UNIVERSIDAD POLITÉCNICA DE MADRID ESCUELA TÉCNICA SUPERIOR DE INGENIEROS DE TELECOMUNICACIÓN



# IMAGE ANALYSIS AND MODELLING OF THE INFARCTED HEART RESPONSE AT THE MICROVASCULAR LEVEL

PhD Thesis

Polyxeni Gkontra Electrical and Computer Engineer, MSc

2018

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#### ABSTRACT

The coronary microvasculature comprises the smallest blood vessels of the cardiac tissue. It continuously adapts in response to physiological and pathophysiological conditions to meet tissue demands. Quantitative assessment of the dynamic changes taking place in the coronary microvasculature is therefore crucial in enhancing our knowledge regarding the impact of cardiovascular disease on tissue perfusion and on developing efficient angiotherapies. This thesis focuses on deciphering the structural and functional changes that occur at the microvascular level, at various stages after myocardial infarction (1, 3, and 7 days following damage). Towards this aim, we have adopted an interdisciplinary approach which combines confocal microscopy, fully automated 3D image analysis and mathematical modelling.

We used thick cardiac tissue sections labelled for nuclei, endothelial cell junctions and smooth muscle cells and we imaged them by confocal microscopy. Firstly, we developed a novel method for the segmentation of labelled structures from confocal images as well as an innovative approach for the accurate 3D reconstruction of the microvasculature based on endothelial cell junction and smooth muscle actin staining. Subsequently, we designed a fully automated image analysis pipeline to extract parameters that quantify all major features of the microvasculature, its relation to smooth muscle actin-positive cells and capillary diffusion regions. The novel pipeline was applied to the analysis of the coronary microvasculature from healthy tissue and also tissue at various stages after myocardial infarction. We used the pig animal model, whose coronary vasculature closely resembles that of human tissue. We discovered alterations in the microvasculature, particularly structural changes and angioadaptation resulting in altered capacity for oxygen diffusion in the aftermath of myocardial infarction. In addition, we evaluated the extracted knowledge's potential in terms of predicting the pathophysiological condition of the tissue. The high accuracy achieved in this respect, demonstrates the ability of our approach not only to quantify and identify pathology-related changes of microvascular beds, but also to predict complex and dynamic microvascular patterns. Lastly, the anatomical data obtained regarding the microvasculature were used to feed a continuum perfusion model in order to calculate physiologically meaningful permeability tensors. By using this theoretical blood flow modelling approach, we

were able to obtain insights into tissue perfusion and to demonstrate the functional effect of the structural changes occurring as a result of myocardial infarction.

Overall, this work is a step forward towards increasing our understanding of microvascular alterations after myocardial infarction, modelling microcirculation at different stages after tissue damage and it also provides an unbiased means for the evaluation of potential treatments.

#### RESUMEN

La microvasculatura coronaria comprende los vasos sanguíneos más pequeños del tejido cardíaco. Se adapta continuamente en respuesta a las condiciones fisiológicas y fisiopatológicas para satisfacer las demandas de los tejidos. La evaluación cuantitativa de los cambios dinámicos que tienen lugar en la microvasculatura coronaria es, por lo tanto, crucial para mejorar nuestro conocimiento sobre el impacto de las enfermedades cardiovasculares en la perfusión tisular y en el desarrollo de angioterapias eficientes. Esta tesis se centra en descifrar los cambios estructurales y funcionales que ocurren a nivel microvascular, en varias etapas después del infarto de miocardio (1, 3 y 7 días después del daño). Con este objetivo, hemos adoptado un enfoque interdisciplinario que combina la microscopía confocal, el análisis de imágenes 3D totalmente automatizado y el modelado matemático.

Utilizamos secciones gruesas de tejido cardíaco teñidas para núcleos, uniones de células endoteliales y células de músculo liso vascular y obtuvimos imágenes mediante microscopía confocal. En primer lugar, desarrollamos un método novedoso para la segmentación de estructuras marcadas a partir de imágenes confocales, así como un enfoque innovador para la reconstrucción 3D precisa de la microvasculatura basada en la unión de células endoteliales y la tinción de actina de músculo liso. Posteriormente, diseñamos una pipeline de análisis de imágenes completamente automatizada para extraer parámetros que cuantifican todas las características principales de la microvasculatura, su relación con las células de músculo liso que expresan actina y las regiones de difusión capilar. La nueva pipeline se aplicó al análisis de la microvasculatura coronaria de tejido sano y también de tejido en diversas etapas después del infarto de miocardio. El modelo animal utilizado fue el cerdo, cuya vasculatura coronaria se parece mucho a la humana. Descubrimos alteraciones en la microvasculatura, particularmente cambios estructurales y angioadaptación que resultan en una capacidad alterada para la difusión de oxígeno después del infarto de miocardio. Además, evaluamos el potencial del conocimiento extraído en términos de predecir la condición fisiopatológica del tejido. La alta precisión lograda en este sentido, demuestra la capacidad de nuestro abordaje no solo para cuantificar e identificar cambios patológicos de lechos microvasculares, sino también para predecir patrones microvasculares complejos y dinámicos. Por último, los

datos anatómicos obtenidos para la microvasculatura se usaron para "alimentar" un modelo de perfusión continuo con el fin de calcular tensores de permeabilidad fisiológicamente significativos. Al utilizar este enfoque teórico de modelado de flujo sanguíneo, pudimos obtener información sobre la perfusión tisular y demostrar el efecto funcional de los cambios estructurales que ocurren como resultado del infarto de miocardio.

En general, este trabajo es un paso adelante para aumentar nuestra comprensión de las alteraciones microvasculares después del infarto de miocardio, modelar la microcirculación en diferentes etapas después del daño tisular y también proporciona un método imparcial para la evaluación de posibles tratamientos.

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## NOMENCLATURE

#### **Acronyms / Abbreviations**

- 3D Three-Dimensional
- BC Box Counting
- BCs Boundary Conditions
- BISQUE Bio-Image Semantic Query User Environment
- BM Basement Membrane
- CFM Continuum Flow Model
- CLSM Confocal Laser Scanning Microscopy
- CMs Cardiomyocytes
- CTA Computed Tomography Angiography
- CT Computed Tomography
- CVD Cardiovascular disease
- ECs Endothelial Cells
- ED Extravascular Distance
- I1MI Infarcted area 1 day post MI
- I3MI Infarcted area 3 day post MI
- I45MI Infarcted area 45 day post MI
- I7MI Infarcted area 7 day post MI

#### Nomenclature

- R1MI Remote area 1 day post MI
- R3MI Remote area 3 day post MI
- R45MI Remote area 45 day post MI
- R7MI Remote area 7 day post MI
- FBs Fibroblasts
- GCV Great Cardiac Vein
- I/R Ischaemia-Reperfusion
- IVUS Intravascular Ultrasonography
- KESM Knife-edge Scanning
- Knn K-nearest Neighbours Classifier
- LAD Left Anterior Descending
- LDx Left Circumflex
- LMCA Left Main Coronary Artery
- LV Left Ventricle
- LVPV Left Ventricular Posterior Vein
- MBF Myocardial Blood Flow
- PET Photon Emission Tomography
- MCV Middle Cardiac Vein
- MF Minkowski Functional
- micro-CT Micro Computed Tomography
- MI Myocardial Infarction
- MIP Maximum Intensity Projection
- MMT Multi-scale Multi-level Thresholding
- MRA Magnetic Resonance Angiography

- MRI Magnetic Resonance Imaging
- NLMF Non-Local Means Filtering
- NSTEMI Non-ST Elevation Myocardial Infarction
- OCT Optical Coherence Tomography
- OMERO Open Microscopy Environment Remote Objects
- PDA Posterior Descending Artery
- PDGFRB Platelet-Derived Growth Factor Receptor Beta
- RBC Red Blood Cells
- RCA Right Coronary Artery
- **REV** Representative Elementary Volume
- SCV Small Cardiac Vein
- SEM Scanning Electron Microscopy
- SMC Smooth Muscle Cells
- SPIM Selective Plane Illumination Microscopy
- STEMI ST-segment Elevation Myocardial Infarction
- SVM Support Vector Machine

## CHAPTER 1

#### INTRODUCTION

### **1.1 Motivation**

Cardiovascular disease (CVD) is the leading cause of deaths worldwide, despite significant progress having been made in its prognosis, treatment and medical management [76]. The socio-economic burden associated with CVD, including myocardial infarction (MI) and its complications such as heart failure [70], has led an important amount of research to focus on unravelling its causes and developing therapeutic approaches [240]. Dysfunction of the coronary microvasculature, even after successful reperfusion of coronary arteries, is increasingly being recognized as the decisive factor for inadequate myocardial tissue reperfusion which can lead to prolonged ischaemia and adverse clinical outcomes [221]. Therefore, the coronary microvasculature has been proposed as a potential prognostic biomarker and therapeutic target [159, 28].

The microvasculature represents the anatomy of microcirculation and comprises the smallest blood vessels of the tissues with diameters of only few microns; capillaries, arterioles and venules. Its architecture is a major determinant of blood flow, oxygen transport, wall shear stress and distribution of pressure in microvessels [177, 163]. Nonetheless, its *in vivo* visualization in humans remains a bottleneck. For this reason, *ex vivo* imaging and different animals models have been used [230, 22, 10, 138, 12, 104]. Among them, the pig animal model has attracted considerable attention due to the similarity of its coronary network to that of humans. Pioneering work by Kassab [104] in this area has provided a wealth of data regarding diameters, length and topology of the porcine microvascular network, through the use of coronary corrosion casts. These data provided significant insight into porcine microvascular structure and enabled the subsequent modelling of hemodynamics at basal conditions [123, 105].

However, the microvasculature is not a static system, but rather a dynamic one and it continuously adapts in order to meet the tissue demands in response to physiological and

#### Introduction



Fig. 1.1 Schematic illustration of the possible forms of angioadaptation. From right to left, the initial state of the microvasculature and the possible vascular patterning processes that can occur during angioadaptation, i.e. sprouting and splitting angiogenesis, structural remodelling and regression.

pathophysiological conditions, such as MI and other CVD, such as diabetes or hypertension. Thanks to its dynamic nature, the microvasculature is capable of adding new vessels by sprouting [32] or intussusceptive (splitting) angiogenesis [48], altering the structure of existing vessels (remodelling [168]) and pruning of abundant vessels (regression [115]). These processes of vascular patterning, termed angioadaptation [236] (Fig. 1.1), affect the architecture of the microvasculature. Despite significant attempts to develop approaches that would promote therapeutic remodelling or revascularization [221], no drug has so been approved for clinical use for MI [39, 33]. There is, therefore, a growing quest to develop a better understanding of the microvascular dynamic changes in MI and how to control them. Towards this aim, quantitative data on the anatomy of coronary microcirculation in pathology and not only under basal conditions are essential [229]. These data are the key challenge for understanding structure-function relation through modelling approaches, and thus, developing and evaluating more efficient therapeutic approaches. In addition, studies are expected to recognize the inherently three-dimensional (3D) and complex structure of the microvasculature.

Obtaining such structural 3D data that span several stages of pathology is nowadays more feasible than ever thanks to revolutionary advancements in imaging systems, including x-ray phase-contrast synchrotron radiation-based micro computed tomography [83], knife-edge scanning [143] and confocal laser scanning microscopy (CLSM) [154]. CLSM has the advantage over other imaging techniques of being a widely available imaging system in biomedical laboratories and of allowing the visualization of the microvasculature with sub-micrometer resolution, at increasing imaging depths. In addition, when combined with

fluorescent dyes, it permits the simultaneous study of the microvasculature in relation to other key players in tissue healing after MI.

However, today's microscopes, produce a vast amount of data whose complexity preclude traditional manual and supervised analysis methods [144]. A work-around adapted by the scientific community during several years has been the assessment of the microvasculature from 2D slices or maximum intensity projections produced from 3D volumes. Nonetheless, it is increasingly apparent that this approach might lead to errors, misinterpretations and loss of important information of biological significance. Therefore, the quest for 3D automated bioimage analysis tools has steadily increased [58]. This exciting area of research and development, that forms part of the emerging field of "bioimage informatics" [155], is already revolutionizing biomedical sciences by offering the possibility to obtain unprecedented insights into biomedical problems, while dealing with subjectivity problems as well as time restrictions and labour requirements in both manual and supervised analysis.

In the case of microcirculation, the rise of 3D automatic image analysis approaches has the additional potential to enable the study of the function of the microvasculature which is hampered by today's imaging limitations. More precisely, *in vivo* measurement of blood flow or pressure in the smallest microvessels embedded in the deeper layers of organs, such as the heart, remains a particularly challenging task or it is feasible only for epicardial vessels [112]. Theoretical models have therefore emerged as a way of going beyond experimental observations and of obtaining novel insight into perfusion and structure-function relation by exploiting structural data [206, 82]. Moreover, despite the challenging nature of the task, reconstruction and quantitative structural information of detailed 3D microvascular networks promises to allow the transition from modelling based on idealized networks to realistic modelling based on anatomical data, i.e. image-based modelling [213, 97, 96, 160]. Blood flow models have been further complemented by models that simulate oxygen transport [201, 80, 64], structural adaptation [172, 168] and blood flow regulation [197].

Overall, it is crucial to quantitatively assess the coronary microvasculature and its changes after myocardial infarction since such knowledge will enhance understanding of the mechanisms of myocardial infarction as well as the design and objective evaluation of new angiotherapies. In a clinical context, a thorough and unbiased evaluation of therapy effectiveness in protecting or restoring the microvasculature can positively affect the management of MI patients. Toward quantitative assessment of the microvasculature and its infarction-related functional and structural changes, fully automated image analysis approaches, combined with mathematical microvascular models, can serve as powerful tools for obtaining unprecedented conclusions from experimental data, as well as complementary solutions to experimental approaches.

#### Introduction



Fig. 1.2 Overview of the study design and goals.

## 1.2 Objective

The main aim of this PhD thesis is to **decipher cardiac microcirculation pathophysiology in infarcted and remote areas after myocardial infarction in an unbiased and reproducible manner**. Towards this aim, we have adopted an interdisciplinary approach combining confocal microscopy, fully automated 3D image analysis and mathematical modelling approaches. Moreover, we have performed our study using an animal model that bears close similarities to humans; the pig. We are particularly interested in the early stages after MI, when tissue salvage might be optimized and maladaptive physiology might be reduced. Quantitative assessment of structural and functional changes occurring to the microvasculature in infarcted and remote cardiac areas after MI, as well as their evolution during disease progression, will enhance our understanding of the pathological process. Concomitantly, this will contribute to the development and efficient evaluation of potential therapeutic approaches which can assist in the acceleration of the translation of findings from basic research to the clinic.

The following main objectives and relative sub-tasks had to be fulfilled to achieve the aim of the thesis:

- i. Acquisition of imaging data of cardiac porcine tissue at different stages after myocardial infarction and at basal conditions with adequate resolution to resolve the microvasculature as well as other cell types.
- ii. Design and development of a fully automated 3D image analysis approach that permits quantification of all major microvascular features and revelation of microvascular changes in the aftermath of myocardial infarction.
- iii. Use of the extracted microvascular features in the prediction of the diseased or healthy state of complex microvascular patterns.
- iv. Modelling of the microvascular behaviour after infarction, and in particular, of its functional properties, such as blood flow in microcirculation, based on the detailed structural patterns of the microvasculature, as well as identification of structural-functional associations.

It is worth noting that this thesis was undertaken as part of the Marie Curie Initial Training Networks program "Next generation training in cardiovascular research and innovation" ("CardioNext", grant Agreement 608027). "CardioNext" was an innovative program that ran from 2013 until 2017 with the aim of training the next generation of translational scientists by combining extensive multi-disciplinary training and innovative individual research projects focused on ischaemic heart disease. More precisely, as part of "CardioNext", cardiac ischaemia/reperfusion (I/R), which is an experimental model of myocardial infarction, and post-infarction remodelling were investigated using the pig preclinical model. The projects aimed at enhancing our understanding of the response of the heart to I/R and at developing novel preventive, diagnostic, prognostic and therapeutic strategies.

## **1.3** Study design & Strategy adopted

CLSM was selected as the imaging system of this work to acquire images with adequate spatial resolution to resolve the microvasculature. As noted previously, CLSM offers submicrometer resolution and allows the simultaneous visualization of the microvasculature with other cell types, such as smooth muscle cells. Here, we used thick slices ( $\sim 100\mu m$ ) of tissue from remote and infarcted areas of porcine hearts. The hearts were obtained from animals that had suffered MI and had been sacrificed at 1, 3, 7 days following MI. Hearts were also obtained from animals at basal conditions. The tissues were stained by means of immunohistochemistry for nuclei, endothelial cell junctions and smooth muscle actin positive (SMA<sup>+</sup>) cells. In total, 126 multi-channel confocal images were acquired for these time-points. Given the impact that microvascular changes at later time points may have in long-term cardiac remodelling and function, the dataset was later augmented with imaging data from subjects sacrificed at 45 days post MI, a stage in which cardiac remodelling has already taken place. It is worth noting that the preclinical experimental model of myocardial infarction is 30-minute occlusion of the left anterior descending coronary artery followed by reperfusion. Moreover, the pig animal model was selected due to its high translational value.

To achieve the second objective of the thesis, we designed a 3D fully automatic bioimage analysis pipeline that allows us to reconstruct the complete microvasculature from stained endothelial cell junctions and SMA<sup>+</sup> cells. Reconstruction of the cardiac microvasculature is based on a novel multi-scale, multi-level thresholding (MMT) algorithm for the segmentation of labelled structures from microscopic images and an ad-hoc "filling" approach. The development of the "filling" approach is of utmost importance because VE-Cadherin is a marker of endothelial cell junctions and it does not label the vessel lumen. It was selected as an endothelial marker over other commonly used markers of the vessel lumen due to its preciseness, even in infarction. However, staining with VE-Cadherin results in the presence of gaps in the reconstructed blood vessels. The size of these gaps is not homogeneous and depends on the type of microvessel i.e. capillaries, arterioles or venules. Thus, the "filling" method was developed to fill gaps of heterogeneous size depending on the vessel type. Following application of MMT and "filling", an accurate reconstruction of the complete microvasculature was produced.

The pipeline subsequently extracts essential biological information about all major characteristics of the microvasculature, the relation with SMA<sup>+</sup> and capillary diffusion regions. This was not possible in the past when using a single software. We demonstrated that by using confocal microscopy and our pipeline, we are able to reveal fine alterations of the cardiac microvasculature in the aftermath of MI. The fully automatic nature of the approach ensures reproducibility, reliable significance and objectiveness of the findings.

Furthermore, we evaluated the potential of the extracted knowledge to be used for predicting the pathophysiological condition of unseen tissue. A high accuracy (higher than 80%) in distinguishing infarcted from basal tissue and in recognizing the stage of the infarcted tissue was achieved. Lastly, we also investigated the imaging data from the later time point post MI (45 days) to understand whether early microvascular alterations persist and/or change during cardiac remodelling.

Towards the last objective of the thesis, we took advantage of the developed bioimage analysis tools and a continuum perfusion model [205, 56]. We developed an image-based modelling framework that uses the reconstructed 3D microvasculature and its structural properties as input and provides us with estimations regarding tissue-scale flow properties, such as the permeability tensor. The permeability tensor encapsulates the reverse flow resistivity of the network and can be calculated by volume-averaging the flow solution over sub-units of spatially periodic networks. In an effort to tackle the spatial periodicity requirement, a mirroring approach is firstly applied on each fully connected sub-network of the reconstructed microvasculature. The tensors of the sub-networks are calculated and they are fused to provide a tensor for all the microvascular network. Following this approach, we were able to obtain a physiologically meaningful solution for the permeability tensors by using the anatomical data directly instead of using artificially generated networks. We then defined the effect of phase separation on the tensors. Lastly, using the permeability tensors, the expected pressure drop along an arteriole-venule path given data from magnetic resonance imagining (MRI) as well as the myocardial blood flow assuming constant and varying pressure drop according to tissue condition. The results of the simulations suggest that permeability tensors and tissue perfusion can be closely approximated using our image-based mathematical modelling approach. Moreover, statistical change analysis of the permeability tensors at different tissue conditions allowed us to reveal the progressive impact of MI at the microvascular perfusion.

A brief overview of the study's design and milestones achieved at each step is provided in Fig. 1.2.

### **1.4 Document Organization**

The current document is organized into six chapters. The current chapter (Chapter 1) introduces the motivation for the development of this PhD thesis, its principal objectives and the strategies adopted to achieve them.

Chapter 2 introduces the biomedical background related to the present work. In particular, the anatomy and physiology of the cardiovascular system, the basics of myocardial infarction

#### Introduction

and the microvasculature are presented. Subsequently, the chapter provides an overview of the imaging technologies available for the visualization of 3D vascular networks with a particular focus on CLSM which is the technique used in the present work. Lastly, an introduction to the basic concepts and the state of the art of the areas of automated image analysis for achieving quantitative microscopy and of modelling of blood flow in microcirculation is provided.

Chapters 3, 4 and 5 contain the main contributions of this thesis. More precisely, Chapter 3 firstly provides a detailed overview of the methodology adopted for the acquisition of the dataset used in this study. Subsequently, the algorithm developed for the accurate segmentation of microscopic 3D images, which are labelled with fluorescent markers, is presented. Lastly, we present the pipeline developed to permit the accurate reconstruction of the complete microvasculature, even in cases where large gaps were present in the labelled structures.

Chapter 4 describes the 3D fully automated bioimage analysis pipeline that was developed to obtain quantitative insight into the cardiac microvasculature and the alterations taking place to it at several stages following MI. The findings obtained by means of the developed pipeline are presented in detail along with geometric models for describing capillary supply areas. Moreover, it is demonstrated that the pool of microvascular features extracted can be incorporated into different classification schemes to predict the healthy or diseased state of the tissue.

Chapter 5 presents the image-based computational framework developed to model and analyse blood flow in the cardiac microvasculature, using real anatomical data. Application of the framework on the imaging data of our dataset and the findings regarding infarction-related alterations in permeability tensors, pressure drop and tissue perfusion are presented.

Chapter 6 summarizes the conclusions and contributions of the thesis and also outlines the limitations of the work. Moreover, it provides examples of the application of the developed approaches in the in-depth analysis of the microvasculature in other biomedical contexts, such as tumour and cardiac mouse tissue. The chapter concludes with a brief presentation of possible future research lines.

### CHAPTER 2

#### BACKGROUND AND STATE OF THE ART

### 2.1 Introduction

This chapter provides an overview of the biomedical context that inspired and formed the basis upon which the present thesis was developed. In addition, it introduces state-of-art approaches in imaging, bioimage analysis and microvascular mathematical modelling. Therefore, this chapter allows us to delve further into the biomedical problem posed, the challenges that need to be tackled and the scientific gaps that need to be bridged by this and future works.

Firstly, a brief overview of the anatomy and physiology of the cardiovascular system and its key components at the macroscopic and microscopic levels is provided. In continuation, we focus on the pathophysiology of myocardial infarction (MI), the main pathology investigated in this thesis. The focus of the chapter subsequently shifts to the microvasculature, a major determinant of the outcome of MI, and to its adaptive nature in health and pathology. Subsequently, we review imaging techniques for the visualization of 3D vascular networks. State-of-art techniques that can be used to image vascular networks from the epicardial vessels down to the capillary level are presented, while special attention is paid to confocal microscopy, the imaging system adopted in this work. We review tools and tasks involved in bioimage analysis, and particularly in the field of automated analysis of microscopic images, an indispensable approach in exploiting the huge amount of data produced by imaging systems in order to extract deep quantitative knowledge. The chapter concludes with a short summary of the basic rheological events involved in microvascular blood flow and of current approaches to model blood flow in microcirculation.

### **2.2 Biomedical Context**

#### 2.2.1 Anatomy and physiology of the cardiovascular system

The cardiovascular system consists of the heart and the circulatory system [1]. The heart is an organ that functions as a muscular pump which propels blood throughout the body to provide oxygen and vital nutrients to all cells. The transport of blood is performed by the circulatory system that is formed of blood and lymphatic vessels [164]. The blood vasculature provides the pathway for the blood to flow, while the lymphatic vasculature provides the pathway for extravasated interstitial fluid and macromolecules to return to blood circulation. Hierarchical networks of arteries and veins form the main components of the blood vasculature. Arteries transport blood rich in oxygen form the heart to the body, while veins transport de-oxygenated blood towards the heart. Arteries and veins are connected by a network of smaller arteries, arterioles, interconnected tiny vessels, i.e. the capillaries, venules and veins. Blood itself is a mixture of red blood cells (RBC), white blood cells or leukocytes, platelets and blood plasma, which contains various dissolved proteins, molecules and ions [163].

At the organ level, the heart in mammals consists of four chambers: the right and left atrium which are the highest chambers and collect blood from the body, and the right ventricle and the left ventricle which are the lowest chambers and pump blood to the body. More precisely, the right atrium receives de-oxygenated blood from the upper and lower body through two large veins; the superior and inferior vena cava respectively. The left atrium receives oxygen-rich blood from the lungs through the pulmonary veins. Subsequently, the atria contract and the blood flows from each atrium towards the ventricle of the same side. Once the ventricles are full, the atrioventricular valves that separate the atria from the ventricles close and the semilunar valves at the bottom of the aorta and pulmonary arteries open. Blood in the right ventricle is pumped during the subsequent contraction of the ventricles through the pulmonary arteries to the lungs where it will be enriched with oxygen. On the other side of the heart, blood already rich in oxygen in the left ventricle is pumped to the rest of the body through the aorta.

Contraction of the heart is achieved through its wall which is divided into three layers: the epicardium which is the outer layer of the heart that protects it, the myocardium which is the inner most layer of the heart. The area between the myocardium and the endocardium is known as subendocardium. Similarly, the area between the myocardium and the epicardium is referred to as the subepicardium. The wall layers differ in terms of fibre orientation [210] and perfusion, in both healthy and pathological conditions [41], as well as in terms of the pressure exerted to them and their susceptibility to myocardial ischaemia, with the subendocardium

Table 2.1 Classification of coronary vessels based on their diameters. Data reproduced from [122]. Ranges are based on reported literature values and the authors have mainly used data regarding the human heart. Values might be different for other species.

Vessel type	Diameter
Major arteries (LAD, RCA etc)	3 – 5 <i>mm</i>
Arteries	$> 300 \ \mu m$
Small arteries	$150 - 300 \ \mu m$
Arterioles (muscular)	$20 - 150 \ \mu m$
Terminal (pre-capillary) arterioles	$10 - 20 \ \mu m$
Capillaries	$5 - 10 \ \mu m$
Venules	$10 - 50 \ \mu m$
Small veins	$50 - 300 \ \mu m$
Veins (muscular)	$> 300 \ \mu m$
Greater veins (GCV, MCV etc.)	1-7 mm
Coronary sinus	10 mm

being at higher risk [2]. The three layers of the heart wall are enclosed in a membrane known as the pericardium.

The heart has its own circulatory system, known as the coronary circulation system [211]. Its function is to provide oxygen and nutrients to the heart tissue itself and to remove waste products. As in the case of other tissues, the coronary circulation system consists of an interconnected network of numerous vessels of different types and sizes (Table 2.1). The coronary vessels can be further categorized based on their size and position within the heart into epicardial vessels (number less than 10), intramural vessels (number less than  $10^6$ ) and vessels of the microcirculation (number larger than  $10^9$ )[142, 122].

Epicardial vessels are the largest vessels of the coronary circulation with diameters ranging from  $500\mu m$  up to few *mm*. As their name implies they can be found at the outer level of the heart wall, the epicardium. The primary coronary epicardial arteries include the left main coronary artery (LMCA) and the right coronary artery (RCA) along with their branches [225, 211] (Fig. 2.1). Both arteries originate from the tricuspid aortic valve located at the root of the aorta. In particular, RCA typically originates from the right sinus of Valsalva and branches out to smaller branches, including the right posterior descending artery (PDA) and the acute marginal arteries. RCA and its branches perfuse the free wall of the right ventricle, the sino-atrial and atrioventricular nodes and part of the left ventricle. The LMCA originates from the left sinus of Valsalva and it branches into the left anterior descending (LAD) and left circumflex (LDx) arteries that further branch off and perfuse the free wall of the left ventricle (LV) and the septum. The coronary epicardial arteries work as conduit vessels. At the surface of heart, apart from the major arteries, the greater veins can be found. The anatomy of the veins might vary, but generally it is formed by the great cardiac vein (GCV) that initially runs parallel at the start of the LAD artery, the middle cardiac vein

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Fig. 2.1 The heart and the composition of its vessels. (a) Schematic representation of the human heart with the main epicardial arteries roughly annotated. Dotted white lines depict the continuation of arteries on the posterior view of the heart. (b) A schematic illustration of a microvasculature plexus along with the composition of the wall of the different vessels that comprise the coronary blood circulatory system. Wall composition variability is related to the distinct function of each type of vessel.

(MCV) that follows the PDA, the small cardiac vein (SCV) that is parallel to the RCA in the posterior view and the left ventricular posterior vein (LVPV) that follows the LCx artery [86, 122]. The greater veins (GCV, MGV, SCV, LVPV) join together in the coronary sinus, a large vessel of diameter 10*mm* that drains blood low in oxygen to the right atrium. Moreover, in the anterior orientation of the heart the anterior cardiac veins of the right ventricle, which drain the right atrium, are found.

Intramural vessels arise as branches of the large coronary arteries and penetrate the myocardium starting from the epicardium towards the myocardium. These intramural arteries with diameters within the range of  $100 - 500\mu m$  are usually referred to as pre-arterioles [28]. Pre-arterioles divide into several orders of smaller arteries until they give rise to the arterioles (diameters  $20 - 150\mu m$ ) and subsequently to the terminal arterioles  $(10 - 20\mu m)$ . Pre-arterioles and arterioles are the main sites of coronary resistance [65]. Pre-arterioles are responsible for flow and myogenic-dependent auto-regulation which is achieved by adapting their diameter according to changes in stress and pressure respectively. Arterioles are directly connected to the smallest vessels of the tissue, the capillaries. The capillaries play a major role in tissue perfusion as they are responsible for oxygen and nutrient transport, as well as for the removal of waste products, a function that is facilitated by their thin walls. The capillary bed is connected to the venules on the other side. These are subsequently connected to small veins. Some of these veins (Thebesian veins) drain directly into the heart

chambers, while others are connected to the greater veins or posterior veins that drain into the right atrium as mentioned earlier [85]. Venules and veins function as capacitance vessels and their walls are hence thinner compared to that of arteries because they have to be able to resist lower pressure. It is worth noting that the capillaries, along with arterioles and venules, comprise the microvasculature and are usually referred to as microcirculation, while the rest of the vessels are referred to as macrocirculation.

