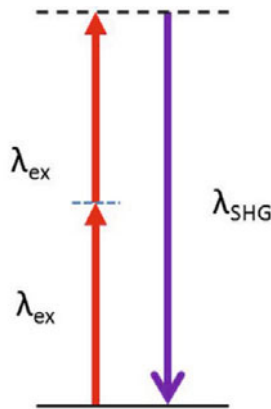


Figure 2.2: Schematic Jablonski diagram of SHG (Larson, 2011). (Original figure in colour freely available to download from our website www.springer.com)

The resulting beam of light, having twice the energy, usually travels in the same direction. As SHG is a coherent process, the resulting beam is also in phase with the exciting light.

In order to generate second harmonics, the electric field of the exciting light has to be strong enough to deform a molecule, and the anisotropy resulting from a non-symmetrical molecule creates an oscillating field at twice the frequency (Cox & Kable, 2006). Hence, the ability to generate second harmonics is peculiar to molecules that lack a center of symmetry. Collagen, being non-centrosymmetric and anisotropic, meets these requirements. Therefore, collagen enclosed structures such as the heart muscle fibers can be visualized by SHG (Campagnola *et al.* , 2002). Moreover, collagen type I is highly crystalline making it even more favourable for SHG, as molecules arranged in a crystalline array generate a much stronger second harmonic response. Even though collagen I and III, both abundant in the ECM surrounding the heart muscle fibers, give a SHG signal, the signal from collagen I is much stronger due to its crystallinity. Besides, SHG imaging allows the differentiation between collagen type I and III by using collagen specific stains. (Cox *et al.* , 2003).

Setup of an multi-photon microscope Basically, a microscope for SHG imaging consists of a scanning microscope (confocal laser scanning microscope) coupled to a pulsed infrared laser, both mounted to an optical table (Cox & Kable, 2006). Ideally, pulsed lasers with short pulse lengths (femtosecond or picosecond lasers) are used for SHG and are raster-scanned across the sample by means of mirrors to scan the focal point. The short laser pulse lengths enable the high power needed for SHG without destroying the sample. High photon densities are necessary to guarantee the concurrent arrival of two photons in the focal point. In order to focus the laser light on the sample, which may scatter a second harmonic, the laser passes through the objective (Kumfer, 2009). SHG microscopy enables the construction of three-dimensional images of specimen by imaging deeper into the tissues by using near infrared wavelengths for the incident light beams.

Generally, the resulting beam of light travels in the same direction as the

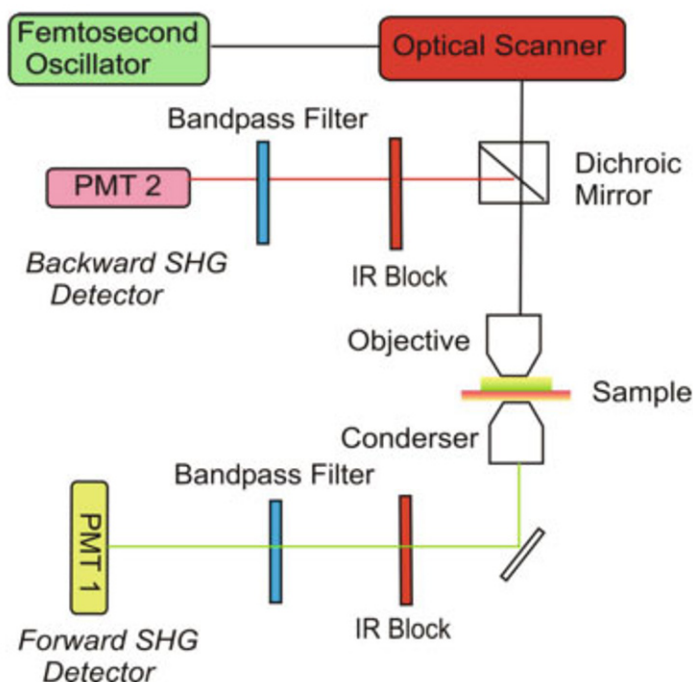


Figure 2.3: Schematic diagram of an SHG microscope (Han *et al.* , 2005). (Original figure in colour freely available to download from our website www.springer.com)

incident beam. However this may alter depending on the illumination conditions, the transparency, and the structure of the specimen. Basically, there is a distinction between forward and backward propagated signals, both the forward and backward SHG detector are depicted in Fig. 2.3. The second harmonic scattered signal is collected by either the objective lens or condenser lens. The immersion objective focuses the exciting beam and collects the backward SHG signals, whereas the condenser collects the forward (transmission) SHG signal through the forward SHG detector. The numerical aperture (NA) of the condenser lens, describing the range within

which light can be accepted or emitted, has to be larger than or at least equal to that of the objective (Cox & Kable, 2006). The NA of the objective influences the efficiency of the backward SHG signals (Han *et al.*, 2005). The amount of room light entering the system is reduced due to the immersion of the system which is an important fact considering nondescanned detectors. However, room light reaching nondescanned detectors should be prevented (Cox & Kable, 2006).

Infrared block filters and bandpass filters in both the forward and backward light paths are in charge of filtering out the illumination light so that only SHG signals are recorded (Han *et al.*, 2005).

Physical background As electromagnetic radiation (light) passes through a sample (a dielectricum) the electric field E influences the charge distribution, namely separates the positive and negative charges. The subsequent redistribution of charges generates an additional field component, namely the polarization (Cox & Kable, 2006). The total polarization (electric dipole moment per unit volume) of a material interacting with light induced by an electric field E can be expressed as a sum of linear and nonlinear terms, given by

$$P = \chi^{(1)} E^1 + \chi^{(2)} E^2 + \chi^{(3)} E^3 + \dots \quad (2.1)$$

where P is the induced polarization, $\chi^{(n)}$ is the n -th order nonlinear susceptibility tensor and E is the electric field. While the first term of the equation describes linear absorption of light, scattering and reflection, SHG is described by the second term of the equation as the nonlinear effect is a polarization of second order in the electric field (Campagnola *et al.*, 2002). These second order effects vanish in materials that have an inversion center (centrosymmetric) or that are isotropic. The nonlinearity between the polarization P and the electric field E in nonlinear media only occurs at very high light intensities induced by pulsed lasers.

3D Analysis of the Myocardial Microstructure
Determination of Fiber and Sheet Orientations

Ticar, J.M.

2016, XV, 76 p. 39 illus. in color., Softcover

ISBN: 978-3-658-11423-7