At the cellular level, the heart comprises many cell types. The major cell types are cardiomyocytes (CMs), fibroblasts (FBs), endothelial cells (ECs), and peri-vascular cells, such as vascular smooth muscle cells (SMCs) [239]. The exact values reported in literature for the relative percentages of these cells vary depending on the species under investigation and the method used to count them, i.e stereology or flow cytometry. In fact, although there is an agreement that the cardiomyocytes occupy 70 - 85% of the volume of mammalian heart and their number represents 30 - 40% of all cells, the relative frequency of non-myocytes has long been disputed. Based on different studies, Zhou and Pu [239] summarized ratios of ECs to CMs and of FBs to CMs within the ranges of 0.8 - 1.9 and 0.4 - 0.7 respectively. Elsewhere, the ratio of ECs to CMs has been reported as 3:1 [164]. Moreover, recently the relative frequency of non-myocytes of the mouse and human were revisited using cutting-edge histology and flow cytometry techniques, as well as modern reagents [161]. The study confirmed that ECs, and particularly vascular ECs, are among the most abundant cells in the adult heart, while fibroblasts represent a minor cell population.

The abundance of vascular ECs can be explained by the fact that they line up the interior of the wall of all blood vessels, forming the endothelium. Apart from endothelium, the wall of blood vessels consists of other cellular and extracellular components and its exact composition varies depending on the vessel type (Fig. 2.1)[25, 38, 164]. More precisely, the walls of large arteries and veins consist of three compartments; the tunica intima, the tunica media and the tunica adventitia. The tunica intima consists of a continuous monolayer of endothelium, covered by basement membrane (BM) and an internal elastic layer. The compartment is surrounded by the tunica media that mainly comprises SMCs but also fibroblasts, connective tissue and collagen wrapped by external elastic tissue. SMCs determine the vascular tone of the vessels and play a pivotal role in flow and pressure regulation. In the case of large arteries, the SMCs layer is thicker than the corresponding layer of veins. The outermost layer of the large vessels, i.e. the tunica adventitia layer, is formed by connective tissue with elastic and collagenous fibres and interspersed fibroblasts, macrophages, mast and progenitor cells among others [139]. It is separated by the media by an external elastic membrane. Arterioles and venules comprise the endothelium layer encased in the BM and solely one or two layers of SMCs, in the case of arterioles or few SMCs, in the case of venules. Arterioles have

additionally internal elastic lamina and their adventitia is of similar thickness to that of their media and it merges with the surrounding tissue. Venules do not present laminae and their adventitia merges with surrounding tissue as well. Lastly, capillaries of the heart also consist of a continuous monolayer of endothelium surrounded by a BM and pericytes. It should be noted that although the endothelium of capillaries in the heart is continuous, it can be fenestrated in other organs, i.e. ECs have pores or fenestrae, or discontinuous [164]. It is also worth highlighting that the aforementioned variety in the thickness of the wall among the different types of vessels is related to their function. More precisely, the arteries have the thickest walls to resist high pressure and push the blood, veins have thinner walls compared to the arteries because they have a capacitance function of collecting blood, while capillaries, which are responsible for oxygen transportation, have thin walls.

#### 2.2.2 Myocardial infarction

Myocardial infarction (MI) is an acute coronary syndrome that results from the interruption of blood flow to a part of the heart caused by an abrupt blockage of a coronary epicardial artery. MI results in inadequate blood supply to the tissue, i.e. ischaemia, which leads to oxygen deprivation, cardiomyocyte cell death and degradation of the cardiac extracellular matrix [74]. Clinically it can be manifested either with an ST-segment elevation (STEMI) or without it, i.e. non-ST elevation MI (NSTEMI) [217]. If ischaemia is left untreated, it can lead to global left ventricular damage and chronic heart failure. Heart failure is a major global health problem affecting an estimated number of 38 million people worldwide [20]. Moreover, although the mortality due to acute MI has decreased thanks to techniques such as timely reperfusion, the incidence of heart failure and the associated socio-economical burden might be increasing [77, 223, 59, 27]. In fact, the prevalence of heart failure is expected to further increase as a combination of population ageing and improved survival rates after MI and other cardiovascular diseases. Therefore, MI continues to contribute as a major health problem both directly, through its unacceptably still high incidence [179], and indirectly, through its complications.

Cardiac repair and remodelling after MI can be divided into four phases: (i) cardiomyocyte cell death, (ii) inflammatory, (iv) formation of granulation tissue, and (v) scar formation [40, 141]. The exact time-course of these phases might differ among different species. Cardiomyocyte death due to necrosis or apoptosis starts within hours after MI. Cell injury activates an intense inflammatory response that involves degradation of extracellular matrix, secretion of pro-inflammatory cytokines and chemokines, infiltration/recruitment of inflammatory cells, including neutrophils, lymphocytes, monocytes, macrophages and dendritic cells. The recruited inflammatory cells remove necrotic cell and extracellular matrix debris
from the tissue by means of phagocytosis. The first two phases could be considered as a common phase; the acute inflammatory phase [74, 165]. Transition from inflammation to repair commences on day four and might last for several days, e.g. up to ten days in the mouse species. This proliferative phase is characterised by resolution of inflammation and transition of fibroblasts to myofibroblasts. The latter deposit collagen matrix and granulation tissue is staring to form. This tissue consists of macrophages, myofibroblasts, new blood vessels and extracellular matrix proteins. During the final phase, granulation tissue matures into a fibrous scar. The scar is free of inflammatory cells and rich in cross-linked collagen. This process lasts from several weeks to a few months and it is accompanied by a thinning of the scar, dilatation of the left ventricle and activation of interstitial fibrosis. These mechanisms might initially play a compensatory role, but over time they could contribute to inadequate healing. This has been linked to the presence of myofibroblasts in areas remote to the infarct and, at the macroscopic scale, to further dilatation of the heart and to geometrical changes to its shape [218].

A major determinant of the adverse outcome of the healing process that leads to deteriorated cardiac function and heart failure is microcirculation. After successful restoration of blood flow to the occluded epicardial artery, adequate perfusion of the myocardial tissue depends on microcirculation which includes the smallest vessels of the tissue [98]. It is therefore of utmost importance to develop therapies that inhibit microvascular dysfunction in order to prevent heart failure rather than to just focus on treating it [98, 91, 27]. To achieve this, the impact of reperfusion on the myocardium should be also taken into account because reperfusion causes damage to the myocardium, despite being mandatory in terms of salvaging the myocardium and reducing infarct size [114, 91, 98].

Since microcirculation cannot be assessed *in vivo*, indirect measures have been used to assess its functionality in a clinical environment. These measures include invasive or non-invasive measurement of coronary blood flow velocity, myocardial blood flow and indices, such as coronary flow reverse that is provided by the ratio of hyperaemic to baseline blood flow or by calculating microcirculatory resistance indexes during coronary catheterization [28]. In depth study of the pathophysiology of ischaemia-reperfusion at the microvascular level, however, requires detailed structural descriptions of microvascular networks. This knowledge can be acquired by post-mortem studies or more frequently by the use of preclinical animal models which also allow us to test hypotheses regarding the mechanisms of pathology and of novel therapies.

Pre-clinical models can be divided into small and large. Both types of models have their pros and cons without any one of them being ultimately the best choice for every scenario [98]. Small animal models are of paramount importance in the study of the hypothesis and of

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phenomena in basic research. On the one hand, the possibility of directly translating findings based on these models to the clinics is limited. On the other hand, larger models, such as swans, dogs or pigs are more expensive and difficult to handle, but their translational value is higher, particularly in the case of those models that are closer to humans, such as the pig.

## 2.2.3 Microvasculature in health and disease

The primary function of microcirculation is the supply of oxygen and nutrients to the tissues to meet their metabolic demands. For this to be achieved and by taking into account that oxygen, in contrast to other solutes such as glucose, can diffuse only over very short distances  $(20 - 200\mu m)$ , the microvasculature presents an interconnected and complex structure that ensures minimal diffusion distance from blood to tissue and maximum surface for exchanges [174]. Particularly in the case of coronary microcirculation, the microvasculature must transfer oxygenated blood in very small distances to satisfy the metabolic demands of individual cardiomyocytes that are high due to their role of facilitating myocardial contraction and relaxation.

Each organ has different metabolic needs. Organization of the capillary bed might therefore differ significantly from organ to organ and also within the same organ, in terms of the morphological characteristics of the microvessels, the angioarchitecture of the network, the arteriolar-venular pathway length and its segments [175]. Structural heterogeneity is accompanied by hemodynamic and functional differences. Due to the close link between the structure and function of the microvasculature, as well as because of difficulties in directly observing microvascular function, significant amount of work has focused on describing the structure of the microvasculature as an indirect determinant of its function. Since the beginning of the 20th century, the structure and arrangement of the coronary microvasculature has been studied in various animal models, including rats [12], sheep [22], horses [22], cats [230, 22], rabbits [230, 138], dogs [10, 22], pigs [104], but also in humans in post-mortem studies [230, 60, 101].

The microvasculature has the unique ability to constantly adapt its structure to satisfy the changing hemodynamic and metabolic demands of the heart. Angioadaptation occurs as a response to growth factors, hemodynamic and metabolic stimuli sensed by endothelial and other cells [236, 39]. The onset of these stimuli, such as oxygen tension, shear stress, and subsequent angioadaptation, is triggered by physiological conditions like exercise and growth, but also under pathological conditions, such as ischaemia. Angioadaptation takes place in short and long term. In the shorter run, the microvasculature can alter the diameters of its segments to maintain blood flow. In the longer run, it might change the composition of its vessels, it can develop novel branches through angiogenesis or intussusception or it might have its abundant vessels pruned.

Studies describing the altered structure of the microvasculature, following an ischaemic episode, have reported decreased capillary density and changes in the diameters of the microvasculature of infarcted areas. [5, 6, 228, 21, 30, 92, 204]. In addition, changes have also been observed in the investment of vessels with perivascular smooth muscle actin cells [181, 116, 49] and of the surrounding tissue with non-perivascular smooth muscle actin expressing cells, i.e. myofibroblasts [49, 218]. More precisely, using a rat animal model and confocal microscopy [228], a 3D study of the microvasculature immediately after MI reported that capillaries appeared to have smaller diameters and to be more tortuous after reperfusion. This resulted in an overall decreased capillary volume, compared to control animals. When reperfusion did not take place, subsequent changes were similar, albeit more prominent. On the contrary, other studies reported the opposite effect, i.e. further decrease of the capillary density in the microvasculature after reperfusion was applied (30 minutes ischaemia followed by 90 minutes reperfusion), as well as vasodilation [92]. On the same animal model, it was further reported that 3 days following MI although myocytes appeared enlarged, the capillary network did not follow the same enlarging trend [6]. On the contrary, decreased laminar surface and increased diffusion distances for oxygen were noticed, something that points to worst tissue perfusion and oxygenation [5]. Four weeks after MI, decreased capillarity similar to those described in earlier studies of human ischaemic cardiomyopathy was observed was observed in infarcted areas of porcine cardiac tissue [116]. The changes in capillary were accompanied by a thickening of the smooth muscle actin cells coverage in infarcted areas. In general, structural changes are more prominent in infarcted areas, but alterations have also been reported in areas remote from the infarct. For example, similar morphological changes in terms of reduced diameter and increased tortuosity as in [228] were reported for capillaries at remote areas of rats suffering from ischaemic congestive heart failure, and also increased diffusion distances were observed in post-mortem human studies of NSTEMI [30]. Despite the paramount importance of these studies, overall information regarding the microvasculature and the progressive changes after MI is sporadic. Moreover in some cases findings might be contradictory i.e. reperfusion, with only few and simplistic parameters reported in each study, extracted with human intervention and in some cases based on 2D slices, which limits the number of images analysed but also might incorporate bias and misinterpretations. However, nowadays, there is a unique opportunity to revisit the 3D microvasculature using cutting-edge imaging and automated computational approaches on large datasets. This will allow us to reach reliable and ground-breaking conclusions on the

structural "fingerprint" of the microvasculature and its pathology-related adaptation. This exciting area of research and development is the focus of the present thesis.

## 2.3 Imaging of 3D vascular networks

Imaging has revolutionized every day clinical practice and biomedical research. Without doubt, it is an indispensable tool in the diagnosis, treatment and follow-up of patients and in achieving "P4 medicine: Predictive, Preventive, Personalized and Participatory" [94, 93] which will change the way healthcare is viewed and provided. In the study of biological specimens and processes, imaging, also referred to as bioimaging, is one of the most widely used techniques in biological laboratories [58]. Tremendous technological advances in physics, engineering, chemistry have led to the development of a plethora of imaging modalities that permit the structural, functional, static or dynamic study of systems and biological processes from the organ level down to the molecular level. A detailed review of the main imaging modalities from the point of view of systems biology can be found in [109] and, in terms of the source employed for imaging, in [237]. Such sources include visible light for optical imaging, electrons for scanning and transmission electron microscopy, x-rays for traditional radiography or the more advanced computed tomography, radio waves combined with magnetic fields to generate images based on the magnetic moments of hydrogen atoms for magnetic resonance imaging, positrons emitted by radionuclides administered to the subject for positron emission tomography and sound waves for modalities, such as photoacoustic tomography or ultrasonography.

Different bioimaging modalities should be used depending on the size of the object that we wish to resolve. Leaving aside availability and cost, the final choice of imaging modality is usually a trade-off between the size of the smallest object that needs to be resolved, or more precisely expressed, the spatial resolution, i.e. the minimum distance between two objects so that they can be visualized as separate, and the penetration depth that we wish to arrive to. In the case of structural imaging of 3D vascular networks with multiscale architecture, we can roughly categorize the modalities according to the anatomical scale to which the vessels we wish to visualize belong to. Despite focusing here on coronary networks, multi-scale or hierarchical imaging approaches are also useful for the 3D visualization of complete vascular networks in other tissues, such as tumour [110] or the brain [90].

More precisely, in the case of coronary networks and the assessment of the largest vessels of the system, i.e. the epicardial, methods whose spatial resolution is not high but they present no depth limitations and permit wide-field views, are preferred. These techniques which can be applied both in clinics but also in cardiovascular research, include magnetic resonance angiography (MRA), computed tomography angiography (CTA), intravascular ultrasonography (IVUS) and optical coherence tomography (OCT) [122]. Magnetic resonance and computed tomography angiography are techniques used in the 3D visualization of blood vessels in vivo based on the homonymous techniques, i.e magnetic resonance (MRI) and computed tomography (CT) imaging respectively. Unlike traditional angiography, which involves catheterization and intra-coronary administration of a radiopaque contrast medium to make the vessels visible by x-ray, MRA is non-invasive. It uses radio waves and magnetic fields to produce blood vessel images without ionizing radiation. Moreover, MRA can be combined with contrast agents to achieve a brighter appearance of the vessels in relation to background tissue. In research studies it has been possible to use 11.77 MRI to visualize the microvasculature of rabbit hearts [26] with resolution of few dozens of  $\mu m^3$ . Similar resolution has been achieved in small animals in studies of other vascular networks, such as the visualization of glioma (7T MRA) [50] or vasculature of mice brains (9.4T time-of-flight MRA) [69]. It is worth noting that MRI-based sequences in cardiovascular research have found wide application not only in the study of blood vessel structure, but also in the assessment of vessel blood flow, tissue perfusion, metabolism, tissue characterization, including viability, fibrosis and edema in the case of MI [95, 189]. CTA combines computed tomography technology with the injection of iodinated intravenous contrast agents to achieve 3D images of the coronary arteries and the heart. CTA achieves higher spatial resolution than MRA and is therefore preferred in clinics [52], but in cases of advanced calcification it might be better to substitute CTA with MRA [95]. CT technology has also been used for tissue characterization [113], but in this respect MRI is generally considered to be more powerful. In contrast with MRA and CTA, IVUS and OCT are used in the study of the wall of the vessels rather than their structure and organization. IVUS is an invasive in vivo method based on a catheter on which an ultrasound transducer is attached. It is used to visualize the interior of coronary arteries by acquiring serial cross-sectional images that can be used to reconstruct the artery in 3D. IVUS allows us to distinguish the lumen and the layers, i.e. intima, media and adventitia, of the vessel wall. OCT is similar to IVUS. However, it uses near-infrared light to achieve a ten-fold higher spatial resolution than IVUS;  $12 - 18\mu m$  compared to that of IVUS which is  $150 - 200 \mu m$  [15]. This high resolution has led to consider OCT to be the optimal technique in the study of the wall of coronary vessels at the microscopic level [137].

Intramural vessels, found in the deeper layers of the heart and having intermediate diameters, are usually visualized by CTA, cryomicrotome and micro-computed tomography (micro-CT) [122]. CTA has higher resolution compared to MRA and it could therefore be used in the visualization of the larger intramural vessels. However, cryomicrotome imaging is more efficient in obtaining detailed views of the complete organization of intramural vessels.



Fig. 2.2 Typical arrangements and components of confocal microscopes, compared to standard wide-field fluorescent and conventional microscopes (top row) along with an example image acquired by means of each microscope (bottom row). (a) Confocal microscope permits the illumination of a single point of the sample thanks to an illumination pinhole and a dichroic mirror. Out-of-focus light (dotted line) does not pass through the confocal pinhole and it is, therefore, not detected. This allows acquisition of sharp images with cellular and sub-cellular resolution. The source of illumination is a laser. (b) The arrangement of wide-field fluorescence microscopes is similar to that of a confocal microscope although wide-field fluorescence microscopes do not include pinholes. This results in light excited from out-of-focus areas to be detected by the camera and in the acquisition of blurrier images compared to images obtained by confocal microscopes. Moreover, visible light is used instead of a laser light. (c) Conventional light microscopes involve direct illumination of the sample by a lamp. Emitted light both from the focal plane and out-of-focus planes is subsequently recorded by the camera resulting in blurry images. All image panels are reproduced from [102].

Performance of cryomicrotome imaging requires firstly that the coronary vasculature is injected with a fluorescent plastic replica material which is left to harden [212]. Subsequently, the heart is frozen and it is sequentially cut into slices using a cryomicrotome device. Fluorescent imaging is performed on each exposed slice using a high-resolution digital

camera. It is worth noting that fluorescent imaging is based on the principle that fluorophores, such as fluorescent dyes or proteins, bond to molecules and when excited by electromagnetic radiation, i.e. light of specific wavelength, they absorb part of the light. A few nanoseconds later they emit a higher wavelength light, than that of the light they were excited with. Therefore, by using appropriate excitation and emission filters, molecular processes or structures, such as cells and their molecular interactions, location and dynamics of gene expression etc can be tracked. In episcopic microtome imaging, arrangement of the imaging components is such that it permits illumination of the sample from above. Cryomicrotome imaging was developed and used to image the entire coronary tree of a goat heart with a resolution that permitted visualization of all intramural vessels with diameters up to  $25 - 40 \mu m$  [212, 220]. Cryomicrotome imaging can also be used in conjunction with fluorescent micro-spheres and computational approaches to study functional properties, such as coronary flow distribution [107, 222]. Another technique for the 3D visualization of entire vascular trees including intramural vessels is micro-CT [234, 184]. Micro-CT is a nondestructive technique based on 2D x-ray projections acquired at multiple angles that can be combined to reconstruct a 3D image. It can be used in vivo but in order to achieve resolution smaller than  $50\mu m$  and at the same time images of various planes, it is recommended to be used ex vivo to avoid radiation issues. Currently, it is also becoming increasing popular for the visualization of the microcirculation due to its advantages in terms of increased penetration depths permitting us to image the entire microvascular network of the rat heart ([120]) or tumours ([71, 54]) with  $8.2\mu m$  and  $3.4 - 4.8\mu m$  resolution respectively. However, it should be noted that to achieve optimal resolution, perfusion with a radiopaque contrast agent is necessary [234]. This renders the technique very expensive in the case of hearts of larger animal models, such as the pig. Light-sheet microscopy, and, more specifically, Selective Plane Illumination Microscopy (SPIM), is another fluorescent microscopy technique that, that when used in conjunction with clearing protocols used to make the sample transparent, is proving to be promising in imaging 3D vascular networks of small animals using thick tissue blocks, with image resolution down to a few microns [150]. SPIM and light-sheet microscopy in general are based on planar illumination of the sample from the side instead from above as is the case with episcopic imaging. This results in reduced photo-bleaching and out-of-focus signal.

High resolution optical microscopy remains the gold-standard for the visualization of the microvasculature in 3D despite continuous improvements in the aforementioned approaches, as well as the development of novel techniques which, although promising, can be expensive and not readily available, such as x-ray synchrotron radiation micro-CT [149, 132, 238, 83] which can reach  $1\mu m$  resolution without the need to use contrast agents. More precisely,



Fig. 2.3 The multiple scales of the coronary blood circulation system visualized by means of different imaging techniques. Panels (a)-(c) are reproduced from [65], and panel (b) is reproduced from [211]. Different modalities are necessary to visualize vessels of all scales; (a) Maximum intensity projection (MIP) of micro-CT. (b) MIP of a cryomicrotome image from a fluorescent labelled cast permitting visualization of intramural vessels of diameter down to  $40\mu m$ . (c) MIP of ultra-high resolution micro-CT with resolution of  $8\mu m$  permitting to resolve arterioles and part of the capillary bed. (d) MIP of confocal multichannel image with resolution  $0.379x0.379x1.001\mu m$  enabling the visualization of all details of the capillary bed and surrounding smooth muscle actin cells; on top row vessels labelled with VE-Cadherin and on bottom row cells labelled with smooth muscle actin.

imaging techniques such as knife-edge scanning (KESM) or confocal laser scanning microscopy (CLSM) yield the higher resolution that might reach 300*nm*-400*nm* [229]. This allows resolution of even the smallest capillaries of the coronary microvasculature. KESM [143] involves optical imaging of the tissue that is being physically sectioned serially. On the contrary CLSM [154], does not require physical sectioning of the sample but it rather scans the specimen using a process known as "tissue sectioning" or "optical sectioning" that is common among other fluorescent microscopes as well [151]. This process includes shifting the focus of the lighting source at different depths and acquiring images (z-stacks) from each focal plane which are subsequently combined to study labelled structures in 3D. Within each plane, in CLSM, the sample is illuminated point by point. Moreover, the specimen is usually stained by means of antibodies with more than one fluorescent marker simultaneously. This allows acquisition of images at cellular and sub-cellular resolutions with up to six channels with recent microscopes [55]; one channel per fluorescent marker. The advantage of confocal microscopy over other wide-field fluorescent microscopes is that it uses single-point illuminations instead of illuminating the whole sample. Additionally, it erases out-of-focus emitted signal by using a pinhole that is placed at the same focal position with the point that the sample is being illuminated. Moreover, the light sources used in CLSM are laser sources, instead of arc-lamps (or more recently light emitting diodes) used in wide-field microscopes or halogen lamps used in conventional microscopes. Lastly, the detector in CLSM consists of photomultiplier tubes instead of conventional cameras used in other systems. It should also be noted that a computer is indispensable in the control of confocal microscopes by means of adequate software. The typical arrangement of a confocal microscope, compared to that of wide-field fluorescent and conventional microscopy, is provided in Fig. 2.2. It consists of (i) a laser device for the excitation of the sample, (ii) dichroic mirrors that reflect short wavelengths, but allow longer wavelengths to go through them, (iii) an objective, (iv) a pinhole that permits emission to reach the detector only from the point of focus, while light emitted by out-of focus objects is discarded, and (v) the detector. The resolution that can be achieved is primarily defined by the numerical aperture of the objective and is lower in z-axis [42]. Application of confocal microscopy to the coronary microvasculature include the microvasculature of mice [46], rat [121], pig [204] heart, to name a few. In recent years, the first successful efforts were made in the development of systems that will permit automated acquisition of relatively large image volumes of several  $mm^3$  by means of CLSM [128, 190, 188]. Furthermore, CLSM can be an extremely useful component of hierarchical imaging systems due to its exquisite resolution. It could be combined with other cutting-edge imaging techniques that produce wider field views or for imaging with lower resolution for faster acquisition of images and subsequently zooming in on areas of interest for higher resolution. This is similar to recent approaches where, for instance micro-CT and histology, were combined to visualize murine hind limb vasculature and muscle tissue [193]. Such approaches could enable visualization of the details of capillaries and exposition of their relation to potential molecules or cells involved in the processes/diseases, while permitting larger views and a reduction in the loss of topological information.

Last but not least, it is worth noting that prior to the wide-spread use of these cutting-edge techniques, early studies of the microvasculature have employed vascular corrosion casting with dyes (e.g. [22, 138, 230]) and silicone elastomer ([10, 104]. Later studies have adopted scanning electron microscopy (SEM) which pioneered microvascular research during the '70s and '80s [216, 4]. Its widespread use is related to what its name implies: it uses electrons instead of photons as its source of light. Therefore, by varying the voltage which is inversely proportional to the wavelength of electrons, it is possible to reach wavelengths a lot lower



Fig. 2.4 Basic tasks involved in a typical automatic bioimage analysis pipeline to convert image data into quantitative knowledge.

than that of photons whose visible light at minimum wavelength is around 400*nm*. However, obtaining 3D information from SEM remains extremely challenging and difficult [219].

Fig. 2.3 presents images of the 3D multi-scale coronary vasculature with increasing levels of resolution acquired by means of different imaging modalities. More precisely, a 3D reconstruction of the porcine vasculature is provided, based on micro-CT images depicting epicardial arteries down to arterioles, along with a detailed view of intramural vessels which has been acquired by cryomicrotome imaging. Moreover, images of capillaries visualized using high resolution (ultra-high) micro-CT and confocal microscopy are presented. Confocal microscopy resolution allows us to distinguish tiny microvessels by staining the junctions of the endothelial cells (VE-Cadherin), along with other tissue components, such as smooth muscle actin positive cells.

# 2.4 Automated image analysis towards quantitative microscopy

Imaging is performed with the aim of studying and understanding the system or process under investigation, as well as of identifying changes that might have occurred in response to physiological or pathophysiological conditions either for clinical or research applications. To achieve this, apart from the images per se, quantitative data are of paramount importance to accurately interpret image content and compare images of various conditions, of different subjects or longitudinally. However, the deluge of state-of-the-art imaging data and the information that they include render manual and supervised analysis tedious and time-consuming, if not infeasible and insufficient. This is why automated image analysis approaches have emerged as an indispensable branch of today's medical and biological practice. This exciting and relatively new field, is becoming more and more crucial as the amount of data and their complexity increases. In fact, automated bioimage analysis has emerged as the sole solution towards converting qualitative microscopic observations into quantitative data [58, 8] in the field of biological research where imaging, particularly microscopy, is an everyday tool used in testing hypotheses and analysing systems and processes with the aim of novel discoveries. Bioimage analysis is at the core of the emerging "bioimage informatics" field [155, 144]. It involves adapting informatics and computer vision techniques to transform images into quantitative knowledge and novel scientific insights.

A number of software solutions has been developed over the past years to enable quantitative microscopic image analysis, both proprietary (commercial) and open-source [87, 58, 144]. Popular examples of proprietary software tools include:

- MetaMorph (Molecular Devices) which has been designed for controlling microscopes during image acquisition and for performing simple analysis tasks on the acquired images mostly in 2*D*,
- Volocity (Perkin-Elmer) capable of handling 3D time-lapse volumes,
- Imaris (BITPLANE) known for its interactive environment for quantitative analysis of microscopic images and increased human intervention,
- Amira (Thermo Fisher Scientific) appropriate for 3D and 4D image analysis of several modalities including microscopy,
- Definiens Tissue Phenomics Software (Definiens) popular for biomarker extraction and oncology applications,
- Huygens mostly used for deconvolution purposes, visualization and interactive analysis.

Despite their undeniable utility, commercial software solutions can be expensive and, therefore, unattainable under restricted budgets. Furthermore, because the code for proprietary software is usually not available, the algorithm behind the analysis remains a black-box. Thus, it is difficult to understand how the analysis is performed and the outcome might be easily misinterpreted [103]. Furthermore, without having access to the code, computational

#### Background and state of the art

scientists are not able to build upon existing methods and make improvements or adequate adaptations to the approach based on the needs of the biological problem at hand. The latter is a very common need since we are far from reaching a universal software solution for all types of images and scientific problems. Open-source solutions have therefore started to attract attention and open-source bioimage analysis tools are expected to keep growing in number [58].

Among the most known open-source solutions are ImageJ, the Java-based cross-platform evolution of NIH Image, whose functionality can be extended with numerous purpose-specific functions and plugins, Fiji which is based on ImageJ but includes a number of plugins preinstalled [194], Icy [43] and Bioimage XD [103] which are based on VTK and ITK libraries and offer multi-purpose modules, functions and work-flows, as well as Cell Profiler which includes a comprehensive collection of image processing modules for quantification [34]. Another common solution for quantitative image analysis is the use of collection of modules with interfaces like Farsight, libraries, for instance OpenCV, ITK, VTK, packages, such as ImagePy [227], or single modules. Efforts to create repositories for open-source image analysis tools are also particularly interesting, for example Bio-Image Semantic Query User Environment (BISQUE) [118] or Open Microscopy Environment Remote Objects (OMERO) [3].

A list of the aforementioned software tools along with others, which have been the focus of a series of reviews over the last years [87, 58, 144], is provided in Table 2.2. However, it should be noted that this list is not exhaustive. Moreover, software development is like a living organism that constantly keeps growing and evolving. It should also be taken into account that, apart from the software per se, algorithms for various image analysis tasks are continuously being published. These are usually the result of significant research efforts focused on developing novel approaches or improving existing ones before they can be translated into software or libraries. In fact, the algorithms that are behind a software functionality might be the result of years of research and constant improvements. This is demonstrated by the existence of numerous methods for specific image analysis tasks or work-flows that might not be available as software but are described in scientific papers, as well as by challenges that take place to compare different approaches [144]. Depending on the task and biological problem at hand, different algorithms/software might perform better or worst.

The main tasks that a typical bioimage analysis solution involves are presented in Fig. 2.4. These can be roughly categorized into pre-processing, segmentation, quantification, statistical analysis of extracted features/patterns, classification and visualization [87, 144]. The first step after acquiring an image is usually to enhance its quality by reducing noise and by

removing unwanted artefacts introduced during the acquisition process. This can be achieved by applying different pre-processing approaches. These approaches can be divided into: (i) deconvolution methods that aim at restoring the original signal by removing the blurriness introduced by microscopes due to out-of-focus signal [191], (ii) denoising methods that improve signal-to-noise ratio [23], (iii) registration approaches that can correct misalignment issues between adjacent slices or between tiles of the same slice produced during acquisition [215], (iv) illumination correction methods that deal with uneven illumination caused by light scattering, antibody penetration etc [209], and (v) contrast and colour enhancement approaches. Apart from being used as a pre-processing method, registration can also be used in building atlases or transforming one image into another by rigid or non-rigid methods with the purpose of comparing or fusing them. Depending on the microscope or even the sample, different pre-processing approaches might be more appropriate. For instance, SPIM usually requires the use of specific denoising approaches which are able to remove stripe artefacts [66]. On the other hand, deconvolution methods are indispensable for images produced by wide-field microscopes, while CLSM images often present blobs of dye that must be removed by morphological operations [186] and so on.

After the image has been pre-processed, segmentation is routinely performed. Segmentation refers to the separation/assignment of the voxels of an image into two categories (binary segmentation), i.e. object of interest or background, or more categories (multiclass segmentation), i.e. background or one of the objects of interest. Developing accurate segmentation methods is one of the most difficult steps for quantitative analysis [144]. In the case of vascular networks, a variety of such approaches has been developed and has been extensively reviewed [111, 129]. These include thresholding which is very popular for fluorescent data, energy-minimizing deformable models, model-based methods, among others. Despite the efforts, there exists no segmentation method that is 100 % accurate and applicable to all image modalities. More often than not, algorithms require extensive fine tuning even for images of same modality acquired under different conditions.

Quantitative analysis takes place after the object(s) of interest has been segmented. This task involves calculation of parameters that permit numerical expression of the properties/features (as well as their distributions) of the object(s) using its segmentation. Such parameters might include morphological, textural, functional or other characteristics. It should be noted that in some cases segmentation can be skipped and quantification can be performed directly on the original or the pre-processed images. Usual example of such analyses involve intensity-based measurements such as co-localization analysis [51].

Statistical analysis using the quantified parameters is also commonly applied to compare different groups of images or longitudinal data, and/or with the aim of discovering correlations

or cause-effect relations (regression). Moreover, the extracted features can be used as biomarkers to classify images into categories, for example diseased or healthy (supervised learning), or to uncover relationships, clusters and patterns in the data without pre-specified categories (unsupervised learning). There exist, however, algorithms that do not require a pre-definition of the features. These are commonly known as representation learning. Popular examples of representation-based machine learning algorithms are deep learning approaches, which can be fed with raw data and automatically extract and learn features from several layers in order to perform the desired task [119]. Since the high computational requirements of deep learning are no longer a hurdle, these methods are becoming increasingly popular due to their higher performance in a variety of tasks compared to classic artificial intelligence methods. Use of deep learning approaches in biological imaging is not restricted to classification. In recent years these approaches have also been proposed for performing other tasks, for instance for the segmentation of biological images by using convolutional neural networks [185].

Furthermore, visualization of the image itself, of its properties or its segmentation is one of the most common tasks during several stages of bioimage analysis. This is why although the majority of software suites used for analysis offer visualization options [67, 154], there are software suites that have been developed with a particular focus on visualization or visualization-assisted analysis, such as Vaa3D [157, 156] or the virtual finger [158].

Other tasks that a bioimage analysis pipeline for imaging data with a temporal component i.e. time-lapse images, that were not listed previously include detection and tracking of objects, i.e. cells or particles [145, 37].

Taking into account the number and complexity of the aforementioned tasks involved in converting images into quantitative knowledge, selecting and combining the most appropriate among them can be challenging. A vast amount of time is usually spent on fine-tuning, supervising and correcting the intermediate and final results. For this reason and for increasing reproducibility of biological experiments, the importance of delivering fully automated methods for every task of the analysis work-flow and of designing modular pipelines without need of human intervention is becoming increasingly apparent [133]. This is particularly true in the case of complex systems, such as 3D microvascular networks. Despite the works on this direction [35, 17, 106, 232], dedicated open source solutions for the 3D analysis of microvascular networks are very limited [106]. In reality even ground-breaking and novel biological studies are still based on few traditional parameters and/or metrics extracted manual or semi-automatically. On the other hand, wide application of the existing for 2D analysis ones even when providing a limited number of parameters, such as AngioTool [241], as well as the unprecedented insights offered when 3D approaches are adopted for

Table 2.2 List of software for quantitative analysis of biological images with a particular focus on microscopy data. Data are based on [87, 58, 144] among others.

Software	License	Functionality	Website
Image-Pro	Commercial	Image acquisition and analysis	http://www.mediacy.com/imageproplus
MetaMorph	Commercial	Acquisition, device control, and image analysis of mi-	www.moleculardevices.com
		croscopic images	
Volocity	Commercial	3D image visualization, analysis and processing for	www.improvision.com
		fluorescence images	
Imaris	Commercial	Interactive image analysis	www.bitplane.com
Amira	Commercial	Visualization, processing, analysis of CT, MRI, DTI but	https://www.fei.com/software/
		also microscopy	amira-for-life-sciences/
Definiens Tissue Phenomics Software	Commercial	Biomarker extraction and oncology	http://www.definiens.com/
Huygens	Commercial	Deconvolution, visualization, interactive analysis for	https://svi.nl/HuygensSoftware
		bright-field and all types of fluorescence microscope	
		images	
ImageJ	Open-source	Image visualization, analysis and quantification support-	rhttps://imagej.net/ImageJ
		ing the addition of plugins, functions	
ImageJ2	Open-source	Next-generation ImageJ with improved functionalities	https://imagej.net/ImageJ2
Fiji	Open-source	ImageJ based software distribution with pre-installed	https://fiji.sc/
		plugins	
Icy	Open-source	Plugins, scripts and protocols to visualize, annotate and	http://icy.bioimageanalysis.org/
		quantify bioimaging data	
BioImageXD	Open-source	multi-purpose software for analysis, visualization and	http://www.bioimagexd.net/
		creation of image processing pipelines	
Cell Profiler	Open-source	Modular high-throughput quantitative analysis of bio-	www.cellprofiler.org
		logical images	
Vaa3D	Open-source	3D Visualization-assisted analysis	http://www.alleninstitute.org/what-we-do/
			brain-science/research/products-tools/vaa3d/
KNIME [47]	Open-source	Visual assembly and interactive execution of image pro-	https://www.knime.com/
		cessing workflows	
iCluster	Open-source	Visualization, clustering, statistical analysis and differ-	icluster.imb.uq.edu.au/
		entiation of high throughput sub-cellular localization	
		imaging	
FARSIGHT	Open-source	Toolkit for quantitative analysis of dynamic tissue	http://www.farsight-toolkit.org/
		micro-environments imaged by optical microscopy	
BISQUE	Open-source	Bio-image database and analysis	https://bioimage.ucsb.edu/bisque
OMERO	Open-Source	Visualization, organization, analysis and annotation of	http://www.openmicroscopy.org/
		microscopic data	
Murphy Lab	Open-source	Management and analysis of bioimage datasets	http://murphylab.web.cmu.edu/software/

microvascular networks [18], demonstrate the potential and the impact that 3D automated image analysis of microvascular networks can have in allowing the subsequent modelling of functional properties and therefore in deepening our knowledge about complex biological systems. Furthermore, approaches to identify subtle but biologically significant differences between even similarly looking networks will be of pivotal importance in increasing our understanding of the changes occurring in the microvasculature and of the time that these occurred. This can enhance our understanding of the mechanisms of pathology and can allow us to identify appropriate time windows for therapeutic interventions to evaluate therapies. Therefore, in this work we focus on designing a fully automated pipeline that can be used in the in-depth analysis of the coronary microvasculature and to decipher its changes after MI.

# 2.5 Mathematical modelling of blood flow in microcirculation

Theoretical modelling is a unique tool to study and understand microvascular flow in health and disease, because detailed measurements of flow properties in the microvasculature bed are very difficult or infeasible to perform. For this reason, this field has attracted significant interdisciplinary research over the years. Over the last two decades excellent reviews have been published that summarize findings regarding microvascular blood rheology properties [169] and modelling methods [176, 163, 166, 229, 122, 203, 206, 82, 198, 199]. In this section, a brief overview of the key-points and state-of-the art methods in modelling microvascular blood flow is provided.

The Reynolds number is defined as the ratio of the inertial term to the viscous term and it can be used in the selection of the most appropriate model for simulating blood flow. In contrast to macrocirculation, where the Reynolds number is high and, thus, 3D models of blood flow which take into account turbulent flow are more appropriate [39], in microcirculation the Reynolds number is low (in the range [0.001, 1)) [199, 166]. Therefore, viscous dominated flow can be assumed and the Stokes equations for an incompressible fluid can be applied. These models should not be confused with the lumped models where complete systems are replaced by a single resistance. Lumped models are very important in systems biology but their oversimplification does not allow the study of microvascular perfusion.

Assuming laminar steady-flow, pressure-driven flow of the individual vessels of the network (segments) can be approximated using the Poiseuille equation [162]:

$$Q_i = \frac{\pi}{128} \frac{D^4}{\mu L}, \ i = 1, ..., N$$
 (2.1)

where N is the number of vessels that the network consists of, while  $D, L, \mu$  stand for the diameter, length and viscosity of the segment under investigation respectively.

For the simulation of blood flow the following rheological properties should be taken into account; (i) the Fahraeus effect [61] and (ii) the Fahraeus-Lindvist effect [62]. In particular, the Fahraeus effect refers to the increased outflow of RBC per unit time, i.e. the discharge hematocrit, observed at the exit of a tube in comparison with the total fraction of RBC in the tube, i.e. the tube hematocrit (tube hematocrit<discharge hematocrit). This phenomenon occurs due to the preference of RBC to travel to the centre of tubes where they move faster than other cells/blood. The Fahraeus effect describes different properties when talking about a single vessel or a complete network. The "vessel" Fahraeus effect is associated with reduced

hematocrit in microcirculation compared to that of the systemic hematocrit. Furthermore, the Fahraeus effect is related with the un-even splitting of RBC in branching points such as bifurcations, trifurcations etc, commonly known as the phase separation effect or plasma skimming. The phase separation effect results in higher hematocrit in daughter vessels with higher flow rates, and therefore a higher flow-weighted mean of discharge hematocrit of all vessels in a microvascular network than the non-weighted mean. The latter is known as the "network" Fahraeus effect.

The Fahraeus-Lindvist effect refers to the dependence of effective (apparent) blood viscosity on the vessel diameter. In particular, in *ex vivo* experiments, it has been observed that apparent viscosity decreases with decreasing diameter in tubes of diameter smaller than  $300\mu m$ . Apparent viscosity reaches its minimum for vessels with diameter  $5 - 7\mu m$  that approximates the dimensions of individual RBC. The phenomenon is attributed to the rheological properties of blood and more precisely to the tendency of RBC to migrate towards the centreline of the vessel leaving a cell-free or cell-depleted layer. The thickness of this layer decreases for vessels smaller than  $5 - 7\mu m$  and the viscosity of vessels with diameters smaller than this threshold value increases with decreasing diameter due to the direct contact of RBC and the vessel wall. Based on several experimental measurements on RBC velocity, empirical relationships have been developed that express the relation of *in vitro* apparent viscosity with tube diameter and hematocrit [167].

However, *in vivo* experiments [130, 131] have demonstrated that *in vivo* blood viscosity is higher than the *in vitro*. Several factors are considered to contribute to this effect. These include the network structure that consists of numerous bifurcations and junctions as well as the heterogeneity in vessel diameters as they both affect the thickness of cell-free layers. Another factor associated with the phenomena of elevated *in vivo* viscosity is the existence of an endothelial surface layer, or glycocalyx, resembling a polymeric brush which does not permit plasma flow near the endothelial-cell surface [170] making this way the effective width of the lumen available for the flow of plasma and RBC smaller. Using the experimental observations, the modified empirical equations describing these relationships were deduced [176, 177]:

$$\mu_{app} = \mu_p \left[ 1 + (\mu_{45} - 1) \frac{(1 - H_D)^C - 1}{(1 - 0.45)^C - 1} \left( \frac{D}{D - 1.1} \right)^2 \right] \left( \frac{D}{D - 1.1} \right)^2$$
(2.2)

where  $H_D$  and D are the hematocrit and the diameter of the vessel segment as mentioned earlier,  $\mu_{45}$  is the apparent viscosity for hematocrit 0.45 ( $H_D = 0.45$ ) and C is the coefficient that defines the shape of curve and expresses the dependence on hematocrit.  $\mu_p$  is the viscosity of blood plasma.  $\mu_{45}$  and *C* are defined by:

$$\mu_{45} = 6e^{(-0.085D)} + 3.2 - 2.44e^{(-0.06D^{0.645})}$$
(2.3)

and

$$C = (0.8 + e^{-0.075D}) \left( -1 + \frac{1}{1 + 10^{-11}D^{12}} \right) + \frac{1}{1 + 10^{-11}D^{12}}$$
(2.4)

Alternative equations that also take into account the variation of endothelial surface layer thickness with microvessel diameter can be found at [170].

It is worth noting that both the Fahraeus and the Fahraeus-Lindvist effects have been observed experimentally. In recent decades they have been also confirmed though computational models and detailed simulations of RBC migration, arrangement and deformations in flow which have demonstrated the power of computational modelling to obtain insights into difficult biological problems [82].

Most discrete microvascular blood flow models are based on that the assumption that microvessels form a network of resistances in which flow is governed by Poiseuille's law and rheological properties are expressed by the aforementioned empirical equations. By applying conservation of flow, i.e. blood that enters a branching point equals the blood leaving the point, the system of algebraic Poiseuille-based equations can be solved to predict blood flow distribution, given adequate boundary conditions (BCs). BCs must be specified in the form of pressure or flow values for all boundary nodes. Examples of the application of these discrete models include the study of hemodynamics of microvascular networks in a variety of tissues, including the rat mesentery [175], brain [136], tumour [213], and heart [105].

Nonetheless, a major challenge in relation to the aforementioned approaches is the prescription of adequate BCs. Several work-arounds have been adapted such as assigning constant or scaled pressure values according to the vessel diameter or type [105, 148, 96], imposing no-flow BCs [180] and more recently optimization methods [75, 153]. However, the effect of BCs on the estimated blood flow cannot be underestimated. Lorthois et al. [136] studied this effect and demonstrated the strong dependence of the final flow solution on the chosen BCs for boundary capillaries using the brain microvascular data reconstructed by Cassot et al. [35].

The limitation of prescribing adequate BCs to the thousands of nodes usually present in microvascular networks can be tackled by using continuum flow models (CFMs). CFMs do not allow simulation of blood flow at individual microvessels. They do, however, permit prediction of tissue-scale blood flow properties and have, therefore, started to gain increasing attention as an alternative to discrete models [229]. This type of models is based on Darcy's law which describes fluid flow in porous media and defines flow as the product of pressure gradient with the permeability of the media [13]. In this context, permeability refers to the inverse resistivity of flow (network conductivity) and in 3D structures it is expressed by a tensor. The permeability tensor is a tissue-scale metric that relates the micro-scale topology, i.e. the topology of the microvascular bed, to the macro-scale flow. Thus, it provides a way of quantifying the effect of structural changes that occur as a result of microvascular diseases and of relating these to whole organ flow observations [208]. This, coupled with the computational efficiency of CFMs and their independence of BCs, make them a powerful tool in advancing our understanding of tissue perfusion in healthy and pathological conditions and in elucidating structure-function relations, as well as in scaling-up discrete models, towards using permeability tensors to parametrise multi-scale models of fluid transport (volume-averaged flow) in complete organs, such as the heart [206].

Permeability tensors can be efficiently calculated by taking advantage of volume-averaging, i.e. homogenization, theory. In particular, Shipley et al. [205] adapted homogenization to develop a continuous model of fluid and drug transport for a tumorous spatially-periodic leaky capillary bed. The model was later adapted by Smith et al. [208] and was used to calculate physiologically relevant permeability tensors used in the study of transmural variations in a non-leaky rat myocardium capillary bed, generated statistically from anatomical data [121], to ensure spatial periodicity. A similar approach was taken by El-Bouri and Payne [56] to calculate permeability tensors of different sizes of 3D synthetic spatially-periodic brain microvascular networks leading to a converged permeability tensor that can be used to scale up larger areas of the brain microvasculature in order to understand whole-organ vascular flow. Recently, Hyde et al. [97, 96] compared different approaches to parametrise a continuous multi-compartment model that permits calculation of a flow solution at each compartment and integration of the models towards building a multi-scale continuum perfusion model of the whole heart with applications in the porcine and the canine animal models. The different parametrisation methods were based in approximations of the permeability tensor using various degrees of anatomical information. These works represent the first efforts to use anatomical data instead of idealized structures, statistically or rule-based generated ones. However, tensors were not calculated explicitly based on Darcy's law solution on the anatomical data a rather challenging task of paramount importance in achieving realistic modelling, which is the focus of the modelling part of this work.

# CHAPTER 3

# MICROVASCULAR IMAGING DATA ACQUISITION & RECONSTRUCTION

This chapter is based mainly on the works [79, 78]

# 3.1 Introduction

In this thesis, we aim to enrich the knowledge about cardiac microcirculation pathophysiology with quantitative data spanning different stages following myocardial infarction (MI) (1, 3, and 7 days), as well as with data from basal conditions, in an unbiased manner. Towards this aim, the first step was the acquisition of imaging data that permit the resolution of the microvasculature and its subsequent accurate reconstruction. Since visualization of the microvasculature with sub-micrometer resolution could not be performed non-invasively or *in vivo*, but it would involve tissue sectioning, a preclinical animal model had to be used. With the aim of increasing the translational value of the findings, for the purposes of the study we selected the pig animal model of ischaemia-reperfusion (I/R).

We are particularly interested in I/R because it is the experimental model of MI that best emulates human myocardial infarction, followed by timely restoration of blood flow to the ischaemic region, i.e. reperfusion, through pharmacological or mechanical approaches, such as percutaneous coronary intervention. Early reperfusion remains the main strategy for reducing the impact of infarction and salvaging the myocardium. However, despite its indisputable role in reducing mortality [179], it is nowadays accepted that reperfusion also induces injury per se to the myocardium. This injury is commonly known as reperfusion injury [91, 98]. Moreover, even in the case of successful restoration of blood to the epicardial coronary arteries, the ultimate determinant of the efficiency or impairment of tissue perfusion is the microvasculature. This crucial role of the microvasculature is associated with the fact that it represents the mesoscale of the circulatory system by acting as the interface between

## Microvascular imaging data acquisition & Reconstruction

large scale, i.e. epicardial coronary arteries and smaller scale, i.e. the cardiomyocytes [98, 202]. For this reason, it is of paramount importance to obtain in-depth knowledge of changes that occur to the microvasculature after infarction while also taking into account the reperfusion step.

Furthermore, in this work, the pig was selected over other large animal models as it is considered to be the most translational and reliable model for the study of I/R [98, 68]. This high translational value of the pig is related to the similarity of its coronary artery anatomy and its distribution to that of humans. Moreover, compared to other large animal models, it has limited pre-existing collateral flow as that observed in humans [89].

In this chapter, we firstly present the method used to induce I/R in the pig animal model. Subsequently, we describe the procedures deployed for tissue sampling and preparation, for immunostaining for nuclei, endothelial cell junctions and smooth muscle actin (SMA) positive cells as well as for imaging by confocal laser scanning microscopy (CLSM). In brief, we have acquired a large dataset that consists of hundreds of 3D multi-channel confocal images. Following acquisition of the dataset, the first step was to establish an automated method for the segmentation of the labelled structures. Thresholding is a popular approach in segmentation of fluorescent images [187] due to its simplicity, computational efficiency and speed. However, due to the challenging nature of 3D confocal images derived from thick samples related to sudden fluctuations in image intensity and/or loss of intensity due to antibody penetration and other imaging artefacts, traditional thresholding approaches were proven insufficient [124]. Therefore, we developed a novel, simple yet efficient method, for the segmentation of labelled structures from microscopic confocal images. The method is applied to all channels of the acquired image stacks.

In continuation, an innovative new method is presented that permits accurate 3D reconstruction of the entire microvasculature from the endothelial cell junction- and SMA-stained thick tissue sections by filling gaps present on the segmented microvessels due to the use of VE-Cadherin instead of a laminar marker. Development of such an approach is of paramount importance because it enables the use of VE-Cadherin, an endothelial marker with high specificity even in infarction, to reconstruct microvessels in this and future studies. Moreover, the developed approach can allow the simultaneous study of endothelial junctions, SMA<sup>+</sup> cells and microvessels through the use of two fluorescent markers (VE-Cadherin, SMA<sup>+</sup> cells) instead of three. The latter is noteworthy given the limited number of fluorochromes that can be imaged simultaneously [55]. It also worth noting that the developed method involves a step of distinguishing between capillaries and arterioles/venules. The importance of identifying the type of microvessels automatically is not solely limited to this process, but it is also crucial in the implementation of theoretical modelling approaches [207]. The approach

Subject	1	2	3	4	5	6	7	8	9	10	11
Time after infarction	Basal	Basal	24 hours	24 hours	3 days	3 days	7 days	7 days	45 days	45 days	45 days
Body weight $(Kg)$	34	30.50	34	32.50	40	34	47.50	39.50	56.5	53	57
Left ventricle (LV) mass (g)	55.83	60.50	49.95	57.84	71.18	56.65	88.90	60.60	70.89	60.59	56.88
LV end diastolic volume (ml)	82.63	88.00	95.35	117.3	100.08	96.41	117.51	138.51	128.42	134.9	130.47
LV end systolic volume (ml)	43.20	42.70	53	77.87	44.04	59.61	62.22	87.15	83.85	93.9	87.37
Ejection Fraction (%)	47.74	51.40	44.41	33.61	56	38.17	47.05	37.08	34.71	30.39	33.04
Infarct Size (%)	0	0	29	19.17	16.61	31.01	25.38	41.59	33.37	35.41	29.01
Hematocrit (%)	30.2	32.6	31.1	21.8	29.2	28	30.4	29	33.1	32.3	30.4
Blood Flow LW / R <sup>a</sup> (ml/min/100g)	121.9	86.9	135.5	142.5	N/A <sup>b</sup>	103.50	114.1	159.40	175.5	139.1	134.8
Blood Flow I <sup>c</sup> (ml/min/100g)	-	-	59.7	104.6	N/A <sup>b</sup>	107.00	58.9	50.90	29.9	79.3	88.1

Table 3.1 Characteristics of the subjects used in the study.

<sup>*a*</sup>LW / R refer to measurements taken from the LV lateral wall (LW) for the control subjects one and two, and to measurements taken from the remote areas (R) for subjects that have suffered infarction (subjects three to eleven).

<sup>b</sup>N/A: Information not available due to missing magnetic resonance imaging (MRI) sequence

<sup>c</sup>I refers to measurements taken from the infarcted areas (I) for subjects that have suffered infarction.

could be further extended to separate arterioles from venules, given their morphological characteristics such as branching patterns and tortuosity.

# 3.2 Microvascular data acquisition

## 3.2.1 Animals & Myocardial Infarction

All experiments were approved by the Ethical Committee for Animal Experimentation of CNIC and the Comunidad Autónoma de Madrid in accordance with the Guide for the Care and Use of Laboratory Animals. Six adult male Large-White pigs weighting 30-40 kg were anaesthetized with a ketamine/xyzaline/midazolam mixture in continuous intravenous infusion. Subsequently, acute myocardial infarction was percutaneously induced using an angioplasty balloon with 30-minute occlusion of the left anterior descending coronary artery (distal to the first diagonal branch) followed by reperfusion. The pigs were sacrificed 1, 3 and 7 days after infarction, respectively. Furthermore, two more pigs under basal conditions were sacrificed to serve as the control subjects of the present study.

This work focuses on the microvascular changes occurring at the early time-points after MI (1, 3 and 7 days post MI) when the potential for therapeutic interventions is higher. However, after the analysis of the early time-points (Chapter 4), we decided to extend our analysis at 45 days post MI with the aim of clarifying whether the microvascular changes observed at the early time points persist and/or deteriorate when no treatment is applied. For this purpose, we used available tissue from pigs that had been subjected to a similar

#### Microvascular imaging data acquisition & Reconstruction



Fig. 3.1 Schematic procedure of tissue harvesting and sampling. Three slices of the heart with thickness of approximately  $\sim 1.5cm$  were cut. Transmural pieces of tissue were subsequently cut from each slice clock-wise with a thickness of around 1cm as indicated on slice I. The infarcted areas, highlighted in yellow on the complete heart and on slice II as an example, were distinguished by eye as the whitish areas. Right ventricle (orange) and septum (green) were not used for the analysis. The heart of the diagram belongs to a subject sacrificed at 7 days post MI.

procedure as described above with 40-minute occlusion and sacrificed 45 days following infarction. All subjects' structural and functional characteristics are provided in Table 3.1.

## 3.2.2 Tissue sampling

The porcine hearts were extracted immediately after euthanasia and cleaned with saline. Subsequently, they were cut into three  $\sim 1.5 \ cm$  thick cross-sections, at the basal, mid-ventricular (papillary muscle) and apical levels. The right ventricle was removed and tissue samples from both infarcted and remote areas from each left ventricle were collected. It is worth mentioning that the sample collection was performed by cutting transmural pieces of around  $1 \ cm$  in a clock-wise manner starting from the leftmost part of the left ventricle. The areas corresponding to the septum were excluded. Infarcted areas were distinguished by eye as whitish areas.

## 3.2.3 Tissue preparation & Immunofluorescence histochemistry

The tissue samples were fixed overnight with 0.4% PFA at 4°C, washed with PBS, cryoprotected with 30% sucrose and embedded in OCT with transmural orientation kept. Thick slices of 100 $\mu$ m were cut using Leica AM1950 automated cryostat. Immunostaining was performed in flotation using the following procedure; 2 hours permeabilization in 0.3% Triton-X 100, 0.1% Tween in PBS at RT followed by 1 hour blocking with 0.3% Triton-X 100, 4% FBS in PBS at 4°C. For the staining of the microvasculature, the slices were incubated in blocking buffer with primary antibodies anti-VE-Cadherin (Santa Cruz) and anti- $\alpha$ -SMA (Sigma) with dilution 1:100 and 1:200 respectively, overnight at 4°C. After washing with 0.3% Triton-X 100 in PBS, incubation with secondary antibodies donkey anti-goat Alexa Fluor-568 and chicken anti-mouse Alexa Fluor-647, 1:500 (Molecular probes) and Hoechst 33342, 1:10000 (Life Technologies) was performed for 2 hours in blocking buffer at RT. After washing, slices were mounted on the glass using Fluoromount G (Southern Biotech). All incubations were performed on a nutator.

VE-Cadherin stains the endothelial junctions and not the complete endothelial cell or the lumen of the vessels. This results in gaps in the blood vessels when reconstructed from VE-Cadherin- labelled tissue. However, VE-Cadherin has been chosen in this work as a vasculature marker over other commonly used endothelial markers, such as Isolectin B4 or CD31 (PECAM-1), due to its specificity even in infarction. Isolectin tends to stain not only endothelial cells, but also macrophages [99]. Therefore, its use in infarcted tissue is not appropriate, especially at early time-points, when the inflammation process occurs. In our samples large contamination of macrophage staining was observed in infarct zones when Isolectin was used. A similar problem was presented in the case of CD31 marker, which is present in endothelial cell junctions but it is also expressed in other cell types, e.g. platelets, monocytes and macrophages [178]. For those reasons, the use of VE-Cadherin that is exclusively an endothelial cell marker [224], was preferred.

In this work, we have also used the information from the  $\alpha$ -SMA channel for studying the perivascular and myofibroblastic content of the cardiac tissue as it will be detailed in the next chapter (Chapter 4). In order to validate our approach regarding myofibroblasts, we studied the co-expression of  $\alpha$ -SMA with other markers expressed by myofibroblasts. Towards this aim, we used images of tissues simultaneously labelled with  $\alpha$ -SMA and such other markers. The protocol followed for the preparation and immunohistochemical staining of those tissues is similar to that described earlier for the tissue used for the study of the microvascular changes. More precisely,  $15\mu m$  slices of tissue from infarcted areas from the pigs sacrificed at 7 days following MI were used. After fixation and cutting, the slices were incubated in blocking buffer with primary antibody anti-CD31 (Abcam) with dilution 1:100 and one of the following primary antibodies: (i) anti-Vimentin (Sigma) with dilution 1:100, (ii) anti-Collagen I (Santa-Cruz) with dilution 1:50, or (iii) anti-PDGFRB (eBioscience) with dilution 1:100, overnight at 4°C. After washing with 0.3% Triton-X 100 in PBS, incubation with secondary antibodies goat anti-rabbit Alexa Fluor-488 (Life Technologies) with dilution 1:500 and Alexa Fluor-488 1:500 (Life Technologies), and Hoechst 33342, 1:10000 (Invitrogen) was performed for 2 hours in blocking buffer at RT. After washing with 0.3% Triton-X 100 in PBS, the slices were incubated in blocking buffer with 5% mouse serum for 1 hour at RT. Subsequently, they were incubated with anti- $\alpha$ -SMA-





Fig. 3.2 MIPs of each channel of three example 3D multi-channel confocal images at different conditions; basal, remote and infarcted 7 days post MI. The images were acquired by tissue simultaneously labelled with Hoechst, VE-Cadherin and SMA. Each column represents a channel; (a) MIP of Hoechst channel pseudo-coloured with blue, (b) MIP of VE-Cadherin channel pseudo-coloured with red, and (c) MIP of SMA channel in grey. Scale bar (25  $\mu$ m) applies to all channels.

Cy3 (Sigma) with dilution 1:300 for an additional hour at RT. After a final wash with 0.3% Triton-X 100 in PBS, the slices were mounted on the glass using Fluoromount G. In this case CD31 was used as a marker of the microvasculature instead of VE-Cadherin due to compatibility issues of the species of primary antibody host.

#### 3.2 Microvascular data acquisition



Fig. 3.3 Image quality improvement after preprocessing. (a) From left to right MIPs of VE-Cadherin channel without preprocessing, after denoising by means of the application of Gaussian filter of size [3,3,3], median filter of size [5,5,5] and Non-Local Means Filtering (NLMF). Yellow windows on the MIPs of the first row denote areas to zoom in. (b) Zoomed areas of MIPs presented on top row. (c) The zoomed areas provided in the middle row after the application of contrast adjustment on the original or denoised images respectively. In this work, NLMF and contrast adjustment have been applied for preprocessing the multi-channel 3D images.

## 3.2.4 Image acquisition & Pre-processing

Spectral imaging was performed by means of Leica SP5 confocal microscopy using a  $40 \times$  oil immersion lens of numerical aperture 1.25. Emissions of 405nm, 561nm, and 633nm laser lines were used to excite the Hoechst, VE-Cadherin and SMA fluorophores respectively. Z-stack slices ( $1024 \times 1024$  pixels) were acquired every  $1\mu m$  and by applying the deepness correction set-up provided by the microscope. The final number of slices acquired depended on particular antibody penetration and ranged between 54 and  $95\mu m$ . The resulting voxel size was  $0.3785\mu m \times 0.3785\mu m \times 1.007\mu m$ , or  $0.3142\mu m \times 0.3142\mu m \times 1.007\mu m$  in a few

## Microvascular imaging data acquisition & Reconstruction



Fig. 3.4 Comparative example of three different thresholding algorithms. A slice of a 3D image (from mouse tissue) with contours of segmented objects by means of Otsu's global, multi-level and hysteresis thresholding in green, red and magenta respectively.

cases. In total, 126 multichannel images, 18 per tissue condition (9 per subject), were acquired and they were used for the analysis at time-points 1, 3, 7 days post MI and under basal conditions. Additionally, 36 images, 18 from infarcted (6 per subject) and 18 from remote areas (6 per subject), were acquired in the same manner and they were used for investigating microvascular characteristics and changes at 45 days following MI. Maximum intensity projections (MIPs) of the multi-channel z-stacks acquired for three example images are provided in Fig. 3.2.

The same imaging system and procedure were also used to acquire images from tissue simultaneously labelled using anti-CD31, anti-SMA and anti-PDGFRB/anti-vimentin. However, emissions of 405*nm*, 543*nm*, 568*nm*, and 647*nm* lasers were used to excite the Hoechst, CD31, SMA and PDGFRB or vimentin fluorophores, respectively. In the case of tissue simultaneously labelled with anti-CD31, anti-SMA and anti-collagen I, the image acquisition process followed was the same, with the difference that the Nikon A1R confocal microscopy was used.

Prior to proceeding with the analysis, after experimentation with different pre-processing approaches for enhancing the quality of the acquired images, the 3D Non-Local Means Filtering (NLMF) [24] was adapted due to its higher performance in terms of removing noise but preserving image information. NLMF is based on non-local averaging of all image voxels. The resulting 3D denoised image volumes were further adjusted for contrast. An example of the performance of NLMF in comparison with Gaussian filtering and median Filtering is provided in Fig. 3.3. Please note all processing and analysis described in this and subsequent chapters have been performed in 3D. MIPs are used in some of the figures of the document only for visualization purposes.

# 3.3 Segmentation of microscopic imaging data

The first step towards studying the microvasculature and its infarction-related dynamic changes from 3D confocal images is to automatically segment the labelled structures of the VE-Cadherin channel. Thresholding is a commonly adapted approach for the segmentation of fluorescently labelled images. In this work, we initially applied different approaches, including Otsu's global and multi-level thresholding [152] and hysteresis thresholding (Fig. 3.4). Apart from the thresholding approaches, we additionally investigated the performance of Frangi filtering [72], a well known multi-scale vessel enhancement filter broadly used for the recognition of tubular structures such as vessels. However, the latter required a significant amount of image specific parameter tuning. Therefore, this approach was discarded. Among the different thresholding methods, the one performing better was multi-level thresholding which provided very accurate results for several volumes (Fig. 3.4). However, there were other cases (Fig. 3.6, bottom row), where the spatial heterogeneity of the intensity within the 3D volume was very high, with lateral or in-depth abrupt changes in intensity. This is probably related to photo-bleaching and fluorescent attenuation. In such cases, the algorithm resulted in very poor segmentations and loss of important vascular information due to oversegmentation or under-segmentation of the labelled structures in several areas. To tackle these limitations, we developed a novel algorithm, the 3D multi-scale multi-level thresholding approach, which accounts for the complex nature of the microvasculature and it is not hampered by the high heterogeneity of intensity present between different volumes and also within the same image volume.

## **3.3.1 3D** multi-scale multi-level thresholding (MMT)

3D multi-scale multi-level thresholding (MMT) algorithm is inspired by the box counting (BC) method [19] and Otsu's multi-level thresholding method [152].

Grids of varying size *e* are overlaid onto the image under investigation. Subsequently, the multi-level thresholding is applied to each box  $B_j(e)$ , j = 1, ..., N(e) of the grid with size *e* in order to calculate the *M* intensity classes that maximize the inter-class variance within the box. For every grid, N(e) thresholds  $(t_M)$ , as many as the boxes that composed it, are therefore calculated and applied to the corresponding box. In total, *k* candidate segmentations  $V_i$ , i = 1, ..., k are produced, one per grid size *e*, as a mosaic of the application of the N(e) thresholds on the boxes. Here, only voxels that belong to the two classes with higher intensity



#### Microvascular imaging data acquisition & Reconstruction

Fig. 3.5 Overview of MMT method.

(M-1,M) are considered as parts of the microvasculature. Thus,

$$V_i(u) = \begin{cases} 1, I(u) \ge t_{(M-1)}(B_j(e)) \\ 0, I(u) < t_{(M-1)}(B_j(e)) \end{cases}$$
(3.1)

where voxel  $u = (x, y, z) \in B_j(e)$  and *I* the original image. It is worth noting that in cases of other markers only the higher intensity level might be sufficient.

Subsequently, candidate segmentations  $V_i$ , i = 1, ..., k have to be fused into a single segmentation. To achieve this, majority rule voting is applied:

$$V(u) = \frac{\sum_{i=1,..,k} w_i V_i(u)}{\sum_{i=1,..,k} w_i} , \qquad (3.2)$$

where  $w_i$  are the weights that define the degree to which candidate segmentation  $V_i$  will contribute to the final segmentation. For our dataset,  $w_i$  were set to 1 considering equal contribution of all candidate segmentations.

An overview of the MMT method is presented in Fig. 3.5. It is worth mentioning that in the traditional BC, cubic boxes compose the grid. However, here, boxes of size  $e \times e \times e_z$  with  $e_z = 10 < e = 2^5, ..., N$ , were used in order to accommodate for the smaller size of our images along z-direction and to cover a wide range of scales while ignoring the smaller ones that provide a very limited region for variance calculation.

## 3.3 Segmentation of microscopic imaging data



Fig. 3.6 Example segmentations by means of MMT method (first row) and of traditional multi-level thresholding (second row) from remote (**a**) and from infarcted area (**b**). The segmented vessels are presented with red on the slices along x-y, y-z, x-z (right) and on the 3D reconstructions (left). Scale bar ( $25 \ \mu m$ ) applies to all images.

The proposed approach was initially applied to the VE-Cadherin channel of a subset of our dataset. The subset consisted of fifty-four 3D confocal images that were acquired from three subjects at 1, 3 and 7 days post MI. This subset formed our initial dataset before expanding it with the addition of other subjects that had suffered infarction as well as with subjects, at basal conditions. The outcome of the MMT application was visually evaluated by an experienced biologist. During evaluation, the segmentations were rated using an ad-hoc rating scale from one to four. Four represented "excellent" segmentation outcome, i.e. all microvascular parts were correctly identified. Three represented "good" segmentation, i.e. the overall pattern/shape of the microvasculature was preserved, but slight over-segmentation and/or under-segmentation could be observed in certain parts. Two represented "poor, but acceptable" segmentation, i.e. when the overall pattern/shape was preserved but there were parts of the vasculature that were highly over-segmented and/or under-segmented. One represented "poor and unacceptable" segmentation which meant that the overall pattern had been changed. Only in one case out of fifty-four the segmentation outcome was rated with one. All the other 53 images were rated with either three or four, and no image was rated with 2. The image for which the segmentation was evaluated with one due to high overestimation of the vascular parts resulting in differences between the true and the estimated





Fig. 3.7 Overview of the proposed approach for the reconstruction of the complete microvasculature from thick sections of pig heart tissue labelled for VE-Cadherin (endothelial junctions).

microvasculature, was discarded from subsequent analysis. Moreover, an additional image was acquired and segmented to retain the size of the dataset.

After confirming the accuracy of MMT for the segmentation of labelled structures from confocal images, it was subsequently applied in the segmentation of VE-Cadherin channel of all images. Furthermore, due to the superiority of the proposed method in terms of performance over other segmentation methods, the approach was applied for segmenting the other channels, i.e. the Hoechst and SMA<sup>+</sup>, as well. Lastly, for the VE-Cadherin channel, segmentation was further improved by excluding possible artefacts, i.e. objects that are smaller than 100 voxels and do not have a nucleus.

# **3.3.2 Endothelial cell junction-based 3D reconstruction of the** microvasculature from cardiac tissue

Following the segmentation of structures labelled with VE-Cadherin, major parts of the microvasculature are reconstructed. However, VE-Cadherin is a marker of endothelial junctions and not of the vessel lumen or of the complete endothelial cell (see Subsection



Fig. 3.8 Example segmentations of the SMA<sup>+</sup> cells channel and the resulting 3D guidance maps for the filling procedure for a basal, an infarcted and a remote case. (a) Cross-sections along x-y-z of the SMA<sup>+</sup> cells channel. (b) Cross-sections along x-y-z of the SMA<sup>+</sup> segmentations overlaid on the original images. (c) SMA<sup>+</sup> segmentations in 3D. (d) 3D guidance map. Parts of the cardiac microvasculature that are recognized as capillaries are represented with orange, while those recognized as arterioles/venules are represented with green.

3.2.3). As a result, there are gaps in the reconstructed blood vessels. The size of these gaps is not homogeneous but rather depends on the type of microvessels. Smaller gaps are observed in capillaries and larger ones in larger microvessels, i.e. arterioles and venules. A filling method was developed to deal with this limitation. The method is applied on top of the VE-Cadherin segmentation with the aim of reconstructing microvascular parts which had not been labelled with VE-Cadherin.

An overview of the filling algorithm is provided in Fig. 3.7. The method is based on two types of information; information regarding the spatial relation of the microvessels with SMA<sup>+</sup> cells and information about the physiology of the different types of microvessels that form the microvascular network. In particular, taking advantage of the knowledge about





Fig. 3.9 VE-Cadherin example segmentations and the corresponding reconstructed cardiac microvasculature for a basal, an infarcted and a remote case. (a) Cross-sections along x-y-z of the VE-Cadherin channel. (b) Cross-sections along x-y-z of the corresponding VE-Cadherin segmentations overlaid on the original images presented in column a. (c) Cross-sections along x-y-z of the 3D reconstructed microvasculature. (d) Reconstructed microvasculature in 3D. Segmented areas from the VE-Cadherin channel are shown in red, while areas reconstructed as part of the microvasculature after the application of the filling pipeline are shown in blue.

the existence of a SMA<sup>+</sup> layer that surrounds arterioles and venules [100], Frangi-based filtering [72] is applied to the SMA<sup>+</sup> segmentation to extract tubular elements whose diameter is in the range of that of arterioles and venules (i.e. larger than the diameter of  $7.3\mu m$  of the larger capillary connected directly to an arteriole [104]). This results in a 3D binary image on which regions that might belong to arterioles/venules are on voxels (voxels of value one) and regions belonging to capillaries are off voxels (voxels with value equal to zero). The 3D image is subsequently used to distinguish between microvessels formed by the VE-Cadherin junctions that are capillaries and those that are possible arterioles or venules. For this purpose, element-wise multiplication of the VE-Cadherin segmentation and the 3D image with arterioles and capillaries is performed. The procedure results in the creation of

a 3D Guidance Map (Fig. 3.8) containing information about the type of microvessels and therefore of the size of gaps that should be filled.

Morphological closing is performed using structural elements of various sizes based on the vessel type, as defined by the 3D Guidance Map. More precisely, in order to fill gaps in regions of capillaries, a ball structural element is used whose size is equal to the diameter of the largest capillary ( $8.2\mu m$ ) found in the pig animal model [104]. Similarly, in order to fill gaps in the case of arterioles/venules a larger ball structural element is used whose diameter is equal to the largest reported arteriole/venule of order 2 ( $20.6\mu m$ ). The combination of filled capillaries and arterioles/venules produced by means of the aforementioned work-flow results in the reconstruction of the complete microvasculature, including regions that might not have been labelled with VE-Cadherin, but nonetheless belong to the microvasculature. Fig. 3.9 allows appreciation of the improved identification of the microvascular network by using the proposed filling approach. Fig. 3.10 provides representative 3D reconstructions of the microvasculature at different conditions under investigation (basal, infarcted and remote at 1, 3, and 7 days following MI respectively) used for subsequent analysis.

## Microvascular imaging data acquisition & Reconstruction



Fig. 3.10 Representative 3D reconstructions of the cardiac microvasculature from tissues at different pathophysiological conditions. (a) Microvasculature reconstructed from tissues at basal conditions. (b) Microvasculature reconstructed from tissues at 1 day post MI. (c) Microvasculature reconstructed from tissues at 3 days post MI. (d) Microvasculature reconstructed from tissues at 7 days post MI. In panels (b), (c), (d) the first two volumes correspond to tissues from infarcted areas at the corresponding time-point after MI, while the third and fourth volumes to tissues from remote ones. Scale bar (25  $\mu$ m) is the same for all channels.
### **CHAPTER 4**

## 3D AUTOMATED IMAGE ANALYSIS OF MICROVASCULAR DATA

This chapter is based mainly on the works [79, 78]

## 4.1 Introduction

Understanding and identifying changes in the 3D structure of the microvasculature not only requires the use of state-of-the-art imaging techniques, but also the use of unbiased image analysis methods that allow the translation of qualitative biological observations into quantitative knowledge. Furthermore, automatic 3D image analysis allows extracting information not attainable from traditional manual analysis, and at the same time, diminishes subjectivity problems, time and labour requirements, of both manual and supervised analysis. Nevertheless, even in the case of automatic analysis, the problem of identifying measures that can optimally describe highly complex structures, their changes and structural-function relations remains a challenging task. Traditional analysis regarding diameters and lengths provide information of paramount importance regarding vessel structure and function, but it is insufficient when dealing with complex objects [84]. This work is a first effort to standardize the quantitative assessment of the cardiac microvascular patterns and, thus, identify their progressive changes during pathology.

More precisely, in this chapter, we present an in-depth study of the coronary microvasculature and of the changes that occur to it at different time-points, after myocardial infarction (MI), at remote and infarcted regions, and in comparison with the basal condition. To achieve this, while also accommodating the complex and 3D nature of the microvasculature, we developed a novel bioimage analysis pipeline that permits the acquisition of novel, quantitative insights into its structure and changes at different stages of pathology. The pipeline is applied to the large dataset of porcine cardiac tissue acquired by confocal microscopy that was described in Chapter 3.

In brief, the pipeline first makes use of the segmentation and filling methods presented in Chapter 3 in order to reconstruct the complete microvasculature from stained endothelial cell junctions and smooth muscle actin-positive (SMA<sup>+</sup>) cells. It subsequently permits us to extract parameters that quantify all major features of the microvasculature and its relation to (SMA<sup>+</sup>) cells. Combining the biological knowledge extracted and statistical change analysis we unravel vital infarction-related changes at the microvascular level, particularly structural changes and angioadaptation in the aftermath of MI. Moreover, we identify geometric patterns that describe the altered capillary diffusion area into the tissue. Lastly, we evaluate the potential of the extracted knowledge to be used for predicting the pathophysiological condition of unseen tissue and we achieve high accuracy (higher than 80%) in the task of distinguishing infarcted and remote from basal tissue, as well as in recognizing the stage of the infarcted tissue. This fact demonstrates the ability of our approach not only to describe, but also to predict complex dynamic microvascular patterns.

Overall, our approach allows for accurate quantification and identification of pathologyrelated changes of microvascular beds, as well as for the prediction of the pathophysiological condition of tissue. This method will provide a deeper understanding of microvascular alterations in a variety of pathological conditions as MI, diabetes or hypertension, the evaluation of the outcome of different treatments, and the identification of optimal timing for angiotherapeutic intervention. At the same time it marks a step forward towards modelling microcirculation at different stages after pathology.

## 4.2 Methods

A brief overview of the bio-image analysis pipeline developed in this work is provided in Fig. 5.2. The first step towards quantifying the microvasculature and its infarction-related dynamic changes is to automatically segment the labelled structures of the VE-Cadherin, SMA and of the Hoechst channels. Towards this aim, the multi-scale multi-level thresholding algorithm (MMT) [79] is applied to every channel of the 3D confocal image as described in Chapter 3. In the case of the VE-Cadherin channel, the approach of filling gaps is then applied on the segmented VE-Cadherin channel to reconstruct microvascular parts not labelled by VE-Cadherin.

After the VE-Cadherin, SMA<sup>+</sup> cells, and nuclei are segmented, and the complete 3D microvasculature is reconstructed, the next step is to quantify the parameters that allow description of the characteristics of the microvasculature and assessment of infarction-



Fig. 4.1 Overview of the proposed bioimage analysis pipeline.

related changes. Here we propose to use a variety of parameters that can be roughly divided into the following categories: (i) parameters at global level that characterize the morphology and topology of the network, (ii) parameters at the segment/local level that characterize the angioarchitecture of the network, (iii) parameters that quantify the relation of the microvasculature with SMA<sup>+</sup> cells, (iv) parameters regarding the capacity of the network for oxygen diffusion. Lastly, we examine novel geometric models to describe the capillary oxygen supply region. Table 4.1 provides a detailed list of the parameters extracted to describe the complex microvascular network described in this and following sections. Statistical analysis of the parameters is performed to identify vital changes that occur at different stages following MI.

#### 4.2.1 Structure-global level

Parameters based on fractal analysis and on the Minkowski Functionals (MF) are used for describing the microvasculature as a complete network, and thus, identifying its alterations after MI at a structure-global level. The concept of fractals was introduced by [140] and

#### 3D automated image analysis of microvascular data

ever since they have been applied in a variety of image analysis and pattern recognition problems. Fractal analysis accounts for the multiscale properties of structures that can be self-similar. In the biomedical field, fractals found great appeal in the study of vascular networks [135], and fractal-based analysis is a powerful tool in cardiology [31]. In [79], we presented the first study to apply a complete 3D fractal-based analysis to quantitatively assess progressive MI-related changes of the microvascular patterns. Here, we follow the same approach and we use three fractal parameters calculated directly from the 3D volumes; fractal dimension, lacunarity and succolarity. Fractal dimension provides an estimation of the morphological complexity of structures. Lacunarity describes the heterogeneity in the distribution of gap sizes. Gaps in the case of cardiac tissue represent non-vascularized areas. Succolarity indicates the capacity of a fluid, i.e. blood in the case of the microvasculature, to flow through the structure. Thus, in biological terms, the higher the fractal dimension, the higher the morphological complexity is, i.e. the number of microvessels. The higher the lacunarity, the more heterogeneous the gap distribution and as a result the blood supply within the tissue is. The higher the succolarity, the larger the amount of blood that can flow in the vessels, thus the better the oxygenation.

On the other hand, the Minkowski functionals (MF) [127] are morphological descriptors (volume, surface area, mean integral of breadth and Euler-Poincaré characteristic) that encompass standard geometric and topological (connectivity) properties of structures. Thus, a combination of fractal and Minkowski-based parameters, allows obtaining complementary information regarding the microvascular networks.

#### 4.2.1.1 Fractal-based analysis

**Fractal Dimension** A variety of methods have been proposed for the calculation of fractal dimension [134]. Among them, the box counting method (BC), which is the most popular and widely used approach, was applied in this work. Grids of cubic boxes of size e are overlaid on the original image. Subsequently, the fractal dimension ( $F_d$ ) is defined as the negative slope of the bi-logarithmic plot of the number of boxes  $n_e$  needed to cover the microvasculature as a function of the box size e. Thus,

$$Fd = -lim \frac{ln(n(e))}{ln(e)}$$
(4.1)

We subsequently identify the cut-off scales over which the microvascular structures no longer present self-similarity [14], i.e. can not be considered as fractals. This additional step was considered necessary because real-life objects, such as the vascular patterns, might not present self-similarity over an infinite range of scales but rather over finite scales. However, no

statistically significant difference was observed between the calculation of fractal dimension with and without cut-offs. This might be explained by the limited physical scales of the microvascular patterns in our dataset. Consequently, the fractal dimension presented in this manuscript is without cut-offs.

**Lacunarity** Lacunarity was calculated by using the gliding box method [84]. Boxes of various sizes *e* are glided over the 3D binary volume of the segmented microvasculature by 1 voxel. For every box size *e*, the total number of boxes of size *e* (*N*(*e*)) and the number of vascular voxels (*P*(*n*,*e*)) inside every box n = 1, ..., N(e) are taken into account. The sum of the number of voxels belonging to the microvasculature in every box n = 1, ..., N(e) is calculated ( $Q_1 = \sum_{n=1,...,N(e)} P(n,e)$ ), as well as the sum of the square number of vascular voxels ( $Q_2 = \sum_{n=1,...,N(e)} P(n,e)^2$ ). Lacunarity for box size *e* is then given by:

$$L(e) = \frac{N(e) \times Q_2}{Q_1^2}$$
(4.2)

In order to tackle the dependence of lacunarity on image density and to be able to compare images of various densities as the ones comprising our dataset, in this work, lacunarity was subsequently normalized using the lacunarity of the complementary image (cL(e)) by  $L_{norm}(e) = 1 - \frac{1}{L(e)} - \frac{1}{cL(e)}$ . Lacunarity over all scales, i.e. all box sizes, is finally defined as the mean of the normalized lacunarity of individual scales.

**Succolarity** Although succolarity (*S*) was firstly described by Mandelbrot [140], the first formal definition and method for its calculation, based on an adaptation of BC method, was proposed recently by [146]. In brief, regions that a fluid can flow are represented by 1 while obstacles to the fluid with 0. Thus, in the case of these study, the areas where blood can flow through the microvasculature are the vascularized areas, while the non-vascularized are considered to be obstructing the flow. There are six possible directions of flow in the case of the 3D microvasculature (Fig. 4.2): horizontal flow from left to right and vice versa, vertical flow from top to bottom and vice versa, and in-depth blood flow from the upper to the lower slices and vice versa. The volume of the 3D microvasculature is decomposed in six 3D images, one per flow direction. Each of those images contains only microvascular parts that would allow a continuous flow, if blood was to enter from that particular direction.

Grids of boxes of different sizes, as in the case of the box counting method, are applied on the decomposed images. The occupation percentage of vascular voxels (O(n,e)) inside each box *n* of size *e*, as well as an indicator of pressure (PR(e)), are calculated. PR(e) is calculated based on the coordinates of the centroid of the box along the direction under



Fig. 4.2 Possible directions of blood flow through the segmented microvasculature; horizontal flow from left to right (arrow 1) and vice versa (arrow 2), vertical flow from up to down (arrow 3) and vice versa (arrow 4), and in depth blood flow from upper to lower slices (arrow 5) and vice versa (arrow 6).

investigation. Therefore, in the cases of horizontal, vertical and in depth directions of blood flow respectively, the pressure is given by the x, y and z coordinates respectively. A normalized version of succolarity is given by dividing the pressure in the case of 100% occupation with vascular voxels for all boxes. Therefore,

$$S(e,d) = \frac{\sum_{n=1,\dots,N(e)} OP(n,e) \times PR(e)}{\sum_{n=1,\dots,N(e)} PR(e)}$$
(4.3)

Taking into account all 6 directions, the overall succolarity is calculated by

$$S = \frac{1}{6} \sum_{d=1,\dots,6} \sum_{e} \frac{S(d,e)}{N(e)}$$
(4.4)

where N(e) refers to the total number of boxes of size *e* comprising the grid.

#### 4.2.1.2 Minkowski-based analysis

For an arbitrary object A in the d-dimensional Euclidean space  $\mathbb{R}^d$ , there exist d + 1 MFs  $M_n(A)$  with n = 1, ..., d + 1. Therefore, in 3 - D space, there exist four MFs, which are proportional to the commonly known properties volume ( $M_1$ ), surface area ( $M_2$ ), mean integral of

breadth ( $M_3$ ) and Euler-Poincaré characteristic ( $M_4$ ). The MFs for the 3D microvasculature are calculated by [127]

$$M_{1}(V) = n_{c}$$

$$M_{2}(V) = -6n_{c} + 2n_{f}$$

$$M_{3}(V) = 3n_{c} - 2n_{f} + n_{e}$$

$$M_{4}(V) = -n_{c} + n_{f} - n_{e} + n_{v}$$
(4.5)

where  $n_c$  is the total number of voxels that the microvasculature consists of,  $n_f$  is the number of open faces,  $n_e$  the number of open edges and  $n_v$  the number of open vertices.

In the present work, we normalize the MFs by the volume of the tissue from which the microvasculature was reconstructed to obtain density, surface area density, breadth density and Euler-Poincaré characteristic density. Normalization was performed in order to allow the comparative analysis of the MFs among different images. This would otherwise have not been possible due to the variability found in tissue volume among the images of our dataset.

#### 4.2.2 Segment-local level

Metrics at the structure-global level provide us with paramount information regarding the whole microvascular plexus, but they do not provide insights into the angioarchitecture, i.e. the morphology and arrangement of the individual microvessels that form the microvasculature, on which the functional properties of microcirculation and their modelling critically depend [173]. For this reason, we perform a quantitative analysis at the segment level using the 3D graph-based representation of the microvasculature (Fig. 4.5A), complemented with information regarding the radius, length, surface and volume of every segment.

#### 4.2.2.1 Graph-based representation and related metrics

In order to convert the microvasculature to a 3D graph that encompasses the topological information of the network, the reconstructed microvasculature is firstly skeletonised through the use of a thinning algorithm [125]. The skeleton is converted to a graph consisting of nodes that represent the branching or end nodes that could be possible sprouts (mean and standard deviations presented in Table 4.1) of the microvascular network and the edges (i.e. segments) that represent the skeleton parts between two consecutive nodes [108]. Subsequently, to eliminate false short spurs produced by the thinning procedure, the 3D image volume with possible areas of arterioles/venules obtained after Frangi filtering (G) introduced in the previous chapter, is used. G allows us to guide a local, pruning process during which end segments (segments defined between a branching node and an ending node) are pruned

based on their length and type (capillaries or arterioles/venules). More precisely, segments whose length is smaller than one-and-a-half times the diameter of the smallest capillary  $(4.1 \mu m)$ , found in the LV of the pig heart according to [104], are used in areas that the 3D G indicated that corresponded to capillaries. On the other hand, on those areas that correspond to arterioles/venules according to G, the segments are pruned when their length is smaller than one-and-a-half times the diameter of the smallest arteriole/venule of order 1 (8.96 $\mu$ m) [104]. The pruning procedure is repeated twice to ensure that spur "Y"-shaped segments, resulting from the thinning process, are entirely pruned. The branching and end-nodes of the skeleton are extracted as nodes with more than one and only one neighbouring point respectively by convolving the 3D skeleton of the image with a  $3 \times 3 \times 3$  sized mask of ones. Bubble-like nodes resulting as an artefact of the skeletonisation process are replaced by the central node. The radius of each segment is calculated using the distance of the points on the skeleton to the closest non-vascular element. Among the distances, the radius is set equal to the largest one. Every segment of microvasculature between two nodes i, j is subsequently considered a tube of constant diameter  $(d_{ij})$ , length  $(L_{ij})$  and the following metrics at segment level are calculated as in [213]

Vascular segment volume 
$$(V_{ij}) = \frac{\pi \times d_{ij}^2 \times L_{ij}}{4}$$
  
Vascular segment surface  $(S_{ij}) = \pi \times d_{ij} \times L_{ij}$   
Tortuosity  $= \frac{L_{ij}}{Euclidean \ distance \ between \ i \ and \ j}$   
Vascular length density  $(L_d) = \frac{1}{V} \times \sum_{ij=1,\dots,N} L_{ij}$   
Vascular surface density  $= \frac{1}{V} \times \sum_{ij=1,\dots,N} S_{ij}$   
Vascular volume density  $(V_d) = \frac{1}{V} \times \sum_{ij=1,\dots,N} V_{ij}$   
Diffusion distance  $(D) = \frac{1}{\sqrt{\pi \times L_d}}$ 

$$(4.6)$$

where V is the volume of the tissue and N is the total number of segments that form part of the microvasculature.

#### 4.2.3 Nuclei-Based refinement & Nuclei counting

The number of nuclei first had to be calculated in order to calculate the number of  $\alpha$ -SMA<sup>+</sup> and endothelial cells. For this purpose, 3D watershed transformation [147] is applied to the



Fig. 4.3 Watershed results for nuclei segmentation. (a) 3D Segmentation of nuclei channel by means of MMT. The volume is colour-coded to highlight nuclei that do not overlap neither with the microvasculature or  $\alpha$ -SMA<sup>+</sup> staining (blueish green) and those that do (orange). (b) Watershed segmentation for separation of individual nuclei represented in (a) with orange. Errors in the separation of nuclei can easily be spotted, but the segmentation was considered acceptable for the purposes of this work.

segmentation of the Hoechst channel in order to separate merged nuclei. Nuclei belonging to  $\alpha$ -SMA<sup>+</sup> were considered to be the ones with simultaneous staining of  $\alpha$ -SMA and Hoechst. Nuclei belonging to endothelial cells were considered to be the ones that were overlapping with the surface of microvessels.

It should be noted that although watershed transformation is a popular approach for nuclei segmentation, it also tends to produce errors, such as over- or under-estimation of nuclei (Fig. 4.3). However, as development of novel tools for nuclei segmentation was beyond the scope of this work, watershed performed reasonably well for our approach and we, therefore, just adapted it for the task at hand. Nonetheless it is noted that the number of nuclei in this work might, therefore, not be precise.

# 4.2.4 Assessment of oxygen efficiency & Super-ellipsoids as descriptors of capillary supply area

In an effort to identify a geometric pattern that would describe the area of capillary diffusion so that it closely matches the relative frequency distributions observed in our volumes, we used a super-ellipsoid model. Super-ellipsoids allow description of a variety of shapes, ranging from cylinders (such as in the Krogh model) to more complex ones, by varying only a small number of parameters. They are a type of 3D geometric shape that belongs to the same, more general family of geometric shapes named superquadrics [9]. Super-ellipsoids are implicitly defined by

$$\left[\left(\frac{|x-Cx|}{a}\right)^{\frac{2}{e_2}} + \left(\frac{|y-Cy|}{b}\right)^{\frac{2}{e_2}}\right]^{\frac{e_2}{e_1}} + \left(\frac{|z-Cz|}{c}\right)^{\frac{2}{e_1}} <= 1$$
(4.7)

where *a*, *b*, *c* are scale factors for axis *x*, *y* and *z* respectively, *Cx*, *Cy*, *Cz* represent the centre of the super-ellipsoid, and  $e_1$ ,  $e_2$  control the shape of the super-ellipsoid.  $e_1$  controls the squareness along z-axis and  $e_2$  the squareness along plane x-y. Therefore, a variety of shapes can be determined by varying  $e_1$  and  $e_2$ ;  $e_1 < 1$ ,  $e_2 < 1$  result in cuboids,  $e_2 = 1$  and  $e_1 < 1$  in cylindroids,  $e_1 = 1$ ,  $e_2 < 1$  in pillow shapes,  $e_1$  or  $e_2$  larger than 2 in pinched shapes,  $e_1$  or  $e_2$  equal 2 in flat-bevelled shapes.

Our aim was to identify a shape that the frequency distribution of distances of points inside the shape to the closest capillary (estimated distribution) best matches a given frequency distribution of maximum extravascular distances (target distribution). Toward this aim, the capillary was first approximated by a cylinder whose diameter was considered equal to the minimum extravascular distance observed. The length of the vessel was arbitrary chosen  $(100\mu m)$ . We subsequently set Cx, Cy, Cz as the coordinates of the centre of the vessel, b as the maximum extravascular distance observed, and c as length of the capillary, while the set of parameters a,  $e_1$ , and  $e_2$  as the unknowns to be defined through optimization. The target of optimization was to minimize the mean square error between estimated and target distributions. The parameters were initialized so as to produce a cylinder (i.e.  $e_1 = 1$ ,  $e_2 = 0.2$ ) whose length is equal to that of the vessel ( $c = 100 \mu m$ ) and whose diameter (i.e. (a,b) is equal to that of the maximum extravascular distance. In each step of the minimization procedure, a new set of parameters and, therefore, a new shape was defined by varying the unknown parameters in predefined ranges. In particular, parameter a was allowed to vary in the range between the minimum and maximum extravascular distance of the target distribution. Parameters  $e_1$ , and  $e_2$  were allowed to vary between [0,3] and, therefore, cover all ranges of possible shapes. The minimization procedure stopped after a minimum was achieved in the root mean square error. It should be noted that a simplified version of the target distribution (10 bins) was used. Optimization is based on fmincon MATLAB (Mathworks) solver and sequential quadratic programming.

#### 4.2.5 Classification of microvascular patterns

The quantitative information about the cardiac microvasculature and SMA<sup>+</sup> cells obtained by our pipeline was evaluated with the task of predicting the condition of previously unseen tissue based on the characteristics of the microvascular bed. Towards this aim, we incorporated the parameters at structure level, at segment level as well SMA<sup>+</sup> related metrics (metrics 1 - 28

of Table 4.1) into a classification scheme. Multi-class classification was performed to predict the time that elapsed since the onset of MI for infarcted tissue and remote tissue ("Infarcted over time", and "Remote over time" respectively in Table 4.2). In all other cases, binary classifications were performed. 9-fold cross-validation repeated 10 times was used. k-fold validation is a common model approach in machine learning that helps to avoid over-fitting the training set and it is usually repeated a multitude of times to ensure different separation of folds. The idea behind k-fold cross-validation is to divide the sample in k equal folds of which k-1 are used for training and one is kept for testing.

In this work, three different classifiers were employed [233]. K-nearest neighbours classifier (Knn) assigns an unseen object to a class, based on the labels of the k-nearest training objects that are closest (or more similar) to the object under investigation. Here, we used 1-Knn classifier, e.g. the tissue is classified according to the label of the closest training tissue. The distance metric used was euclidean distance.

Support vector machines (SVMs) are inherently binary classifiers. Standard SVMs define a hyperplane function that guaranties optimum separation, e.g. largest margin, between the (training) data of the two classes. A new object is subsequently assigned to one or the other class based on whether the predefined hyperplane function is positive or negative for that particular object. The idea of reducing the multiclass classification problem to binary classification problems has been introduced and different approaches have been developed in order to perform multi-class classification with SVMs. In this work, we adapted the oneversus-one strategy according to which SVMs are built for all possible pairwise comparisons. Each class is, therefore, compared to each other separately.

Adaboost is based on a cascade of weak classifiers (learners) used to create a strong classifier which has higher accuracy that single classifiers. During each iteration of the training procedure, a learner is added so as to weight higher misclassified examples of the previous iteration and, therefore, to redirect the focus of subsequent weak learners in those examples. The strong classifier produced by a weighted sum of the weak learners is responsible for the assignment of labels to unseen objects. In this paper, we used Knn weak classifiers.

#### 4.2.6 Statistical analysis

Statistical significance of differences in medians for pairwise comparisons was assessed by non-parametric, two-sided Wilcoxon rank sum tests. P-values were corrected for multiple testing with the Benjamini-Hochberg false discovery rate procedure which was applied to the fifteen pairwise tests performed per each quantified parameter. The number of samples per tissue category remained the same throughout the chapter and it equals to 18. Two

sample Kolmogorov-Smirnov tests were performed to evaluate the statistical significance of differences in distributions.

#### 4.2.7 3D Visualization

Visualization of 3D volumes (reconstructions, skeletons, distance maps) was performed with open-source softwares Paraview (http://www.paraview.org/) and ITK-SNAP (http://www.itksnap.org/).

#### 4.2.8 Code availability

The code for all modules of the image processing pipeline presented in this work is opensource (https://bitbucket.org/xenia\_gk/microvasculatureanalysis\_gkontra\_et\_al\_2018), along with the supporting documentation to run the modules separately or the complete pipeline at once. The statistical analysis and the creation of the comparative plots per metric was performed with an additional module of our pipeline, which is also available. Code/libraries from previous works necessary to run the pipeline are also provided and the creators have been credited accordingly. The code was written in MATLAB (Mathworks).

## 4.3 Results

## 4.3.1 Myocardial infarction decreases 3D complexity, integrity, and connectivity of the coronary microvasculature while it leads to altered capacity and heterogeneity of blood flow

The fractal parameters demonstrate significant differences in terms of multi-scale characteristics of the microvascular networks in the infarcted areas, over time (Fig. 4.4a). In particular, when comparing infarcted areas at 1, 3, and 7 days, we noted that the morphological complexity (fractal dimension) and capacity of blood flow (succolarity) of the microvasculature decreased, while the heterogeneity in the distribution of the non-vascularized areas (lacunarity), increased. The reverse relationship between morphological complexity and heterogeneity has been observed in earlier studies [84, 79]. Moreover, the distribution of non-vascularized areas affects blood flow. Thus, higher heterogeneity, as observed here, is expected to result in higher heterogeneity in blood flow within the tissue, and could therefore denote the existence of regions with lower perfusion. Furthermore, complexity and heterogeneity in the infarcted areas at 1 and 7 days are statistically different than in the corresponding remote areas (1



Fig. 4.4 Myocardial infarction decreases 3D complexity, integrity, and connectivity of the coronary microvasculature while it alters heterogeneity in the distribution of non-vascularized areas and capacity for blood flow. (a) 3D fractal-based parameters; Fractal Dimension, Lacunarity, and Succolarity. (b) Parameters based on the Minkowski functionals calculated for complete microvasculature (capillaries, arterioles, venules); Vascular Density, Surface Area Density, Breadth Density, Euler-Poincaré Characteristic Density. (c) Parameters based on the Minkowski functionals calculated for capillaries exclusively; Capillary Volume Density, Capillary Surface Area Density. In all figures of the manuscript, the bars show mean values, while error bars represent standard deviation. Furthermore, \*, \*\* and \*\*\* represent p-value< 0.05, 0.01 and 0.001 respectively. The p-values were calculated by means of Wilcoxon rank-sum tests and corrected with the Benjamini-Hochberg procedure for multiple testing. The number of samples compared is 18 per tissue condition.

and 7 days respectively). In terms of all multi-scale characteristics, the differences of the microvasculature from infarcted areas at 7 days post MI are significant compared with that at basal conditions. It is worth also noting that at infarcted areas 1 day post MI the tendencies compared with basal conditions are opposite to 7 days: higher morphological complexity

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Fig. 4.5 Microvascular angioadaptation occurs in response to MI. (a) Example graph-based representation of a microvascular structure. Microvascular angioadaptation occurs in response to MI. (a) Example graph-based representation of a microvascular structure. Each microvessel, i.e. segment from branching node to branching/ending node, is considered as a tube of constant radius. (b) Analysis of the characteristics of the microvessels in the pig infarcted heart in terms of vascular segment radius, vascular segment length, vascular segment surface, and vascular segment volume. (c) Analysis of vascular length density, vascular surface density, and vascular volume density.

and blood flow instead of lower, and lower lacunarity instead of higher. This could be linked to the first response of the system to MI and the poorer contractility of cardiomyocytes at day 1. Moreover, in contrast with the changes observed in microvascular networks from infarcted areas, no significant differences are found in the remote areas over time and compared with the basal case.

The reduction in the morphological complexity of the microvasculature in infarcted areas can be further explained by the progressive loss of microvascular volume that occurs Table 4.1 List of the parameters (mean  $\pm$  standard deviation) that are extracted automatically for the characterization of the cardiac microvasculature, its interactions with SMA<sup>+</sup> cells and remodelling due to MI. I1MI, I3MI, and I7MI, refer to infarcted areas at 1 day, 3 and 7 days post MI respectively. Similarly, R1MI, R3MI, and R7MI refer to the corresponding remote areas.

	Basal	I1MI	I3MI	I7MI	R1MI	R3MI	R7MI
Fractal-Based Metrics							
1. Fractal Dimension	$2.22 \pm 0.03$	$2.26 \pm 0.05$	2.22±0.04	2.15±0.04	$2.24{\pm}0.04$	2.23±0.03	2.22±0.05
<ol> <li>Lacunarity (×10<sup>-2</sup>)</li> </ol>	$76.92 \pm 5.15$	$72.75 \pm 3.85$	$78.08 {\pm} 5.52$	$83.98 {\pm} 2.96$	$76.33 {\pm} 4.58$	$73.64{\pm}2.73$	76.1±4.03
<ol> <li>Succolarity (×10<sup>-2</sup>)</li> </ol>	$0.33 {\pm} 0.14$	$0.49 {\pm} 0.23$	$0.28 {\pm} 0.13$	$0.19{\pm}0.11$	$0.32{\pm}0.16$	0.3±0.16	0.3±0.2
Minkowski-Based Metrics							
4. Vascular Volume Density (%)	6.75±0.85	8.9±2.82	6.46±1.06	$5.04{\pm}0.92$	7.21±1.34	6.14±1.29	6.64±1.83
5. Surface Area Density (×10 <sup>-3</sup> ) ( $\mu m^2/\mu m^3$ )	$48.36 {\pm} 7.05$	55.06±12.86	$46.45 \pm 6.95$	$27.15 {\pm} 4.05$	$48.2 \pm 6.25$	47.1±6.62	$45.04{\pm}11.28$
6. Breadth Density ( $\times 10^{-3}$ ) ( $\mu m/\mu m^3$ )	$1.47{\pm}0.29$	$1.43 {\pm} 0.27$	$1.42{\pm}0.28$	$0.64{\pm}0.11$	$1.37{\pm}0.19$	$1.52 \pm 0.23$	$1.27 \pm 0.32$
7. Euler-Poincaré Characteristic Density $(\times 10^{-5})(1/\mu m^3)$	$-4.32\pm1.78$	$-4.84{\pm}3.1$	$-3.36 \pm 1.38$	$-0.05 \pm 1.49$	$-3.5 \pm 1.62$	$-4.06 \pm 1.69$	$-3.56 \pm 2.64$
8. Capillary Volume Density (%)	$6.07 \pm 0.9$	$8.04{\pm}2.63$	5.7±1	$4.34{\pm}0.85$	6.55±1.17	$5.43 \pm 1.22$	5.7±1.97
9. Capillary Surface Area Density ( $\times 10^{-3}$ ) ( $\mu m^2/\mu m^3$ )	46.94±7.15	53.47±12.69	44.5±7.15	$25.34{\pm}4$	$46.62 \pm 6.19$	45.11±6.63	$42.5 \pm 11.91$
Graph-Based Metrics							
10. Vascular length density $(\times 10^{-3})(\mu m/\mu m^3)$	3.51±0.85	4.13±1.43	3.51±0.82	1.65±0.3	3.37±0.58	3.54±0.74	3.18±1.21
11. Vascular surface density $(\times 10^{-3})(\mu m^2/\mu m^3)$	$38.87 \pm 6.87$	$51.79 \pm 18.71$	$38.05 \pm 7.24$	$24.37 \pm 4.63$	$40.35 \pm 7.75$	$36.94 \pm 8.29$	36.94±13.86
12. Vascular volume density $(\times 10^{-2})(\mu m^3/\mu m^3)$	3.94±0.66	6.16±2.75	$3.92{\pm}0.93$	$3.33 {\pm} 0.87$	4.48±1.3	$3.59 \pm 1.11$	3.94±1.68
13. Vascular segment radius $(\mu m)$	$2.65 {\pm} 0.31$	2.91±0.33	$2.62{\pm}0.3$	$3.25 {\pm} 0.29$	$2.79 {\pm} 0.33$	$2.48 {\pm} 0.26$	$2.74 \pm 0.26$
14. Vascular segment length $(\mu m)$	$18.85 {\pm} 2.64$	$16.09 \pm 2.83$	$17.65 \pm 2.22$	$15.74{\pm}2.77$	18.15±2.96	$19.89 \pm 3.21$	17.59±2.77
15. Vascular segment surface $(\mu m^2)$	$215.3 {\pm} 49.14$	$200.75 \pm 35.27$	$193.63 {\pm} 27.74$	$231.67 \pm 35.76$	$218.53 {\pm} 42.92$	$207.73 \pm 33.87$	206.2±34.89
16. Vascular segment volume $(\mu m^3)$	224.15±71.59	$234.5 \pm 54.3$	$200.87{\pm}46.54$	313.63±59	$241.77 \pm 63.37$	$200.19 {\pm} 42.08$	$220.22 \pm 50.23$
17. Tortuosity ( $\mu m/\mu m$ )	$1.39{\pm}0.01$	$1.38 {\pm} 0.02$	$1.4{\pm}0.03$	$1.38 {\pm} 0.04$	$1.38 {\pm} 0.02$	$1.39{\pm}0.02$	$1.39 \pm 0.03$
18. Vascular segments $(\times 10^5)^a$	$1.91{\pm}0.6$	$2.77 \pm 1.48$	$2.02{\pm}0.57$	$1.09 \pm 0.3$	$1.91 \pm 0.49$	$1.86 {\pm} 0.68$	$1.9{\pm}0.97$
19. Vessels of diameter $\leq = 6.9 \ (\mu m) \ (\%)$	$94.56 {\pm} 2.94$	$90.14 \pm 5.45$	93.02±4.1	$83.92{\pm}6.01$	$92.14{\pm}5.02$	95.56±2.93	92.94±3.03
20. Vessels of diameter between 6.9 and 8.2 (µm) (%)	$3.01 \pm 1.46$	$4.88{\pm}2.02$	$3.54{\pm}1.83$	$8.46 {\pm} 2.31$	$3.92{\pm}2.06$	2.21±1.38	$3.9{\pm}1.52$
21. Vessels of diameter > 8.2 ( $\mu m$ ) (%)	$2.42 \pm 1.57$	4.98±3.56	$3.44{\pm}2.62$	$7.62 \pm 4.21$	$3.94 \pm 3.07$	$2.23 \pm 1.68$	3.16±1.74
22. Branching nodes $(\times 10^4)^a$	$10.14 \pm 3.43$	$14.79 \pm 8.12$	10.74±3.33	$5.48 {\pm} 1.58$	9.94±2.71	$9.79 \pm 3.84$	9.85±5.31
23. Blind-ends/sprouts $(\times 10^4)^a$	$5.08 \pm 1.42$	7.47±3.93	5.37±1.3	$3.44{\pm}0.89$	$5.64 \pm 1.47$	4.95±1.75	$5.49 \pm 2.38$
24. Branching nodes b	$30.02 \pm 4.03$	35.1±6.26	$31.29 \pm 4.76$	$32.91 \pm 5.61$	$30.54{\pm}5.22$	$28.58 {\pm} 4.57$	31.11±5.37
25. Blind-ends/sprouts b	$18.22 \pm 5.81$	$19.46 {\pm} 4.52$	$16.52 \pm 3.41$	$20.47 {\pm} 4.18$	18.99±4.59	$16.92 \pm 4.32$	$21.04{\pm}6.56$
SMA <sup>+</sup> related metrics							
26. Vessels covered with SMA (%)	80.05±11.57	67.32±19.45	69.34±7.81	54.03±9.82	73.82±9.71	74.5±15.59	78.01±14.33
<ol> <li>SMA<sup>+</sup> layer thickness (μm)</li> </ol>	$2.43 \pm 1.15$	$2.32{\pm}0.77$	4.13±2.4	$10.23 \pm 1.76$	$2.7 \pm 0.69$	$2.75 \pm 0.8$	3.31±1.37
28. Damage Index	$0.01 {\pm} 0.01$	$0.01 {\pm} 0.01$	$0.02{\pm}0.02$	$0.14{\pm}0.06$	$0.01 {\pm} 0.01$	$0.02{\pm}0.01$	$0.02{\pm}0.02$
29. Myofibroblasts $(\times 10^4)^a$	$1.04{\pm}0.51$	$0.9 \pm 0.56$	$0.96 {\pm} 0.42$	$2.12{\pm}0.77$	$0.64{\pm}0.32$	$0.77 \pm 0.34$	$0.67 \pm 0.44$
<ol> <li>SMA<sup>+</sup> perivascular cells<sup>b</sup></li> </ol>	31.71±11.34	24.58±12.6	$24.83 \pm 8.48$	$45.72 \pm 17.49$	$30.95 \pm 6.76$	$22.68 \pm 7.48$	26.19±10.53
Efficiency in oxygen diffusion							
31. Maximal Extravascular Distance (µm)	27.35±6.76	28.29±4.52	33.72±6.8	46.22±8.26	30.37±5.13	28.32±3.71	29.69±4.42
32. Median Extravascular Distance (µm)	14.57±3.56	$15.07 \pm 2.38$	$17.92 \pm 3.58$	24.51±4.35	$16.16 \pm 2.7$	$15.08 \pm 1.95$	15.8±2.33
33. Capillary Density c	1236.16±714.07	$1226.84 \pm 567.85$	$1101.16 \pm 525.2$	394.24±211.981	091.95±452.94	1053.55±781.83	1059.93±514.7
34. Intercapillary Distance (µm)	$16.91{\pm}2.08$	16.93±1.84	$17.44{\pm}2.46$	$25.54{\pm}4.44$	$17.68 {\pm} 1.62$	$17.17 {\pm} 1.85$	19.51±3.64
35. Diffusion Distance $(\mu m)$	9.77±1.43	9.1±1.38	9.72±1.17	$14.07 \pm 1.38$	$9.83 {\pm} 0.83$	9.63±1	$10.55 \pm 2.17$
Additional cell-related metrics							
36. Endothelial cells $(\times 10^5)^d$	22.85±6.83	19.19±5.19	19.36±3.82	18.31±5.97	19.18±4.5	19.17±3.74	15.12±2.76
37. Endothelial cells b	$44.14 \pm 8.53$	40.93±8.16	35.97±5.78	55.47±16.91	$40.44 \pm 6.09$	33.1±4.75	33.13±7.98

<sup>*a*</sup>Number per  $mm^3$  of tissue

<sup>b</sup>Number per *mm* vascular length

<sup>*c*</sup>Number per *mm*<sup>2</sup> of tissue

<sup>d</sup>Number per mm<sup>3</sup> vascular volume

in the infarcted areas as revealed by the decreased vascular volume density (Fig. 4.4b) observed in those areas. Furthermore, the microvasculature appears reduced in the infarcted areas, with less breadth and smaller surface area for the exchange of oxygen and nutrients, while it is progressively converted from a strongly connected network to a less connected one (progressively increasing Euler-Poincaré characteristic). The reduced connectivity and volume are related to the lower capacity of blood flow and probably to its heterogeneity described earlier. An unexpected observation is the significant decrease of the microvascular volume density at remote areas at 3 days post MI compared with remote 7 day post MI and

basal. Further exploration of the lost vascular volume (capillary volume or arteriole/venule volume) led to the observation that the loss is associated with the loss of capillary volume (Fig. 4.4c). Lastly, as with the fractal parameters, in terms of all metrics the infarcted area 7 days post MI presents significant differences with basal areas, as well as with corresponding remote areas.

#### 4.3.2 Myocardial infarction promotes microvascular angioadaptation

The most profound changes are observed at infarcted areas 7 days after MI. By examining Fig. 4.5b it is obvious that the microvascular segments of those regions present larger radius than those of tissues from infarcted areas at earlier time-points, remote areas at 7 days post MI and at basal conditions, despite the reduction of the overall microvasculature described in the previous subsection. This results in segments with significantly larger volume than segments from the other areas, while their surface area is significantly larger than that of segments from infarcted areas at 3 days post MI and their length is shorter than segments from basal conditions. In contrast, the microvessels in both the infarcted and remote areas 3 days post MI become significantly smaller in terms of radius compared to those of the infarcted areas from earlier and later time-points under investigation, indicating a possible vasoconstriction at this time-point. The radii of microvessels from infarcted and remote tissue 1 day following MI, as well as from remote areas 7 days post MI, seem to remain unaffected. No significant difference among any tissue categories is observed in terms of tortuosity and the relative plot was, therefore, omitted. However, the corresponding mean and standard deviations are provided in Table 4.1.

Length, surface and volume density (Fig. 4.5c) calculated by means of the graph-based representation of the microvasculature confirm the microvascular changes revealed by the corresponding Minkowski-based metrics (breadth, surface and volume density). This fact demonstrates that the latter are sufficient in describing those characteristics of microvascular networks and it is therefore feasible to avoid exhaustive extraction of centerlines when characteristics of the individual microvessels are out of interest.

Subsequently, segments were divided into three classes based on the morphological data of [104] in order to study the size of microvessels at each tissue condition. The maximum diameters found in the left ventricle of the pig heart according to the published data are 7.3  $\mu m$  for capillaries fed directly by arterioles ( $C_{oa}$ ), 8.2  $\mu m$  for capillaries drained directly by venules ( $C_{ov}$ ), and 6.9  $\mu m$  for cross-connecting capillaries ( $C_{cc}$ ) and capillaries connected to  $C_{oa}$  and  $C_{ov}$  types ( $C_{oo}$ ). Thus, the three classes created are (i) segments with diameter smaller or equal to 6.9  $\mu m$  corresponding to  $C_{cc}$  and  $C_{oo}$  capillaries, (ii) segments with diameter



Fig. 4.6 Changes in the number and percentages of microvessels in the infarcted pig heart according to their radius and to the physiological condition of the tissue. On the left (**a**), an example microvascular volume color-coded according to the size of its segments. On the right, (**b**) analysis of the number of microvascular segments per  $mm^3$  of tissue for the different tissue conditions. Analysis of the percentage of vascular segments with diameter smaller than  $6.9\mu m$  (**c**), with diameter between 6.9 and  $8.2\mu m$  (**d**) and with diameter larger than  $8.2\mu m$  (**e**). I1, I3, I7, R1, R3, R7 stand for infarcted areas and remote areas at 1, 3, 7 days post MI respectively. Note that statistical hypothesis testing in (**b**) refers to the comparison of the overall number of vascular segments i.e. the sum of the three categories.

between 6.9 and 8.2  $\mu m$  corresponding to  $C_{oa}$  and  $C_{ov}$  capillaries, and (iii) segments with diameter larger than 8.2  $\mu m$  representing arterioles or venules.

A gradual decrease in the overall number of microvascular segments per  $mm^3$  of tissue is observed at the infarcted areas (Fig. 4.5d). The decrease becomes profound and statistically significant at 7 days post MI compared with all other conditions. The reduced number of segments explains the reduced morphological complexity, vascular volume density, surface area and breadth density of this area expressed by the fractal dimension and the Minkowski metrics (Fig. 4.4). Furthermore, a higher number of vascular segments can be also observed in the infarcted area 1 day post MI compared to the basal tissue. This difference, although not significant, matches the higher density and morphological complexity on this tissue category described in the previous subsection (Fig. 4.4).

Looking further into the percentage of vessels in each radius range (Fig. 4.6) the percentage of microvessels with diameter larger than 8.2  $\mu m$ , i.e. arterioles/venules, and "medium"-sized capillaries of the categories  $C_{oa}$  and  $C_{ov}$  (6.9 and 8.2  $\mu m$ ) are increased in infarcted areas 7 days post MI. On the contrary, the smallest vessels ( $C_{cc}$  and  $C_{oo}$ ) are reduced

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compared to the healthy tissue, infarcted tissue at earlier stages after MI as well as with the corresponding remote area. This fact demonstrates a regression of smaller microvessels and probably dilation or even arterialization of the remaining vessels in infarcted tissues from 7 days following MI. The smallest microvessels from infarcted tissue 1 day post MI are significantly decreased as well, while the percentage of capillaries  $C_{oa}$  and  $C_{ov}$  (6.9 and 8.2  $\mu m$ ), is increased when comparing to microvessels belonging to basal tissue, which implies a possible vasodilation effect. Interestingly, at remote areas 3 days post MI the number of smallest capillaries is increased compared with the remote areas at other time-points, and in contrast, the percentage of "medium"-sized vessels is reduced.

Information from the  $\alpha$ -SMA channel and its relation to the microvasculature (Fig. 4.8a) is quantified through an additional module of the analysis pipeline in order to study perivascular cells stained by  $\alpha$ -SMA, i.e. pericytes and smooth muscle cells. We are particularly interested in the coating of microvessels by layer(s) of  $\alpha$ -SMA expressing cells as this could be an indicator of vessel maturation [49, 73] and denote the end of a plasticity window for intervention [181]. A significantly lower percentage of vessels coated with  $\alpha$ -SMA<sup>+</sup> cells is observed in infarcted areas 3 and 7 post MI compared to the basal tissue as well as to the corresponding remote area for the second case (Fig. 4.8b). However, the layers of  $\alpha$ -SMA<sup>+</sup> perivascular cells on those days, i.e. 3 and 7 days post MI, are significantly thicker than in the basal case as shown in the same panel. Furthermore, the thick layer of  $\alpha$ -SMA<sup>+</sup> at the infarcted area 7 days post MI consists of significantly more cells than in the infarcted areas of previous time-points, the basal and remote area 7 days post MI as indicated by the increased number of  $\alpha$ -SMA<sup>+</sup> perivascular cells per *mm* of vascular length (Fig. 4.8c). Thus, we have a proliferation of the perivascular  $\alpha$ -SMA<sup>+</sup>, and not a dilation of the cells.

We also noticed, mainly in infarcted areas,  $\alpha$ -SMA<sup>+</sup> regions that were not associated with microvessels, i.e. they were not localized in the perivascular layers of microvessels. Given that myofibroblasts are non-vascular cells that produce extracellular matrix indispensable for healing after MI and have contractile properties related to their expression of  $\alpha$ -SMA [218], we considered those  $\alpha$ -SMA<sup>+</sup> regions corresponding to myofibroblasts. We further complemented their characterization by their concomitant expression of other markers, such as platelet-derived growth factor receptor beta (PDGFRB)[63], vimentin and collagen I[81] (Fig. 4.7). We then decided to extract quantitative information regarding the myofibroblasts based on the  $\alpha$ -SMA<sup>+</sup> staining. For this purpose, a damage index was defined for the tissue as an estimator of the presence of myofibroblasts. Precisely, the index is given by the ratio of the volume of myofibroblasts is quantified as the number of simultaneously stained nuclei by Hoechst and  $\alpha$ -SMA.



Fig. 4.7 SMA<sup>+</sup> cells co-express other myofibroblast markers. (a) Tissue simultaneously labelled using anti-CD31, anti-SMA and anti-PDGFRB, a marker that is expressed in fibroblasts, myofibroblasts, perivascular smooth muscle cells and that increases during myocardial fibrosis. (b) Tissue simultaneously labelled with anti-CD31, anti-SMA and anti-vimentin, or (c) anti-collagen I. Both markers are expressed in fibroblasts and myofibroblasts during cardiac remodelling. In all cases, the first three columns provide the maximum intensity projections of the original channels (upper row of the panels) and of the resulting segmentations of the channels (lower rows of the panels). In the case of SMA<sup>+</sup> cell segmentation, yellow represents areas automatically recognized as SMA<sup>+</sup> perivascular regions, while green the SMA<sup>+</sup> myofibroblast region. In the last column, 3D reconstructions showing non-perivascular SMA<sup>+</sup> myofibroblast areas that are positive or negative for the other marker in grey or green respectively. Perivascular SMA<sup>+</sup> area is highlighted in yellow with double-positive regions for other markers in blue.



Fig. 4.8 Changes in SMA<sup>+</sup> perivascular cells and myofibroblasts in the aftermath of MI. (a) Example of 3D reconstructions of the microvasculature (red) merged with the segmentation of the SMA<sup>+</sup> cells channel (grey) in a basal and an infarcted 7 days post MI case. (b) Changes in SMA<sup>+</sup> related metrics, i.e. the percentage of vessels with a SMA<sup>+</sup> coat along with the corresponding thickness of the coat. (c) Analysis of the number of SMA<sup>+</sup> cells of which the SMA<sup>+</sup> coat covering the microvessels consists. (d) Analysis of the cardiac tissue by means of indicators of tissue fibrosis, i.e. the Damage Index (percentage of SMA<sup>+</sup> volume that are not associated with vessels), and number of myofibroblasts per *mm*<sup>3</sup> of tissue.

Damage index (Fig. 4.8d) demonstrates a non-significant increase in the presence of myofibroblasts in infarcted tissues at the first time-points after MI (1 and 3 days) followed by a significant increase at the later time point (7 days post MI). This observation is in accordance with findings in the healing human tissue [231], and rat tissue after MI [226]. A lack of increase in the damage index in the remote areas compared to the basal case denotes no increase of myofibroblasts, and thus, no inappropriate deposition of extracellular matrix

Classifier	1	2	3	Classifier	1	2	3
Infarcted - Basal	77	81	74	Remote - Basal	68	72	75
I1MI - Basal	82	75	59	R1MI - Basal	61	70	61
I3MI - Basal	66	77	47	R3MI - Basal	65	63	57
I7MI - Basal	100	100	94	R7MI - Basal	63	59	70
Infarcted over time	75	89	72	Remote over time	48	50	40
I1MI - I3MI	71	84	64	R1MI - R3MI	61	66	57
I1MI - I7MI	100	100	97	R1MI - R7MI	53	60	56
I7MI - I3MI	93	97	93	R7MI - R3MI	56	64	56
Infarcted - Remote	67	71	56				•
I1MI - R1MI	53	68	69				
I3MI - R3MI	62	60	42				
I7MI - R7MI	97	100	88				

Table 4.2 Accuracy (%) in classifying the distinct vascular patterns using different classifiers: (1) Knn, (2) SVM, (3) Adaboost, and 9-fold cross-validation, repeated 10 times. Metrics of 1-28 of Table 4.1 are used as features for performing the classification.

in remote areas [218]. As far as the absolute number of myofibroblasts per  $mm^3$  of tissue is concerned, the trends presented in infarcted areas are similar with that of the damage index. However, additionally a decrease of myofibroblasts in remote areas at 1 and 7 days post MI compared with the basal case can be observed.

## **4.3.3 3D microvascular characteristics predict the healthy or diseased** status of the cardiac tissue

The proposed approach achieves high accuracy in the range 74% - 81% in differentiating between infarcted and basal tissue, as well as in recognizing the day after MI that the infarcted tissue belongs to (72% - 89%). The accuracy in classification drops to levels of 68% - 75% and 56% - 71% in differentiating between remote and basal tissue, and between remote and infarcted tissue respectively. This is expected taking into account the lack of significant differences in the majority of metrics between those categories. It is worth noting that, for the same reason, the worst levels of accuracy in classification (40% - 50%) are achieved in terms of recognizing which day following MI the remote tissue belongs to.



Fig. 4.9 Capillary diffusion distances support altered oxygen diffusion in the infarcted cardiac tissue. (a) Example of 3D extravascular distance maps for a basal and a 7 days post MI case. (b) Histograms of the extravascular diffusion distances for all volumes of the dataset colour-coded according to the tissue condition. (c) Kolmogorov-Smirnov statistical tests for identifying significant differences in the mean relative frequencies of diffusion distances between the different tissue categories. (d) Analysis of the maximum and median extravascular diffusion distances at the different tissue conditions based on all volumes of the dataset. (e) Analysis of metrics traditionally related to oxygen-diffusion distances, i.e. capillary density, and inter-capillary distance calculated from 2D slices of the 3D volume, and of the diffusion distance calculated by using the length density, instead of the detailed 3D maps of panel.

# **4.3.4** Structural changes of the microvasculature support altered oxygen diffusion in the post-infarcted cardiac tissue

The diffusion of oxygen from the microvascular network to the tissue critically depends on the density and the arrangement of the microvascular bed [174]. Simple metrics, such as capillary density, intercapillary distance, diffusion distance as derived from vascular length density, and 3D extravascular distances (EDs), have been proposed in the literature as indices of oxygen diffusion, with the latter having been proven more efficient [35].

In this work, 3D maps of EDs were calculated by getting the distance of every point to the closest vessel (Fig. 4.9a) and they were used to calculate the relative frequency distributions (Fig. 4.9b). From the comparative plot, the most striking difference that we can observe among the different tissue conditions is a long-tail in the distribution of EDs at the infarcted areas 7 days following MI. This tail implies longer diffusion distances, which are associated with longer diffusion times, and thus worst oxygenation [174]. Two sample Kolmogorov-Smirnov tests were used to compare the mean relative frequencies of each tissue category (Fig. 4.9c). The outcome verified the statistical significance of the difference in the shape of the distributions. The median and maximal EDs, calculated as the 50% and 95% quartile respectively [35], also confirm significantly increased diffusion distances in the infarcted areas 7 days following MI. However, significant differences in both metrics in the infarcted areas 3 days following MI, compared to the basal and corresponding remote tissue, highlight that the increase in the EDs had already started in the infarcted areas from day 3 following MI. Moreover, the EDs at remote areas at 1 and 7 days after MI are also affected and are significantly different from that of the basal tissue.

EDs calculated directly from the complete 3D volumes are expected to be more accurate than capillary density and intercapillary distance which are calculated in 2D, or the diffusion distance which is derived from the vascular length density. However, for the sake of complete-ness and for facilitating comparison with past and future works, these metrics are provided in Fig. 4.9d. According to the plots, the capillary density is affected and reduced in infarction, with the reduction becoming significant at 7 days post MI in infarcted area. On the other hand, the intercapillary distance which is associated with an inverse relationship with capillary density, is increased in the infarcted areas at 7 days following MI. The diffusion distance calculated directly from the length density follows the same trend as the EDs calculated from the 3D distance maps, although an underestimation of the distances can be observed.

Lastly, we contrasted the mean relative frequency distributions of 3D EDs for each tissue category with the ones that would be provided by equivalent Krogh cylinder models [117]. The Krogh cylinder model assumes the supply of oxygen by every capillary to a cylindrical region whose radius equals half the intercapillary distance. As a result, the

relative frequency distributions in the Krogh cylinder model are monotonically increasing to the maximum distance to the closest vessel (Fig. 4.10) and are far from describing the distributions observed in the cardiac tissue studied in this work. In an effort to identify a geometric pattern that would describe the area of capillary diffusion so that it closely matches the relative frequency distributions observed in our volumes, we used a super-ellipsoid model. Super-ellipsoids allow description of a variety of shapes, ranging from cylinders (such as in the Krogh model) to more complex ones, by varying only a small number of parameters (see Section 4.2.4). Thus, we formulated a question of finding a set of parameters that would define super-ellipsoids that match simplified versions of our distributions (10 bins) as an error minimization problem between the observed relative frequency distributions and the ones corresponding to different super-ellipsoids (Fig. 4.11). Root mean squared error in the range of 0.0027 - 0.0073 between the relative frequency distributions of extravascular distances in the super-ellipsoid models and the target ones demonstrate the superiority of the super-ellipsoid model over the Krogh cylinder model to represent the region of capillary diffusion in the cardiac tissue studied here.

# **4.3.5** Changes in the microvasculature and in the diffusion of oxygen persist and intensify at later time-points post MI

We next analysed, by means of our image analysis pipeline, the microvascular characteristics of porcine tissues from infarcted and remote areas 45 days post MI (Fig. 4.12) to examine whether the observed microvascular alterations during the first week post MI persisted at later time-points and whether additional changes occurred. The results, which have been summarized in Table 4.3 point to the persistent nature of the extensive microvascular remodelling that takes place 7 days post MI in infarcted areas together with a deterioration in certain metrics at 45 days post MI. Changes, which had been slightly observed at remote areas 7 days post MI, also intensified and became evident at remote areas 45 days post MI.

Both infarcted and remote areas at 45 days post MI present the same increasing or decreasing trends in those parameters which describe the microvasculature (fractal-, minkowskiand graph-based metrics) as the corresponding tissue 7 days post MI compared to earlier time-points post MI and basal conditions. There are several metrics, however, where increases or decreases are so prominent that statistically significant differences are observed between the tissues examined 7 and 45 days post MI. More precisely, infarcted areas at 45 days post MI have less vascular surface, breadth and length density, while they present a higher heterogeneity in distribution of non-vascularized areas compared to 7 days post MI. Concomitantly, there were fewer smaller and more medium and larger microvessels



Fig. 4.10 Equivalent Krogh cylinders and histograms of diffusion distances per tissue category; basal, infarcted and remote 1, 3, 7 days following MI. (a) Equivalent Krogh cylinders pseudo-coloured according to the distance of each point to the central capillary (represented with grey in all sub-figures). The radius of the central capillary for each tissue condition was set equal to the mean vascular segment radius calculated for the particular condition. Similarly, the length of the central capillary, and thus, of the corresponding Krogh cylinder is set equal to the mean vascular segment length of the tissue condition. Finally, the radius of the Krogh cylinder is set equal to the half the intercapillary distance which was measured from capillary centre to capillary centre using 2D slices of the volumes of each tissue condition. (b) Histograms of relative frequencies of distances of the tissue points to the central capillary calculated for the equivalent Krogh cylinders of panel **a**.



Fig. 4.11 3D super-ellipsoids are adequate geometrical models to represent the capillary diffusion area. (a) 3D super-ellipsoids estimated to best fit the mean distributions of extravascular distances extracted from histograms of 10 bins for the different tissue conditions (one per row; basal, infarcted and remote 1, 3, 7 days following MI) from x-y (first column), x-z (second column) and y-z views (third column). The tissue points inside the 3D shapes are pseudo-coloured according to the distance to the central capillary (represented with grey in all sub-figures). The radius of the central capillary for each tissue condition was set equal to the minimum extravascular distance found for the particular case. (b) Accuracy of the fitting between the mean distributions of extravascular distances for the different tissue conditions (target represented with red) and the distributions produced using the estimated 3D super-ellipsoids of panel **a** to best fit them (represented with blue). RMSE stands for the root mean square error, while  $a, b, c, e_1, e_2$  are the estimated parameters that define the 3D super-ellipsoids. In particular, a, b, c, are the scale factors for axis x, y and z respectively, and  $e_1, e_2$  control the shape of the super-ellipsoid.



Fig. 4.12 Representative 3D reconstructions of the cardiac microvasculature from tissues at 45 days post MI. The first two volumes correspond to tissues from infarcted areas, while the third and fourth volumes to tissues from remote ones.

with increased intercapillary and diffusion distances at 45 days. Altogether this remodelling indicates worse vascular perfusion and oxygen supply in infarcted areas at late time-points. In the remote areas at 45 days similar changes were observed for the microvasculature which was characterized by a further loss of its morphological complexity, breadth and connectivity. These changes are accompanied again by enlarged vessels, reduction in smaller and increase in larger microvessels along with higher heterogeneity in the distribution of non-vascular areas as well as a trend to poorer diffusion. Moreover, the SMA<sup>+</sup> perivascular layer is thinner, while the number of myofibroblasts decreases in both remote and infarcted areas compared to day 7 post MI. This suggests deceleration or termination of extracellular cardiac remodelling at this late stage.

### 4.4 Discussion

The link between the anatomy of microcirculation and CVDs' onset and progression had led to considerable efforts in unravelling the fine perturbations of the microvascular networks. This is particularly true in the case of MI were an amount of important works had been performed prior to our work [6, 181, 49, 228, 21, 30, 116, 92, 204]. However, there is a high variability among the quantification methods used in those studies, with each study reporting a different set of parameters to characterize the anatomy of microcirculation and some of them being based on the 2D analysis. Works more closely related to ours [204] do not provide the wealth of data which are indispensable in describing the complications of MI, but are rather based on only a few traditional metrics.

This work was designed to deal with these shortcomings by allowing the in-depth study of all major characteristics of the microvasculature in 3D through a single pipeline. Our pipeline consists of modules that permit translation of image information to quantitative biological

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Table 4.3 Mean  $\pm$  standard deviation of all parameters for tissue from infarcted and remote areas at 45 days following MI and their change (increase or decrease) with respect to infarcted and remote areas at earlier time-points after MI and under basal conditions. 11MI, I3MI, I7MI, and I45MI refer to infarcted areas at 1 day, 3, 7 and 45 days post MI respectively. Similarly, R1MI, R3MI, R7MI and R45MI refer to the corresponding remote areas. Up-arrows and down-arrows indicate increase and decrease respectively of the parameter value at 45 days post MI in infarcted (columns 2 to 5) or remote areas (columns 7 to 10) compared with the tissue category under investigation. Red (for increase) and blue (for decrease) colours are used to denote that the difference is statistically significant. \*, \*\* and \*\*\* represent p-value< 0.05, 0.01 and 0.001 respectively. The p-values were calculated by means of Wilcoxon rank-sum tests and corrected with the Benjamini-Hochberg procedure for multiple testing. The number of samples compared is 18 per tissue condition.

		I45MI	MI I45MI I45MI I45MI			R45MI	R45MI	R45MI	R45MI	
	I45MI	vs	vs	vs	vs	R45MI	vs	vs	vs	vs
		I1MI	I3MI	I7MI	Basal		R1MI	R3MI	R7MI	Basal
Fractal-Based Metrics										
Fractal Dimension	$2.12{\pm}0.06$	↓***	↓***	$\downarrow$	↓***	$2.17{\pm}0.04$	↓***	↓***	↓**	↓**
Lacunarity ( $\times 10^{-2}$ )	89.17±4.05	<b>↑*</b> **	^***	<b>↑*</b> **	^***	$85.17 \pm 5.52$	<b>^**</b> *	^***	<b>↑**</b> *	<b>^**</b> *
Succolarity ( $\times 10^{-2}$ )	$0.14{\pm}0.08$	↓***	↓**	$\downarrow$	↓***	$0.24{\pm}0.16$	$\downarrow$	$\downarrow$	$\downarrow$	$\downarrow$
Minkowski-Based Metrics										
Vascular Volume Density (%)	$4.88 {\pm} 1.24$	↓***	↓***	$\downarrow$	↓***	$7.03 \pm 1.11$	$\downarrow$	^*	1	1
Surface Area Density (×10 <sup>-3</sup> ) ( $\mu m^2/\mu m^3$ )	$21.48 {\pm} 5.33$	↓***	↓***	↓**	↓***	$39.94{\pm}5.92$	↓**	↓**	$\downarrow$	↓**
Breadth Density (×10 <sup>-3</sup> ) ( $\mu m/\mu m^3$ )	$0.5 {\pm} 0.13$	↓***	↓***	↓**	↓***	$1.05 {\pm} 0.15$	↓***	↓***	↓*	↓***
Euler Characteristic Density ( $\times 10^{-5}$ ) ( $1/\mu m^3$ )	$0.97 {\pm} 0.99$	^***	<b>^</b> ***	$\uparrow$	<b>^</b> ***	$-1.98 \pm 0.91$	^**	^**	^*	^***
Capillary Volume Density (%)	$4.45 \pm 1.22$	↓***	↓**	$\uparrow$	↓***	6.01±1.33	$\downarrow$	$\uparrow$	$\uparrow$	$\downarrow$
Capillary Surface Area Density (×10 <sup>-3</sup> ) ( $\mu m^2/\mu m^3$ )	$20.78 {\pm} 5.36$	↓***	↓***	↓**	↓***	$38.23 \pm 6.44$	↓**	↓**	$\downarrow$	↓**
Graph-Based Metrics										
Vascular length density $(\times 10^{-3})(\mu m/\mu m^3)$	$1.18 \pm 0.39$	↓***	↓***	↓**	↓***	2.51±0.62	↓**	↓***	$\downarrow$	↓**
Vascular surface density $(\times 10^{-3})(\mu m^2/\mu m^3)$	$21.09 {\pm} 6.38$	↓***	↓***	↓*	↓***	$35.68 {\pm} 8.84$	$\downarrow$	$\downarrow$	$\downarrow$	$\downarrow$
Vascular volume density $(\times 10^{-2})(\mu m^3/\mu m^3)$	$3.56{\pm}1.02$	↓**	$\downarrow$	↑	$\downarrow$	$4.61 \pm 1.24$	↑	^*	$\uparrow$	$\uparrow$
Vascular segment radius $(\mu m)$	$3.89 {\pm} 0.29$	<b>^**</b> *	<b>^**</b> *	<b>^**</b> *	<b>^**</b> *	$3.28 {\pm} 0.21$	<b>^**</b> *	^***	^***	^***
Vascular segment length $(\mu m)$	$14.38 {\pm} 3.24$	$\downarrow$	$\downarrow^*$	$\downarrow$	$\downarrow^*$	$16.38{\pm}1.88$	$\downarrow$	↓*	$\downarrow$	$\downarrow^*$
Vascular segment surface $(\mu m^2)$	$261.44{\pm}64.16$		^**	↑	↑	$232.39{\pm}25.64$	↑	$\uparrow$	^*	$\uparrow$
Vascular segment volume $(\mu m^3)$	$444.73{\pm}119.39$	<b>^**</b> *	<b>^**</b> *	<b>^**</b> *	<b>^**</b> *	$299.56{\pm}40.88$	^*	^***	^***	^**
Vascular segments $(\times 10^5)^a$	$0.88 {\pm} 0.41$	↓***	↓***	$\downarrow$	↓***	$1.56{\pm}0.46$	$\downarrow$	$\downarrow$	$\downarrow$	$\downarrow$
Vessels of diameter $\leq = 6.9 \ (\mu m) \ (\%)$	67.49±7.19	↓***	↓***	↓***	↓***	$86.02 \pm 4.61$	↓**	↓***	↓***	↓***
Vessels of diameter between 6.9 and 8.2 ( $\mu m$ ) (%)	12.77±3.2	<b>↑**</b> *	<b>↑</b> ***	<b>↑**</b> *	<b>↑</b> ***	$7.09{\pm}1.9$	^***	<b>↑**</b> *	^***	<b>^**</b> *
Vessels of diameter > 8.2 ( $\mu m$ ) (%)	19.75±7.09	<b>^**</b> *	<b>^**</b> *	<b>^**</b> *	<b>^**</b> *	$6.9 {\pm} 3.05$	^*	^***	^**	^***
SMA <sup>+</sup> related metrics										
Damage Index	0.13±0.06	<b>^**</b> *	<b>^</b> ***	$\downarrow$	<b>^</b> ***	$0.01 {\pm} 0.01$	$\downarrow$	$\downarrow$	$\downarrow$	$\downarrow$
Vessels covered with SMA (%)	$54.87 \pm 13.87$	$\downarrow$	↓**	$\uparrow$	↓***	$81.53 {\pm} 8.04$	$\uparrow$	$\uparrow$	$\uparrow$	$\uparrow$
SMA <sup>+</sup> layer thickness ( $\mu m$ )	9.44±4.39	<b>↑**</b> *	^***	$\downarrow$	^***	$2.21 \pm 0.51$	$\downarrow$	↓*	↓*	$\downarrow$
Myofibroblasts $(\times 10^4)^a$	$1.51 {\pm} 0.88$	^*	$\uparrow$	↓*	$\uparrow$	$0.38 {\pm} 0.18$	$\downarrow^*$	↓***	↓*	↓***
SMA <sup>+</sup> perivascular cells <sup>b</sup>	$52.78 \pm 31.2$	^**	^**	$\uparrow$	^*	$29.89 {\pm} 8.79$	$\uparrow$	^*	$\uparrow$	$\downarrow$
Efficiency in oxygen diffusion										
Maximal Extravascular Distance $(\mu m)$	56.32±11.2	<b>^**</b> *	<b>^**</b> *	<b>^</b> *	<b>^**</b> *	30.91±3.43	1	^*	$\uparrow$	<b>^*</b> *
Median Extravascular Distance $(\mu m)$	$29.82{\pm}5.9$	<b>^**</b> *	<b>^**</b> *	^*	<b>^**</b> *	$16.45 \pm 1.8$	↑	^*	↑	^**
Capillary Density c	$482.47{\pm}190.43$	↓***	↓**	$\uparrow$	↓*	$770.16{\pm}450.86$	$\downarrow$	$\downarrow$	$\downarrow$	$\downarrow$
Intercapillary Distance $(\mu m)$	$32.19 {\pm} 8.52$	^***	<b>^**</b> *	^*	<b>^**</b> *	$19.54{\pm}2.28$	^*	^**	$\uparrow$	^**
Diffusion Distance $(\mu m)$	17±2.43	<b>↑*</b> **	^***	^**	^***	$11.51 \pm 1.47$	^**	^***	$\uparrow$	^**

<sup>*a*</sup>Number per *mm*<sup>3</sup> of tissue

<sup>b</sup>Number per *mm* vascular length

<sup>*c*</sup>Number per *mm*<sup>2</sup> of tissue

knowledge regarding (i) the multiscale and geometric properties of the microvasculature, (ii) topological characteristics and biologically important morphological information about the segments the microvascular structure consists of, (iii) the microstructural relationship with SMA<sup>+</sup> cells, which comprise myofibroblasts -key players in tissue healing after MI- as well as perivascular cells -relevant for vessel maturation and stabilisation-, and (iv) diffusion efficiency of the microvascular bed and adequate geometric models of the capillary supply region. We show that each class of parameters offers complementary information in understanding the effects of MI and that it is indispensable to study all classes in order to understand changes to a system as complex as the microvasculature.

Among the main findings is an intensive structural remodelling of the microvasculature at 7 days post MI, which persists and even deteriorates 45 days post MI, in combination with an abundant presence of myofibroblasts, a higher percentage of microvessels which lacked a SMA<sup>+</sup> coating, but with the coated ones having a thick SMA<sup>+</sup> layer. The latter might mark the end of the plasticity window for intervention [181]. By quantifying the effect of the structural remodelling in functional terms, we were able to identify a low capacity for blood flow (succolarity) and long diffusion distances which is linked with worse oxygenation. This might be related to the regression of vessels non-coated with SMA<sup>+</sup> resulting in a less complex and connected network that, as a result, requires longer diffusion times. Moreover, despite the decrease in the capacity of blood flow at the tissue level, observed also at organ level (Table 3.1) at infarcted areas 7 days post MI, the larger diameter of the individual microvessels and the SMA<sup>+</sup> hyperplasia, together with increased number of endothelial cells per length (Table 4.1), likely reflects adaptation to persistent/chronic high flow in these remaining vessels. Interestingly, in terms of structural characteristics (density, size of microvessels) the infarcted area at 3 days post MI, seems closer to the basal one than at 1 or 7 days post MI. This could indicate a normalization of the effect of infarction 3 days following MI after a first inflammatory response at day 1 and, therefore, it denotes a time window for therapeutic intervention around day 3 post MI when the microvasculature is not structurally so different from the basal but functionally it already starts the misadaptation as implied by the larger extravascular distances and higher heterogeneity in non-vascularized areas observed in infarcted areas 3 days post MI. Moreover, our results imply a connection between the appearance of constriction or dilation and the time which elapsed following MI. In particular, we observed a tendency for an initial dilation of the vessels in infarcted area at 1 day post MI, a subsequent constriction at 3 days, followed by possible pruning of microvessels, and maybe dilation/arterialization of the remaining microvessels at day 7. It is also worth noting that apart from the vasodilation effect the structural changes at infarcted areas at 1 day post MI are opposite to that of 7 days. Specifically, an increased number of microvessels, vascular density and complexity at infarcted areas at 1 day post MI were described. Thus, we cannot rule out angiogenesis (either sprouting or intussusceptive) at that time-point but it is clear that there is an overall loss of microvasculature afterwards, mostly of capillaries. In addition, as expected [40], changes at remote areas are not significant or

they are milder than the ones found in the infarcted areas, but they are not entirely absent as pathology progresses.

In conclusion, our fully automated image analysis pipeline permitted the accurate reconstruction of the 3D microvasculature and subsequent acquisition of novel, quantitative insights into its structure and changes at different stages after MI. The study was performed in the pig model, whose coronary microvasculature bears strong similarities to that of humans. Our method also provides an automated and unbiased means of evaluating therapeutic approaches and of diagnostic classification of unseen cardiac tissue. Furthermore, the wealth of quantitative data on the anatomy of microcirculation provided can serve as reference for comparisons for future studies in the field of cardiac microvascular research as well as enable modelling microcirculation at different stages of pathology. Lastly, the open-source nature of our approach makes it reusable in future studies of the cardiac microvascular tree and its changes are in focus.

## CHAPTER 5

## IMAGE-BASED MATHEMATICAL MODELLING OF THE INFARCTED HEART RESPONSE AT THE MICROVASCULAR LEVEL

## 5.1 Introduction

Despite the crucial role of blood flow in microcirculation, its assessment is hampered by today's imaging limitations. Although advances in imaging systems, in combination with computational image analysis approaches, have facilitated the reconstruction of detailed 3D microvascular networks [35, 101, 121, 78], *in vivo* measurement of blood flow or pressure in cardiac microvessels remains a challenging task [112]. Mathematical models of blood flow in microvascular networks have emerged as a complementary solution to experimental approaches, in an attempt to go beyond experimental observations. These models have the potential of offering unprecedented insight into microvascular blood flow and structure-function relation by exploiting structural data. Blood flow models have been further complemented by models that simulate oxygen transport [201, 80, 64], structural adaptation [172, 168] and blood flow regulation [197].

As described in Chapter 2, microvascular blood flow models can be roughly categorized into discrete and continuum flow models (CFMs). Models within the first category provide important information regarding distribution of hemodynamics. However, they require prescription of adequate boundary conditions, i.e. flow or pressure, at all boundary nodes of the network. This is why continuum perfusion models have emerged as complementary solutions. CFMs do not allow simulation of blood flow at individual microvessel level. They do, however, permit prediction of tissue-scale blood flow properties and they have, therefore, been gaining increased attention as an alternative to discrete models [229]. These types of models are based on Darcy's law. CFMs coupled with homogenization theory

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permit calculation of permeability tensor by solving the tissue scale fluid mechanics problem (Darcy's law) on spatially periodic networks.

We have adapted a CFM approach to assess the effect of myocardial infarction (MI) and its subsequent progression (1, 3 and 7 days post MI) on microvascular perfusion. Our aim is to unravel functional implications of microvascular structural changes which occur in response to MI by calculating permeability tensors. In this respect, we used anatomical data from thick slices of cardiac tissue extracted from the pig animal model. We subsequently introduced a mirroring approach that allowed us to deal with the spatial periodicity requirement of previous CFM in order to obtain a physiologically meaningful solution by using anatomical data and not statistically or ruled-based generated networks. Furthermore, the phase separation effect was incorporated into the calculation of the permeability tensors and its effect was quantified. We subsequently used the calculated permeability tensors and values from magnetic resonance imaging (MRI) data to estimate the expected pressure drop along an arteriole-venule path. Lastly, we calculated the myocardial blood flow (MBF) in two scenarios: (i) assuming the same pressure drop for all tissue conditions, and (ii) pressure drop values defined according to the condition of the tissue. In the first case, the pressure drop was adapted from literature values. In the second case, we used the mean value of the previously calculated pressure drop per tissue condition. The simulation results indicate the existence of alterations in the permeability tensors, in pressure drop and therefore in MBF related to the structural remodelling and altered angioarchitecture of the microvasculature after MI. We also investigated whether these changes persisted 45 days following MI.

Overall, the computational tools developed as part of this work and their application to a porcine myocardial infarction ischaemia-reperfusion (I/R) model allow a deeper understanding of microvascular alterations in MI by modelling of microcirculation at different stages after pathology. To the best of our knowledge, this is the first study where tissue scale blood flow properties have been predicted at different stages following MI and compared to tissue from basal conditions based on real 3D anatomical data with sub-micrometer resolution and on a continuum homogenization model. The permeability tensors extracted as a result of this work can additionally be used to parametrise organ scale models of the heart at different time-points after MI. Future work could include combining the continuum model with a discrete model in order to explore changes taking place in the distribution of hemodynamics, something that is not possible to measure on individual microvessels with today's imaging systems.

### 5.2 Methods

#### 5.2.1 Continuum blood flow modelling

Acquisition and processing of the 3D image data used in this work were described in detail in Chapters 3 and 4 respectively. In a nutshell, we used thick slices of cardiac tissue ( $\sim 100\mu$ m) that had been harvested from the left ventricle of pigs in basal conditions and of pigs that had been sacrificed 1, 3 and 7 days following MI. In the case of subjects that had suffered MI, tissues were obtained from infarcted and non-infarcted (remote) areas. These samples were subsequently stained for cell nuclei (Hoechst), endothelial junctions (anti-VE-Cadherin) and smooth muscle cells (anti- $\alpha$ -SMA) and were imaged by confocal microscopy.

The confocal images were subsequently segmented by using the multi-scale multithresholding (MMT) algorithm [79]. The filling approach from [78] was applied on top of the segmentations of the VE-Cadherin channel in order to accurately reconstruct the complete microvasculature from the endothelial junctions. Lastly, the graph-based representation of the microvasculature, along with the radii and length of the microvessels, were extracted from every connected sub-network that made up the network.

It should be noted that a sub-set of the complete dataset, which has been described in previous chapters, was used. The subset contains solely images of tissue samples that belong to the myocardium, excluding images of samples from the endocardium and the epicardium, as the orientation of vessels [126] in those areas would render simulations meaningless. Therefore, the subset used in this work consists of 12 3D images in basal conditions, 13 images of infarcted condition on days 1 and 3 post MI respectively, 15 images of infarcted conditions 7 days post MI, 16 images for remote condition on day 1 post MI, 11 images of remote areas 3 days post MI, and 9 images of remote areas 7 days post MI. The dataset was enriched with data from infarcted and remote areas 45 days post MI that had been acquired and analysed in a similar manner. It should be noted that the categorization of the volumes into the three regions of the heart was performed by two experts biologists post-acquisition and based on the orientation of the vessels on the images.

#### 5.2.1.1 Calculation of permeability tensors and perfusion in microvascular networks

Calculation of permeability tensors was performed by applying a continuous two-phase porous perfusion model that has been adapted from [56]. Well-separated micro- (microvas-cular network) and macro- (tissue) scales are assumed, as well as non-leaky vessels. At the micro-scale, it is additionally assumed that the tissue consists of spatially periodic micro-cell units that contain two phases; (i) the microvasculature, which permits the blood to flow and,

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(ii) the interstitium, which consists of cells and extravascular matrix and behaves as a porous medium. Furthermore, blood flow of the microvessels is considered incompressible and dominated by viscous forces (Stokes flow). Taking these assumptions into account and the fact that volume averaged tissue-scale blood velocity equals the surface mean blood velocity  $(u_s)$  [7], the homogenization technique of [205] is applied and the volume averaged form of Darcy's law is deduced to:

$$u_s = -\mathbf{K}(I)\nabla p \tag{5.1}$$

where  $\mathbf{K}(I)$  is the permeability tensor of the image volume *I* and given the 3D nature of *I* it is of size  $[3 \times 3]$ .  $\nabla P$  stands for the pressure gradient applied on the tissue, while  $u_s$  is the surface mean blood velocity and it is thus given by:

$$u_s = Q_s/S \tag{5.2}$$

where S is the area of the surface and  $Q_s$  represents the sum of the flow rates of the capillaries on that surface.

By combining equations 5.1 and 5.2, the elements of each row of the permeability tensor can be then calculated by applying a pressure gradient along one of the three possible directions, i.e. x-direction, y-direction or z-direction in Cartesian coordinates, and by calculating the sums of flow rates on the surfaces of the image volume in each case. The diagonal elements account for the link between the flow rate in one direction and the pressure gradient in the same direction, while the off-diagonal elements capture the link between the flow rate in other directions (cross flow) and the pressure gradient in the one direction. More precisely, each element of the permeability tensor with position (i, j) is calculated by:

$$K_{ij}(I) = \frac{\sum_{m=1}^{M_j} q_m^j}{S^j \nabla p^i}, \ i, j = 1, 2, 3$$
(5.3)

where  $\nabla p^i$  stands for the pressure gradient applied at direction *i*,  $M_j$  is the number of vessels on the outflow surface at the direction *j*, and  $S_j$  is the surface area of the outflow surface at direction *j*.  $q_m^j$  stands for the blood flow rate of vessel  $m = 1, ..., M_j$  at direction *j*. By assuming axial flow, the flux at each microvessel can be calculated according to the Poiseuille law by:

$$q_m = \frac{\pi r_m^4 \Delta P_m}{8\mu_m l_m} \tag{5.4}$$

where  $r_m$ ,  $l_m$ ,  $\mu m$  and  $\Delta P_m$  represent the length, radius, viscosity and pressure drop respectively of microvessel *m*. When constant hematocrit is assumed for all vascular segments,



Fig. 5.1 Mirroring of the original image colour-coded with light green (7). 2D slices along x,y and z directions of the 3D resulting mirrored image that contains 8 copies of the original image and periodic boundaries on opposites faces.

viscosity is calculated according to equations of [176, 177]. It is, however, a fact that due to the uneven splitting of red blood cells in branching points, the hematocrit is not uniform in all segments of microvascular networks. It is rather heterogeneous with higher values in daughter vessels with higher flow rates and lower ones in those with lower flow rates. This effect is known as phase separation effect or plasma skimming (Section 2.5). In order to take it into account, Pries and Secomb [166] have established equations that allow calculation of the varying hematocrit of each microvessel segment to be subsequently used for calculating segment viscosity, as described in Chapter 2.

Lastly, taking into account the relation between perfusion and the permeability tensors, and the relation between perfusion and MBF, the latter can be calculated using the permeability tensors by:

$$MBF(mL/min/100g) = \frac{k_{ii} \times \Delta p \times 0.133 \times 10^{-3} \times 60 \times 100}{\rho \times l^2}$$
(5.5)

where  $\Delta p$  stands for the pressure drop in *mmHg* over a microvascular path of length  $l \ \mu m$ ,  $\rho$  represents myocardial density ( $\rho$ ), while  $0.133 \times 10^{-3}$ , 60 and 100 are conversion factors for pressure units from *mmHg* to  $kg\mu m^{-1}s^{-2}$ , minutes to seconds, and 1g to 100g respectively.

#### 5.2.1.2 Dealing with real anatomical data: Mirroring & The complete pipeline

Completely connected networks are required in order to perform blood flow simulations. Therefore, when dealing with anatomical data the first issue that needs to be tackled is the presence of disconnections in microvascular networks due to sample preparation, imaging and/or reconstruction processes. In recent years, different algorithms have been proposed for correction of missing vascular connectivity [182, 195, 11]. These algorithms are adequate when dealing with data from healthy tissues and they have even been used in tumour models where usually the networks present high connectivity. Nonetheless, when dealing with the cardiac microvasculature post MI, safe assumptions cannot be made for recovering connectivity as the disconnections that are present might be pathology-related and they should, therefore, be preserved. For this reason, a different approach has been developed. This approach uses connected components analysis which is applied to the microvasculature to label the fully connected sub-networks that comprise it. Sub-networks that represent at least 15% of the complete vascular density are retained, while the rest are not considered in subsequent simulations. Permeability tensors could then be calculated separately for each connected sub-network. However, additional issues must be overcome prior to the calculation of the permeability tensors.

Firstly, anatomical data do not present spatial periodicity, as assumed by CFM. In an effort to tackle this limitation, previous works have either used synthetic networks that had been generated by the anatomical data based on statistics in a manner that assured periodicity [208, 56], or, alternatively, parametrisation methods were deployed instead [97]. This work presents a novel mirroring solution that permits direct use of continuum perfusion models on anatomical data without there being a need for the use of the aforementioned approaches. More precisely, the initial volume *I* of size [Nx, Ny, Nz] which contains a fully connected microvascular sub-network is firstly mirrored along x-direction. The resulting volume is then mirrored along y-direction and, lastly, mirroring of the already-mirrored volume, along the first two dimensions takes place along z-direction. This procedure results in an image volume of size  $[2 \times Nx, 2 \times Ny, 2 \times Nz]$  that contains 8 flipped duplicates of *I*. Nodes on the opposite face of *I* along all directions are thus periodic (Fig. 5.1).

Furthermore, blind-ends, i.e. branches connected to only one branch and do not touch volume borders, were eliminated from the microvascular networks. This was done because blind-ends were possible sprouts that would not contribute to the permeability tensors or were imaging/skeletonisation artefacts. It should be noted that, prior to this pruning step, end-branches located within a small distance from volume borders were elongated to reach the border and to thus avoid being pruned as blind-ends. This additional step was incorporated into the process to avoid discrepancies in imaging and skeletonisation procedures that lead


Fig. 5.2 Overview of the proposed pipeline for the calculation of the permeability tensors and MBF from microvascular 3D anatomical data.

to branches that do not touch image borders, but rather end within a few micrometers from them.

After the skeletons of the components have been mirrored and non-contributing blindends have been removed, the permeability tensors of each sub-network are calculated and they are subsequently fused to produce the permeability tensors of the volume. After examining different approaches of fusing the permeability tensors, we concluded that the weighted average of the components, corrected for overlapping areas among the components, would physiologically be the most accurate approach. More precisely, i.e.

$$k_{ij}(I) = \sum_{cc=1,..,m} w_{ij}(cc)k_{ij}(cc)$$
(5.6)

where  $k_{ij}(cc)$  stands for the permeability tensor element i, j = 1, ..., 3 of the sub-network cc and m is the number of all sub-networks that comprise the image volume I. The weights  $w_{ij}(cc)$  are defined by taking into account the surface of the sub-network and the complete surface of the image that contains sub-networks:

$$w_{ij}(cc) = \frac{S_j(cc)}{\sum_{cc=1,...,m} S_j(cc)} - \sum_{\substack{n, \ k=1,...,m \\ k>n}} S_j(n) \cap S_j(k)$$
(5.7)



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Fig. 5.3 Dependency of the permeability tensor on Representative Volume Element. Three different sizes were investigated:  $256 \times 256 \times Nz$ ,  $512 \times 512 \times Nz$ ,  $1024 \times 1024 \times Nz$  voxels, with Nz standing for the size of the image along z-axis. The original image volume was therefore decomposed into 16, 4, and one volume(s)/unit(s) respectively depending on the unit size used. Tensors for the sub-volumes under investigation were calculated by applying the proposed approach for the calculation of permeability tensors from anatomical data on each sub-volume. The asterisk size is proportional to the volume of the units normalized by a volume size of  $0.0074mm^3$  corresponding to  $1024 \times 1024 \times 50$  voxels of size [0.379, 0.379*m*, 1007]. The different asterisk sizes are due to the fact that the resulting connected network inside the unit might be smaller than the initial unit to which the image was decomposed. For each time point, the plots on the left show the maximum element of the permeability tensor in relation to volume size of the sub-networks of all images available in our dataset. On the right, there are plots for every image (one line per image) after fusing the tensors of the units in which it was decomposed. The permeability tensor here is given as the median of the different sub-volumes for simplicity reasons.

where  $S_j(cc)$  stands for the outflow surface of the sub-network *cc* along direction *j*. These weights will be scaled further using the resulting vascular volume of the fully connected sub-network (V(cc)) and the actual vascular volume in the same region ( $V_{init}(cc)$ ), as will be described in Section 5.3.1.

In the case of confocal data, volume orientation compared with heart orientation is lost. Therefore, in order to compare different images, the direction i = 1 is considered to be the direction of maximum flow, direction i = 2 is the direction with medium flow and i = 3 is the direction with the lowest flow among the three. To achieve this, elements  $K_{11}, K_{22}, K_{33}$  are firstly sorted in descending order. The rest of the elements of the tensor are then rearranged accordingly.

Figure 5.2 provides an overview of the complete pipeline.

### 5.3 Results

#### 5.3.1 Analysis of the proposed framework

Firstly, we studied the permeability tensors in relation to the size of the resulting subnetworks, after they had been adequately processed for subsequent simulations. This was done to identify sizes of volumes below which calculation of permeability tensors could lead to errors. Such a step is important because application of homogenization techniques is valid only when the length of the micro-cell unit is large enough to permit calculation of a converged permeability tensor that remains constant within the volume. In fact, the smallest size of spatially periodic units that results in the calculation of the converged/effective permeability tensor is known as the Representative Elementary Volume (REV) [13]. In [160], a hybrid model was developed and applied on cubic volumes of brain microvascular data. It was observed that errors increased with decreasing ratio of length of volume side per capillary length and stabilized at a ratio of about four, which corresponds to volumes of size  $200\mu m$ . In [207] increased permeability tensor values for micro-cell units with length smaller than  $200\mu m$  were also observed, while in [56] the REV was estimated at  $375\mu m$ .

To define this cut-off size, we considered the capillary bed of each volume as the mesoscale which is homogeneous inside smaller areas of itself. More precisely, the volume was divided into smaller microscopic units with a size of  $256 \times 256 \times Nz$ ,  $512 \times 512 \times Nz$ ,  $1024 \times 1024 \times Nz$  voxels, where Nz stands for the size of the original image *I* along z-axis (in voxels). We applied the developed framework at each unit to calculate permeability tensors for each one of the them. We concluded that the permeability tensor for volumes below 512 were frequently overestimated, with the larger element of the permeability tensor being an order higher than values reported in literature [207] (Fig. 5.3). Therefore, in our subsequent analysis, we excluded connected components whose size was smaller than this cut-off limit. This means that the permeability tensor of a 3*D* image was calculated as the weighted sum of the connected components whose side was larger than 170  $\mu m$  prior to application of the mirroring procedure.

We then investigated different forms of fusing the permeability tensors of the subnetworks which comprise the microvasculature of the image volume being studied to produce the equivalent permeability tensor of the image. The fusing methods can be adapted from developments for heterogeneous media. More precisely, although theoretically, homogenization theory assumes a homogeneous medium, this is rarely the case with real life objects. In fact, the medium can be heterogeneous and consist of several phases. This is why different methods have been developed to scale-up from the permeability tensors based on the tensors of individual phases [192]. Such approaches include the harmonic average for serial flow,

the weighted average for parallel flow, geometric average for random flow among the most popular. Here, we have adapted a weighted average because the flow inside a volume is considered to be the sum of the flow of the sub-networks present in the image volume and independent from each other, while the contribution of each sub-network depends on the surface area it covers over the surface occupied by all sub-networks. An alternative approach that provided similar solutions was the use of median flow. This is expected when taking into account the similarity of sub-networks within our relatively small volumes.

We observed components, where deletions of the blind-ends resulted in extremely oversimplified versions of the network compared to the original, with the final one representing only 15% of the original vascular network within the volume occupied by the sub-network. We therefore performed correlation and regression analysis and discovered a statistically significant relation of the permeability tensor to the ratio of sub-network vascular volume (V(cc)) to the vascular volume in the corresponding volume of the image  $(V_{init}(cc))$  prior to any post-processing having taken place. More precisely, the radii and length of all vascular segments, prior and after repetitive elimination of the blind-ends until the microvascular network under investigation is fully connected and has no blind-ends, had already been calculated. Therefore, we used them to calculate the two volumes, i.e. V(cc) and  $V_{init}(cc)$ , by considering each microvessel as a tube of constant radius. Using the basal condition, we plotted the permeability tensor as a function of the ratio of volume of the sub-network prior and after the elimination of the blind ends, and we calculated the Kendall's Tau coefficient which was equal to 0.47 (p-value = 0.008) and 0.66 (p-value =  $8 \times 10^{-5}$ ) for  $k_{11}$  and  $k_{22}$ respectively. We subsequently performed regression analysis and found that we could fit a model, for instance a linear one  $(y \sim \beta_0 + \beta_1 x, \text{ with } \beta_0 = -8.3 \times 10^{-4} \text{ (p-value} = 0.13),$  $\beta_1 = 5.95 \times 10^{-5}$  (p-value= $3.59 \times 10^{-9}$ )) could describe the dependency of the permeability tensor on the ratio. We finally decided to apply a scaling factor equal to the reverse of the ratio  $\left(\frac{V(cc)}{V_{init}(cc)}\right)$  to the permeability tensors in order to adjust them on the basis of this dependency. The scaling factor was incorporated by multiplying the weights  $w_{ij}(cc)$  by it. Following application of the scaling factor, permeability could no longer be predicted by  $\frac{V(cc)}{V_{init}(cc)} (y \sim \beta_0, \beta_0 = 4 \times 10^3 \text{ (p-value} = 9.49 \times 10^{-28})).$ 

Furthermore, we validated that our mirroring approach did not affect calculation of the permeability tensors. We performed a second mirroring of the mirrored volumes to achieve that and tensors for the two cases were compared. This resulted in volumes of size  $[4 \times N_x, 4 \times N_y, 4 \times N_z]$ , where  $[N_x, N_y, N_z]$  are the dimensions of the original columns and  $[2 \times N_x, 2 \times N_y, 2 \times N_z]$  of the mirrored volumes. The results indicate that the permeability tensors are almost identical in both cases, indicating the appropriateness of the approach in dealing with the lack of periodicity in anatomical data and in also paving the way to

	I1MI	I3MI	I7MI	I45MI	R1MI	R3MI	R7MI	R45MI	Basal
k11	$0.0052 \pm 0.003$	0.0033±0.0024	0.0043±0.0019	$0.0098 \pm 0.0068$	$0.003 \pm 0.0025$	$0.0022 \pm 0.001$	$0.0032 \pm 0.0022$	$0.0061 \pm 0.0023$	$0.0035 \pm 0.0032$
$(mm^3 \ s \ kg^{-1})$									
k22	$0.0026 \pm 0.0013$	0.0013±0.0021	$0.0019 \pm 0.0014$	$0.0039 \pm 0.0033$	$0.0011 \pm 0.0007$	$0.0009 \pm 0.0004$	$0.0016 \pm 0.0014$	$0.0024 \pm 0.0013$	$0.001 \pm 0.0008$
$(mm^3 \ s \ kg^{-1})$									
k33	$0.0013 \pm 0.0012$	$0.0006 \pm 0.0011$	$0.0007 \pm 0.0008$	$0.0014 \pm 0.0016$	$0.0005 \pm 0.0004$	$0.0004 \pm 0.0003$	$0.0007 \pm 0.0008$	$0.0013 {\pm} 0.0008$	$0.0006 \pm 0.0005$
$(mm^3 \ s \ kg^{-1})$									
MBF <sub>K11</sub>	295.72±167.16	186.66±133.4	242.59±107.91	550.63±383.62	169.19±139.06	121.7±56.24	182.31±124.75	341.27±130.64	197.66±179.11
(ml/min/100g)									
MBF <sub>K22</sub>	149.3±74.15	71.42±117.29	106.86±78.7	219.27±188.57	63.78±39.05	48.16±21.97	87.65±77.65	133.04±74.75	57.07±45.98
(ml/min/100g)									
MBF <sub>K33</sub>	73.06±66.09	31.17±59.56	39.4±45.2	78.38±91.84	27.28±20.63	19.81±15.6	38.69±43.52	72.4±47.7	34.54±28.93

23.18±12.86

21.31±13.36

20.41±11.78

9.88±4.27

26.31±25.9

2.85±1.84

(ml/min/100g)

DP (mmHg) 16.94±10.81

7.16±4.13

5.1±2.83

Table 5.1 Mean and standard deviation of diagonal elements of permeability tensors, pressure drop and MBF according to  $k_{11}$ ,  $k_{22}$  or  $k_{33}$ 

blood flow simulations by using continuum Darcy flow models in the case of such datasets. Moreover, it should be noted that, theoretically, the direction of mirroring (left/right) does not have an impact on the results. Only slight differences might be observed due to boundary segments that are connected to the same node.

# 5.3.2 Altered permeability tensors, pressure drop and myocardial blood flow after myocardial infarction

Following formulation of a complete framework for image-based microvascular perfusion modelling, we applied the proposed approach to the porcine cardiac volumes described previously. Images for which the permeability tensor was not computed resulted in additional images being excluded from subsequent analysis, for instance images where no component represented more than 15% of the image vascular density or images for which the resulting size of all of its sub-networks were smaller than 170  $\mu m$ . Therefore, the final number of images comprising our dataset was 12 in basal conditions, 11, 12, 9 and 10 for infarcted areas 1, 3, 7 and 45 days post MI respectively. 13, 7, 8 and 12 images were included from remote areas 1, 3, 7 and 45 days post MI respectively.

Mean and standard deviation of the main elements of the permeability tensor for each tissue condition are provided in Table 5.1. For simplicity reasons, and similarly to the previous chapter, images corresponding to tissue from infarcted and remote areas, 1, 3, 7 and 45 days post MI respectively were abbreviated as I1MI, R1MI, I3MI, R3MI, I7MI, R7MI and R45MI on tables of the current Chapter. In basal conditions, the permeability tensor element along the direction of maximum flow is  $(3.5 \pm 3.2) \times 10^{-3} mm^3 s kg^{-1}$ . This indicates that permeability tensors estimated by means of our proposed framework is within physiological ranges according to previous work, and, in particular, is of the same order to those estimated for the rat myocardium, where  $k_{11}$  was found equal to  $(3.3 \pm 0.8) \times 10^{-3} mm^3 s kg^{-1}$  [207].



Fig. 5.4 Permeability tensor elements after sorting directions by larger permeability. \*, \*\* and \*\*\* represent p-value< 0.05, 0.01 and 0.001 respectively. Calculations have been performed considering constant hematocrit of 0.4 in all vessel segments. The p-values were calculated by means of Wilcoxon rank-sum tests and corrected by the Benjamini—Hochberg procedure for multiple testing. The same annotations apply to all figures of the chapter.

Smaller values have been, however, calculated for the brain  $(4.28 \times 10^{-4})$  [56]. Permeability tensor elements along the direction of medium  $(k_{22})$  and lower  $(k_{33})$  flow direction are between less than half of  $k_{11}$  and an order smaller, therefore, confirming that there is a preference of flow along a main direction. This is in accordance with previous findings regarding the myocardium of rats where permeability in the same direction as longitudinal capillaries was estimated 10.8 larger that the direction of cross-connecting capillaries [207]. It should also be noted that variability among the volumes is particularly high. However, this is expected taking into account the heterogeneity of the microvasculature and therefore microvascular flow between different regions of the heart [41]. The off-diagonal elements of the permeability tensor are practically zero, therefore, confirming that cross flow is minimal.

It is worth noting that the direction of maximum flow was the direction along the z-axis only on one volume. This is expected since orientation of capillaries in the myocardium is longitudinal and not transversal. Moreover, even if orientation was lost during tissue harvesting, effort was made during acquisition to acquire images of areas where orientation of vessels was longitudinal and not transversal. This way we ensured a larger field of view of the vessels and their cross-connections, which would otherwise be more limited given that the maximum depth of our volumes is about  $100\mu m$ , while it is  $387.50\mu m$  along x-y the length of our volumes.

Moreover, with a view to quantifying alterations, statistical change analysis was performed in the same manner as in the case of the image-based features described in the previous chapter (Section 4.2.6), i.e. by means of Wilcoxon rank sum tests (Fig. 5.4). The permeability tensor elements along all directions at infarcted areas 1 day post MI have increased compared to the basal condition and also to the corresponding remote area. In case of  $k_{22}$  element the differences become statistically significant. These findings in conjunction with the increased



Fig. 5.5 Altered pressure drop after myocardial infarction. Pressure drop was calculated using the larger element of the permeability tensor and different arteriole-venule path length. Pressure drop for a path length  $349\mu m$  (**a**),  $512\mu m$  (**b**) and  $675\mu m$  (**c**).

vascular density and complexity produced during the early vascular response to MI described in the previous chapter, point to a possible reaction of the network to the ischaemic event and subsequent reperfusion. Permeability at infarcted areas on day 7 seems to increase to levels close to the one of the basal case. Taking into account vessel dilation described in the previous chapter, we conclude that this is possibly a system mechanism to compensate lack of adequate oxygen by increasing flow. However, since the distribution of the network does not permit adequate oxygen diffusion as indicated by the large extravascular distances (Fig. 4.9), it appears that increased flow cannot compensate for proper heart function. An adequate angioarchitecture is necessary instead as described in [174]. It should be noted that when analysing these results, we have to take into account the high dependence of the permeability tensors on vessel diameters (fourth power). Remote areas seem closer to the basal and infarcted 3 days post MI. A single value regarding MBF at the organ level cannot express microvascular blood flow variability. Nonetheless, we can use such measurements -for instance, measurements provided by MRI systems- to estimate the expected pressure drop along arteriolar-venular paths whose flow properties are encapsulated by permeability tensors. Therefore, we used MRI-based data regarding MBF (Table 3.1) of each subject and we calculated the pressure drop (equation 5.5) for different arteriolar-venular pathways, each described by the permeability tensor of an image volume of the corresponding subject of our dataset. In an earlier work on the pig animal model [104], the path length was found to be equal to  $512 \pm 163 \mu m$  when defined as a linear distance between the centre of mass of the arteriolar mass and the venular domains. Since deviation from the mean was large and the path length has an important effect on pressure drop, we estimated the pressure drop for three possible lengths: (i)  $349\mu m$  (mean-standard deviation), (ii)  $512\mu m$  (mean) and (iii)  $675\mu m$  (mean+standard deviation) (Fig. 5.6). Moreover, due to lack of MRI data for one of our subjects regarding day 3 post MI, we have used data from the other subject at the same



Image-based mathematical modelling of the infarcted heart response at the microvascular level

Fig. 5.6 Myocardial blood flow calculated using sorted  $k_{11}$ ,  $k_{22}$  or  $k_{33}$ . (a) MBF considering constant pressure drop of 19.5*mmHg* and a path length of 512 $\mu$ *m* independently of the tissue condition. (b) MBF considering varying pressure drop according on the tissue condition and a path length of 512 $\mu$ *m*. The pressure drop for these simulations are the mean values presented in Table 5.1. In both cases, calculations have been performed considering constant hematocrit of 0.4 in all vessel segments.

time-point. It should be noted that, if the path length during pathological conditions changes, then the resulting pressure drops would have been different. However, due to lack of such data, we assume that path length between arteriolar and venular domain of a network remains unchanged.

Estimated pressure drops in remote areas were close to the ones in basal areas. On the contrary, pressure drops in infarcted areas 1 and 7 days post MI infarction were very low and significantly different than those at basal areas. This indicates non physiological values on those conditions. It should be noted that pressure drop on day 3 post MI was closer to the estimated values for volumes in basal conditions. This indicates a possible normalization effect at this specific time-point, as demonstrated in the previous chapter by means of other biomarkers. When grouping values by subject, pressure drops in basal conditions for the smallest ones was very close to expected values for pressure drops in healthy conditions, i.e. 5-20*mmHg* according to [163]. In addition, high variability was observed in the estimated values, something that was expected taking into account the variability of the permeability tensor elements among different volumes.

We subsequently calculated the MBF using the main elements of the permeability tensors. Initially, we assumed the same pressure drop (19.5mmHg) under all tissue conditions. We noticed high MBF on day 1 compared to MBF in basal conditions or in the corresponding remote ( $k_{22}$ ). A similar increasing trend might be observed day 7 in infarcted areas. Although the difference is not significant, the increasing trends indicates that if we could control the pressure drop between the arteriolar-venular path and retain the pressure drop on day 7 to levels as high as that prior to ischeamia then MBF could return to levels close to basal. In an effort to incorporate varying pressure drop per tissue condition, we also calculated MBF by using the mean pressure drop estimated previously for each time-point. In this case, contrary to when using constant pressure drop, infarcted areas 7 days post MI have statistically significant lower MBF as calculated using  $k_{11}$ . The trend is similar when using  $k_{11}$  and  $k_{33}$  although it is not significant.

Lastly, we analysed permeability tensors for volumes 45 days post MI. Permeability tensors calculated 45 days post MI were even higher than the ones calculated for volumes 7 days post MI. The results are presented in Table 5.1. Structurally, 45 days post MI, the volumes of infarcted areas are more similar to those 7 days post MI compared to the ones in basal conditions or in earlier times after MI. Nonetheless, microvascular networks have been further remodelled and have even larger diameters and a further decreased number of small segments (capillary rarefaction) compared to basal microvascular networks (see section 4.3.5). Moreover, remodelling of the remote areas 45 days post MI is also noticeable as expressed by statistically significant differences in several features compared to the ones from basal and other remote areas (Table 4.3). Networks are more disconnected, as demonstrated by the elevated Euler characteristic number, compared with basal conditions, corresponding to the one for the remote and infarcted areas at earlier times. This resulted in small subnetworks being used for calculation of permeability. Therefore, given the strong dependency of permeability tensors on diameter, along with the smaller size of volumes 45 days post MI, due to disconnections, it is possible that permeability tensors might be overestimated for 45 days.

For comparison purposes, the aforementioned blood flow properties when calculated without the application of the scaling factor to account for the simplification of the network after deletion of blind-ends are provided in Table 5.2.

#### **5.3.3** Effect of phase separation phenomenon on permeability tensors

We then sought to incorporate the phase separation effect into the calculation of the permeability tensors in an effort to define the effect on its calculation. For this reason, we

Table 5.2 Mean and standard deviation of diagonal elements of permeability tensors, pressure drop and MBF according to  $k_{11}$ ,  $k_{22}$  or  $k_{33}$  when the scaling factor to account for the oversimplification of the network is not applied.

	I1MI	I3MI	I7MI	I45MI	R1MI	R3MI	R7MI	R45MI	Basal
k <sub>11</sub>	$0.0037 \pm 0.0023$	$0.0018 \pm 0.0012$	$0.0022 \pm 0.0009$	$0.0038 \pm 0.002$	$0.0017 {\pm} 0.0021$	$0.001 \pm 0.0006$	0.0021±0.0017	$0.004 \pm 0.0018$	$0.0028 \pm 0.0027$
$(mm^3 \ s \ kg^{-1})$									
k <sub>22</sub>	$0.0019 \pm 0.0011$	$0.0006 \pm 0.0009$	$0.0009 \pm 0.0006$	$0.0017 \pm 0.0014$	$0.0006 \pm 0.0004$	$0.0004 \pm 0.0002$	$0.001 \pm 0.001$	$0.0016 \pm 0.0011$	$0.0008 \pm 0.0007$
$(mm^3 \ s \ kg^{-1})$									
k <sub>33</sub>	$0.0009 \pm 0.0009$	$0.0002 \pm 0.0004$	$0.0004 \pm 0.0004$	$0.0006 \pm 0.0007$	$0.0003 \pm 0.0003$	$0.0002 \pm 0.0002$	$0.0005 \pm 0.0006$	$0.0008 \pm 0.0006$	$0.0005 \pm 0.0004$
$(mm^3 \ s \ kg^{-1})$									
MBF <sub>K11</sub>	210.04±129.1	101.54±69.18	121.55±50.69	211.79±112.97	93.38±116.72	57.41±34.83	116.12±94.79	225.27±102.69	159.5±152.62
(ml/min/100g)									
MBF <sub>K22</sub>	107.25±61.7	35.68±48.67	53.46±34.88	95.48±81.25	34.33±25.08	23.07±13.29	57.34±57.52	89.47±59.42	44.1±41.68
(ml/min/100g)									
MBF <sub>K33</sub>	50.52±49.15	13.77±19.89	21.41±21.93	34.02±37.01	15.11±14.61	$10.35 \pm 8.84$	26.06±32.72	47.71±32.96	26.75±24.74
(ml/min/100g)									
DP	12.09±11.06	31.18±19.23	10.35±6.06	6.77±4.27	54.05±30.46	50.2±34.12	39.43±31.56	16.24±9.12	44.99±52.12
(mmHg)									

incorporated an iterative procedure in the calculation of the tensors for each sub-network as show in Fig. 5.7.

In brief, a constant hematocrit (0.4) was initially assumed and used along with the morphological data of the network after post-processing with our framework had taken place in order to calculate the permeability tensor along the x-direction. The pressure solution that resulted in the calculation of the permeability tensor elements  $k_{11}$ ,  $k_{12}$ ,  $k_{13}$  was used as an input to an iterative publicly available algorithm (https://physiology.arizona.edu/ people/secomb/netflow) for the calculation of varying hematocrit when separation effect on junction points is taken into account [171]. Once the solution converges or reaches the maximum number of permitted iterations (100), the permeability tensors are calculated with the new varying values of the hematocrit. Once the new permeability tensors differ less than  $2 \times 10^{-17} mm^3 s kg^{-1}$  in all elements from the last iteration, the procedure is considered as converged and the calculated tensor as the final one. Due, to convergence not reached for some volumes, we additionally set a limit of 300 iterations, that would be reached for few volumes.

Simulations under basal conditions point to a change in the calculated sub-networks tensors when phase separation is incorporated. When comparing the permeability tensors after their fusing for each volume, the percentage change of the diagonal elements of the permeability tensor  $k_{11}$ ,  $k_{22}$  and  $k_{33}$  was  $-23.51 \pm 49.22\%$ ,  $-24.89 \pm 44.71\%$  and  $-40.77 \pm 20.59\%$ . This is in accordance with earlier studies that have found an effect of the varying hematocrit incorporation on blood flow rates of 20% [214]. Therefore, it demonstrates the importance of incorporating non-Newtonian properties in the calculation of permeability tensors.



Fig. 5.7 Overview of the approach for incorporating the phase separation effect in the calculation of permeability tensors.

# 5.3.4 Unravelling the effect of vascular remodelling on permeability tensors

Earlier studies on statistically generated and synthetic networks have confirmed that changes in diameter have a severe effect on the permeability tensors as theoretically expected if one takes into account the dependency of tensors on fourth power of diameters. In this work, we have also observed the effect of structural changes on the microvasculature after MI, in terms of permeability tensors. However after MI, as described in the previous chapter, several forms of vascular remodelling take place simultaneously: changes in vessel diameters, pruning of microvessels, alterations in the connectivity of microvessels among others. Therefore, in an attempt to isolate the effect of dilation or constriction of vessel diameters as well as pruning of immature vessels, the following case scenarios were studied:

- i. dilation of all microvessels by incrementing their diameter by 10%, 20%, 30%,
- ii. dilation of 10%, 20% and 30% of the microvessels by incrementing their diameter by 30%,
- iii. constriction of all microvessels by decreasing their diameter by 10%, 20%, 30%,
- iv. constriction of 10%, 20% and 30% of the microvessels by decreasing their diameter by 20%,

v. pruning of 10%, 20% and 30% of the microvessels with the smallest radii

For our study, we used volumes under basal conditions and applied the aforementioned remodelling scenarios to the major sub-network of the image. We subsequently calculated the permeability tensor for the sub-network for the different remodelling scenarios and compared the result with the tensor obtained without applying remodelling. Dilation of all vessels of the network by only 10% produced a major increase to the elements of the permeability tensor. More precisely, an increase of  $99.64 \pm 117.77\%$ ,  $159.24 \pm 384.30\%$ ,  $73.10 \pm 43.29\%$  was observed for  $k_{11}$ ,  $k_{22}$  and  $k_{33}$  respectively. An additional increase of the diameters by 10% the  $k_{11}$ ,  $k_{22}$  and  $k_{33}$  increase by  $520.41 \pm 441.2$ ,  $704.54 \pm 1300\%$  and  $363.46 \pm 129\%$  respectively, with the tensors of few images changing order of magnitude and other being close. When the percentage reached 30% the increase was so high that the tensors of all images changed order of magnitude. These results demonstrate that vessel dilation has a major impact on the permeability tensors. When the change is applied to a percentage of vessels, the permeability tensor always increases, but does not arrive 10-fold increase as it does when all vessels are enlarged.

Constriction of diameters of all microvessels by 10%, resulted in a decrease of the diagonal elements ( $k_{11}$ ,  $k_{22}$ ,  $k_{33}$ ) by a mean percentage of 24.97%, 1.35% and 26.20% respectively. Further constriction of the vessels by an additional 10% led to a decrease of the elements by 67.34%, 50.40% and 67.10% respectively. When constriction reached 30%, the decrease of the permeability tensor values was almost 100% for all three elements ( $k_{11}$ : 95.78 ± 1.66%,  $k_{22}$ : 94.12 ± 8.88%,  $k_{33}$ : 95.63 ± 1.47%). When 20% constriction is applied to a varying percentage of vessels randomly chosen, the direction of the change expressed by the mean remains the same, i.e. the tensor is decreasing. However, interestingly, there are volumes where the tensor has increased. This indicates that there are cases where constriction in certain vessels could cause an increase in blood flow.

In the case of pruning of a percentage of the smallest vessels of the microvasculature, the results were highly variable and the direction of the change of the permeability tensors was either positive or negative depending on the angioarchitecture of the network. This implies that by pruning either effect, i.e. increase or decrease of the tensors depending on the location and the pruned vessel, could be attained. Nonetheless, the mean values of percentage of change were positive. For instance, pruning of 10% of the microvessels resulted in increase for  $k_{11}$ ,  $k_{22}$ ,  $k_{33}$  equal to  $1.40 \pm 101.63\%$ ,  $144.59 \pm 348.61\%$ , and  $44.2200 \pm 100.56\%$  respectively. Pruning of 20% of the microvessels produced a  $106.77 \pm 286.96\%$ ,  $239.88 \pm 577.49\%$ , and  $56.2600 \pm 106.24\%$  increase to the diagonal elements. Lastly, when the 30% of the smallest microvessels were pruned, the tensor diagonal elements increased by  $517.65 \pm 990.66\%$   $240.04 \pm 467.50\%$ ,  $48.99 \pm 161.30\%$ .

Fig. 5.8 provides an example image following remodelling on the basis of the aforementioned scenarios and the resulting diagonal elements of the permeability tensor.  $k_{11}$ ,  $k_{22}$  and  $k_{33}$  before any remodelling taking place were  $3.7 \times 10^{-3}$ ,  $1.59 \times 10^{-4}$ ,  $1.55 \times 10^{-4} mm^3 s kg^{-1}$  respectively.

### 5.4 Discussion

Advancements in imaging technologies, combined with automated image analysis approaches, are nowadays making acquisition of detailed structural data of microvascular networks feasible. Therefore, the development of frameworks that permit a move from simulations on idealized networks to image-based simulation of microvascular perfusion is of paramount importance, if we are to take full advantage of these novel tools to advance our knowledge regarding microvascular blood flow and how this relates to whole organ measurements. In an effort to bridge this gap, we present the first study of microvascular perfusion based on performing simulations using 3D detailed anatomical data of the cardiac capillary bed in healthy conditions and spanning several stages after MI. The data span both remote and infarcted areas.

In this work, we adopted a CFM to perform perfusion simulations. The potential and strengths of such models have already been demonstrated and discussed in innovative studies of coronary [180, 207, 96] and brain microvasculature [56, 57, 160]. As previously noted, these models do not permit simulation of blood flow at the individual vessel level. Their advantage lies in that the final solution is independent of boundary conditions and their output can contribute to enhancing our understanding of structural-functional relation as well as of measurements at organ level. According to Darcy's law, the permeability tensor can be defined by dividing fluid velocity with pressure gradient. When homogenization techniques are adopted, the permeability tensor is calculated by solving Darcy's law on spatially periodic assumed sub-units. In this work, we have adopted this approach for every sub-network forming part of the microvasculature captured in a confocal image z-stack. The tensor of the image under study is approximated by properly fusing the tensors of the sub-networks that comprise it.

The developed framework allowed us to simulate blood flow at different time points. We discovered increased values for all diagonal elements of the permeability tensor 1 day post MI. This increase is related to the altered angioarchitecture of the network and particularly, to the network diameters. More precisely, the highly complex and slightly dilated microvasculature of infarcted areas on day 1 post MI is accompanied by increased permeability. On the contrary, constricted vessels on day 3 post MI resulted in reduced permeability tensor values,

while dilated vessels on day 7 show a tendency for increased permeability tensor values. The latter might be a possible mechanism to compensate for tissue damage.

Estimated MBF values imply that capillary rarefaction and the intense vascular remodelling occurring in infarcted areas 7 days post MI do not limit the capacity for blood flow if the pressure drop is kept at the same level as that prior to MI. Nonetheless, non reduced blood flow 7 days post MI contradicts our findings by means of succolarity. Succolarity, as introduced in the previous chapter, encapsulates fluid capacity to flow within a network and was found to be reduced in infarcted areas 7 days post MI. This unexpected difference could be related to the fact that, during calculation of succolarity, blood rheological properties and the strong dependence of blood flow on diameter are not taken into account. However, it is also possible that it is related to the fact that the pressure drop used to calculate MBF was not proper. This is also implied by the fact that when using pressure drop adjusted to the time point and area post MI, we found that MBF 7 days post MI would decrease compared with basal areas or the corresponding remote. Unfortunately, lack of topological information and measurements at voxel-level in our MRI data did not permit a direct validation of the approach and subsequent necessary adjustments. However, literature values regarding MBF, although variable, show that the estimated MBF values by means of our approach (Table 5.1) are generally close to reported values. Moreover, earlier studies on MBF confirm that a high variability and different trends post MI should be anticipated depending on the transmural layer, i.e. myocardium to epicardium, the anterior or posterior position as well as the level from base to apex of the heart [41, 53].

More precisely, using micro-spheres the MBF was estimated in pigs in a recent study [196]. Mean and standard deviation for MBF was found to be  $122 \pm 92$  and  $99 \pm 31$  ml/min/100g using 1.5 Tesla ad 3 Tesla MRI respectively during rest. The corresponding values during hyperaemia were  $221 \pm 167$  and  $208 \pm 0.81$ , while during reduced flow (half-flow) were  $0.56 \pm 0.37$  and  $0.55 \pm 0.26$ . It is worth noting that similar values could be calculated with contrast-enhanced cardiac MRI. In [29], data from photon emission tomography (PET) studies on healthy humans were summarized with the highest reported MBF values being  $120 \pm 30$  at baseline and  $440 \pm 90$  ml/min/100g during hyperaemia. In an effort to reduce variability in MBF reported in literature, in [36] a large cohort of human volunteers was used. Mean and standard deviation for baseline MBF corrected for workload was calculated equal to  $133 \pm 31.6$  ml/min/100g, with values ranging from 73.6 to 242.8. Using the dog animal model, a study with microspheres injected at 15 second, 15 minutes, 4 hours and 3 days post 2-h coronary occlusion and reperfusion as well as control subjects demonstrated that immediately after reperfusion the MBF was increased and even higher than in non-infarcted subjects, but 3 days post MI the flow reduced [41]. Moreover, there

was a high variability on the estimated values depending on the region from the epicardium towards myocardium. In the same animal model, a study of 5-minutes repetitive occlusion and subsequent reperfusion found that 1 week post MI the MBF returned to normal levels in both infarcted and remote areas [45].

There are some limitations to this work that could be addressed by future research. Firstly, due to the challenging nature of the task of simulating perfusion with anatomical data and the particular nature of our relatively small volumes, several simplifications were necessary to be made in the post-processing of the volumes to convert the initial microvascular data into fully connected networks without blind-ends. Another important limitation of the present study is that the opportunities for validation are limited. It would be of particular interest to study the relation of the presented results with measurements from MRI. However, to achieve this, information regarding volume topology would be required along with voxel level measurements from MRI. Also, the voxel size of a typical MRI is of the order of millimetre or sub-millimetre, which means that resolution would not be enough to resolve volumes of  $300\mu m$  length. Nonetheless, it might be possible for future studies to apply the developed framework to obtain insights to the relation between blood flow at micro-scale and macroscale, by uniting several sequential volumes. This would be of paramount importance in clinics as it could lead to translation of measurements at organ scale to conclusions regarding alterations of the microvasculature, a major determinant of the outcome after successful reperfusion of the epicardial arteries, as detailed in previous chapters. In addition, we used the same pressure drop under all conditions for MBF estimation. However, it is possible that this assumption does not reflect reality, as indicated by the estimated pressure drop per tissue condition (Fig. 5.6) calculated using the permeability tensors and the MRI-based data that we had available for our subjects. In an effort to address this shortcoming, we have also performed simulations using the mean pressure drop that was previously estimated. However, this is not an ideal approach because pressure drop calculation had already been based on the permeability tensor. Lastly, high variability in the estimated values and the small number of samples after exclusions of several images of our dataset reduce the power of statistics and possible statistical significant differences might not have been detected.

Overall, this work marks the first effort to simulate tissue-scale properties of blood flow using anatomical data directly and not idealized or statistically generated data. We used data from basal conditions and from different time-points after MI, and both from infarcted and remote areas from a highly translational animal model. The permeability tensors calculated for the basal condition are within the physiological limits according to previous work. Concomitantly, the estimated pressure drop using MBF obtained from MRI points to an altered function of the microvasculature on day 1 and 7 days post MI. In addition,

according to data 45 days does not improve without the application of therapy. Despite the presence of increased blood flow, the remodelling compensatory mechanisms are not adequate enough to allow efficient oxygenation. It should be noted that permeability values presented here can be used in future studies to parametrise multi-scale models of coronary blood perfusion, where it is not feasible to simulate flow in the complete capillary bed or to achieve image resolution that permits visualization of both capillaries and large epicardial vessels.



Fig. 5.8 Example image with modified radii and number of segments used in the study of the effect of vessel dilation, constriction and pruning on the permeability tensors. (a) From left to right, increase by 10%, 20%, 30% of vessel diameter of all microvessels that comprise the microvasculature. (b) 30% increase of vessel diameter applied to the 10%, 20% and 30% of vessels. (c) Decrease of vessel diameter of all microvessels or, (d) a percentage of them: 10%, 10% and 30% (from left to right). (e) Pruning of 10%, 20% and 30% of the capillaries with the smallest radius. Below each image, the diagonal permeability tensor elements are provided. In parenthesis, the percentages of decrease (negative sign) or increase (positive sign) in respect with the original values. i.e. without any remodelling. Image corresponds to tissue from basal conditions. Some example cases of remodelled vessels (b, (d)) or areas of pruned vessels (e) are highlighted inside yellow circles.

## **CHAPTER 6**

### CONCLUSIONS

### 6.1 Contributions

This PhD thesis offers contributions both in terms of the development of innovative computational approaches for the analysis and modelling of microvascular data as well as in terms of novel biological findings. In terms of development of computational approaches, the main contributions of this thesis are:

- A novel 3D multi-scale multi-level thresholding (MMT) approach for segmenting labelled structures from confocal images.
- A novel algorithm for the reconstruction of the complete microvasculature by using information regarding endothelial junctions and smooth muscle actin+ cells.
- A simple, yet efficient, approach to distinguish between capillaries and arterioles/venules.
- An innovative open-source bioimage analysis pipeline for the in-depth study of microvascular networks after myocardial infarction (MI) that is scalable to other diseases and tissue types.
- A scheme that allows recognition of microvascular tissue condition to serve as a powerful tool for the diagnosis of tissue condition in biopsies, as well as in the evaluation of the outcome of potential therapeutic approaches.
- A framework for modelling blood flow in microcirculation based on anatomical 3D imaging data.

In terms of biological significance, the main contributions of this thesis are:

• Enrichment of the database of knowledge about microcirculation physiology with quantitative data spanning different stages after MI.

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- Quantification of the changes occurring in the cardiac microvasculature in an unbiased and reproducible manner respecting its inherently 3D and complex structure:
  - The cardiac microvasculature progressively loses its integrity and complexity in infarcted areas.
  - Intensive structural remodelling of microvessels 7 days post MI, in combination with thicker smooth muscle cell layer might mark the end of the plasticity window for therapeutic intervention.
  - As expected, structural remodelling of the infarcted area 7 days post MI leads to a deterioration in microcirculation and also adversely affects efficiency in oxygen supply.
  - Infarcted area 3 days post MI appears closer to the basal compared to 1 or 7 days post MI. This finding indicates a normalization of the effect of infarction on day 3 post MI.
  - Existence of a possible relation between the appearance of dilation or constriction of microvessels and the time that has lapsed since infarction. In particular, we observe a tendency for an initial dilation of the vessels in infarcted areas 1 day post MI, a subsequent constriction 3 days post MI followed by possible pruning of microvessels and dilation of the remaining microvessels.
  - As expected, changes at remote areas are not significant or they are milder than the ones observed in the infarcted areas, but as pathology progresses they appear intensified.
  - Changes at later time points following MI (45 days) persist and intensify in infarcted but also remote areas when no therapy is applied.
- Identification of an optimal time frame for therapeutic interventions about 3 days post MI.
- The first microvascular data on permeability tensors, pressure drop and tissue perfusion before and following MI obtained through image-based simulations.

# 6.2 Impact

The response of the microvasculature to tissue injury is crucial to limiting damage and promoting repair. Nonetheless, therapies involving manipulation of the microvasculature have been found to have limited, if any, effectiveness and applicability in the clinic. This

is particularly true in the case of Cardiovascular Disease (CVD). Failure of such methods is in part related to a lack of knowledge about the profound changes that occur in the microvasculature in response to CVD. To overcome this limitation, an important component is imaging data, that permit detailed visualization of the microvasculature, coupled with computational methods for unbiased quantitative analysis and subsequent modelling of the biological processes or of properties that cannot be measured experimentally.

In an effort to advance this exciting area of science and cover the knowledge gap regarding the microvasculature with quantitative and reliable data, we presented an in-depth study of the coronary microvasculature based on a newly developed fully automated image analysis pipeline. The pipeline permits the 3D reconstruction of the microvasculature and subsequent acquisition of novel, quantitative insights into its structure and into changes occurring at different stages of pathology. The pipeline was applied on a model of MI, one of the leading complications of CVD. The findings reached by the quantitative analysis were complemented with image-based computational simulations of tissue scale blood flow properties. This permitted us to go beyond experimental measurements.

Previous studies in the quantification of microvascular changes after MI have relied merely on a few traditional metrics extracted manually or via supervised analysis from a limited number of images, 2D sections or 2D maximum intensity projections. On the contrary, the fully automated nature of our approach ensures that reliable and reproducible conclusions can be reached while respecting the inherently 3D nature of the microvasculature. This would have been infeasible or would have required a tremendous amount of time and effort if manual or supervised analysis had been applied instead. In addition, the outcome would have been susceptible to subjectivity and human error. Therefore, studies based on fully automated approaches are of pivotal importance in the growing quest for quantitative biology and reproducible findings.

Moreover, the tools and data provided within this work are of interest to computational and experimental scientists but also to medical researchers. In particular, the quantitative data can serve as reference and basis for comparisons for future studies in the field of cardiac microvascular research, as well as for studies modelling microcirculation at various stages of pathology. In addition, the permeability tensors of this work can be used to parametrise multi-scale models of coronary microvascular perfusion and also to obtain unprecedented insight into myocardial blood flow and its relation to the microvasculature.

Lastly, although our study was performed in a model of MI, the applicability of the developed open-source solutions for the reconstruction, analysis and recognition of complex 3D microvascular networks is not limited to this field, but could also facilitate the in-depth study of any condition that has an impact on the microvasculature and impairs tissue perfusion,

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including brain disease, tumours and diabetes. In fact, our automated image analysis pipeline or its modules have been successfully applied in other biomedical problems, while other novel applications are under investigation. More precisely, by means of our developed tools we have analysed the following:

• Breast tumor microvasculature to elucidate the effect of different stromal Caveolin-1 (CAV1) expression levels in Triple Negative Breast Cancer.

In-depth characterization of tumor xenograft vasculature labelled with CD31 and imaged by confocal microscopy was performed by means of our 3D fully automated image analysis pipeline to achieve unbiased quantification of microvascular networks. We analysed images under different stromal CAV1 expression levels and acquired by different microscopes. The pipeline, as described in this thesis, permits quantification of the major features of the vascular networks, including their morphology and angioarchitecture, without human intervention. Here, we solely used modules of the pipeline that permit the extraction of parameters with physiological meaning with regard to tumour vasculature which only partially consists of functional vessels. Adequate modifications/additions to the pipeline regarding the segmentation method were developed. Necessary relevant adaptations of the pipeline and results can be found at [44].

• Mouse microvasculature after MI with and without applying a therapeutic approach.

The application of our pipeline permitted identification of subtle, yet biologically significant, changes in the microvasculature after a novel angiotherapy was applied. Modifications and an additional module have been incorporated to the pipeline to account for the differences in behavior of the staining markers between mouse and porcine tissue, and for the particular nature of the mouse microvasculature. The adaptations and the subsequent results of this work can be found at [235].

• Comparison between microvascular networks in regenerating skeletal muscle 5 and 7 days post injury from wild-type and knock-out mice lacking p38α in myeloid cells.

3D confocal images of blood vessels labelled by ICAM2 have been analysed. The images were acquired by using different imaging systems. The work is currently under development.

## 6.3 Limitations

Despite the importance of our findings, there are some limitations to the current work. One of the strengths of this study is the choice of the highly translational porcine animal model, as mentioned earlier. However, due to the cost of and difficulty in handling large animals, we were able to use only a limited number of subjects.

Furthermore, the chosen ischaemia model might influence the microvascular changes described in this study. This means that the same changes may not be necessarily anticipated in other ischaemia models, such as permanent coronary artery ligation or ischaemia with longer duration. Nonetheless, this limitation could motivate future research as the presented quantitative analysis approach paves the way for standardized analysis and future comparison of different experimental ischaemia set-ups.

Another limitation of the present work is the loss of precise topological information at the organ level during tissue processing and confocal microscopy imaging. Also, in spite of the fully automated and un-biased analysis pipeline, human bias could potentially be introduced in terms of selecting the areas to be imaged by confocal microscopy and of adjusting microscope settings.

In addition, since no automated method can ensure 100% accuracy in terms of segmentation, the exact metric numbers might deviate slightly from reality, although the trends would remain uninfluenced given that the same method was applied to all images. Moreover, although the improvement provided by our developed segmentation algorithm was confirmed visually, it would be interesting to have compared our automated segmentations to manual segmentations so as to have a quantitative metric of the deviation between both approaches. However, given the amount of work and time required for such a comparison and as the improvements provided by our method were so obvious, such a comparison was not considered cost and time efficient.

It should also be noted that running the complete image analysis pipeline on large images can be computationally and time demanding, but this drawback can be balanced out because it does not requires human intervention and it, therefore, minimizes costs and labour requirements while facilitating calculation of complex metrics.

Some additional limitations exist in terms of the modelling framework. These are similar to those of theoretical models in general, i.e. we have had to make a series of assumptions in order to perform the simulations. Nonetheless, when possible we tried to make sure that the effect of our assumptions was minimal. Such examples include the assumption that deletion of blind-ends has minimal effect on the calculation of the permeability tensors as well as the assumption of independent flow among sub-networks, the cut-off size of sub-networks to be excluded etc. Lastly, we have assumed steady laminar flow. Although the assumption

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Fig. 6.1 Software under development for the open-source bioimage analysis pipeline presented for the analysis of microvascular data.

stands true due to the very small Reynolds number in microcirculation, it would have been interesting to have also incorporated pulsatile flow.

### 6.4 Future work

Directions for future research have been, in part, indirectly described by the contributions, impact and limitations of the present thesis, but they are also summarized in this section. An area of future work involves the application of the proposed computational tools in image analysis and image-based microvascular perfusion modelling in future studies of MI and other biomedical problems. As mentioned previously, the approaches developed as part of this thesis can be applied to any biomedical problem where the microvasculature is a diagnostic or therapeutic target. Research in this direction has already been initiated, as noted in section 6.2.

Moreover, within the context of research related to MI, an area for future work in terms of quantitative image analysis could involve evaluation of possible angiotherapies, as stated earlier. The first effort in this direction was the evaluation of the response of the microvasculature to a combined VEGFA/S1P gene-cell therapy to promote revascularization and microvascular salvage after MI in mice [235].

Furthermore, it would be interesting to increase the number of subjects used in this work to further strengthen the findings and to enable comparison of the estimated blood flow properties at subject level. It would be also particularly interesting to combine the developed quantitative analysis approaches with multi-scale study of the microvasculature. This could be achieved by combining imaging modalities that permit wide field-views such as micro-CT, with confocal microscopy for the assessment of particular regions of interest with higher resolution. Such an approach would further enlighten our knowledge of the coronary microvasculature and its microvascular response after MI. Furthermore, a deeper understanding could be achieved by performing a detailed study of different regions within the same heart. Towards this, the areas could be sampled by the use of surgical biopsy punch during sample collection, as it was done for the study of tumorous networks [183]. Such an approach would permit preservation of rough topological information.

In addition, although the pig animal model was the focus of this work, another exciting area of future research could be the application of the developed methods on other animal models to perform a comparative, quantitative study of the microvascular response of MI in different preclinical animal models . Our method could be also deployed in the study of different experimental models of MI, e.g. permanent coronary artery ligation, versus ischaemia with perfusion, so as to further advance our knowledge regarding MI. Moreover, the approach could be used to compare the effect of ischaemia duration and cardioprotective strategies, such as preconditioning [88].

Regarding specifically the modelling framework of this work, it would be of paramount interest to compare the tissue perfusion results with measurements from magnetic resonance imaging (MRI) data. This would be a challenging task because the voxel size of a typical MRI is larger than the volume that can be visualized by a confocal system and due to the loss of the topological information during acquisition. However, if we could overcome these limitations, the comparison between modelling results and MRI measurements would enable us to enhance our understanding of how microvascular flow is related to whole organ measurements. In this respect, retaining part of the topological information by biopsy punch during acquisition, as described earlier, could be helpful. Moreover, several areas could be imaged to cover a voxel or sub-voxel of an MRI.

Another area for future research could include extension of the developed image-based mathematical modelling framework to simulate blood flow in individual vessels of the porcine cardiac tissue volumes of our dataset. To achieve this, we would have to tackle the lack of functional data for setting appropriate boundary conditions at the inlet and outlet nodes of the network. For this purpose, we envision to use the output of the continuum model and, more precisely, the estimated myocardial blood flow, to solve an optimization problem [75] for estimating boundary conditions, such as pressure or flow, given the tissue scale blood flow properties. Combination of such an optimization routine with a discrete blood

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flow model, similarly to [213], will enable subsequent simulation of hemodynamics of each segment of the microvasculature and it would therefore allow us to study their distributions. Furthermore, structural information, combined with blood flow simulations, at segment level would allow detailed simulation of transport of oxygen at the cardiac tissue spanning several stages after MI. Once blood flow and oxygen will have been simulated, a step further would have involved theoretical simulations of angiogenesis, structural adaptations and pruning [200], to unravel the mechanism or combination of mechanisms that control the ischaemic tissue phenotype or that could be applied as a therapeutic approach to reverse the effect that MI has had on the tissue. Moreover, future integration of imaging data regarding the microvasculature with the molecular information obtained by omics analysis performed within "CardioNext" [16] can revolutionize our understanding of MI by shedding light to phenotypic and molecular mechanisms.

Last but not least, the development of a graphical user interface for the algorithms developed within this work is another interesting area of future work. The automated image analysis pipeline code is open-source and freely available at https://bitbucket.org/xenia\_gk/microvasculatureanalysis\_gkontra\_et\_al\_2018 in order to enable application and adaptations of the pipeline in future research. The code is organized in modules and documented in detail. However, the lack of a graphical interface precludes its use by users without programming experience. For this reason, a software suite for the image analysis pipeline is currently being developed. The general design of the software is provided in Fig. 6.1. The code will remain publicly available in order to enable potential adaptations and extensions that are usually necessary to tackle other biomedical problems. Efforts will also focus on improving the speed and computational requirements of the initial code. This, along with its open-source nature, will enhance applicability and extensibility of our approaches for future studies.

### PUBLICATIONS

#### Journal articles

Díez-Sánchez, A., Martin-Padura, I., Sánchez-Alvarez, M., Catalá-Montoro, M., Osteso, M., Palacios, I., Albacete-Albacete, L., Acin-Perez, R., Jiménez-Carretero, D., Gkontra, P., Pavón, D., Montoya, M., Enriquez, J., Bjorkoy, G., and Del Pozo, M. Caveolin-1 expression in Cancer-Associated Fibroblasts determines functional proangiogenic signaling through organelle homeostasis to support ordered tumor growth. *In preparation*.

Żak, M. M., Gkontra, P., Squadrito, M. L., Ferrarini, A., Mota, R. A., Rocha, S., Agüero, J., Vázquez, J., Palma, M., Ibáñez, B., and Arroyo, A.G. Combined VEGFA/S1P genecell therapy improves vascularization and limits adverse cardiac remodeling after myocardial infarction in mice. *Submitted*.

Gkontra, P., El-Bouri, W., Norton, K-A., Santos, A., Popel, A.S., Payne, S., Arroyo, A.G. Image-based mathematical modelling of the infarcted heart response at the microvascular level. *Submitted*.

Gkontra, P., Norton, K.-A., Żak, M. M., Clemente, C., Agüero, J., Ibáñez, B., Santos, A., Popel, A. S., and Arroyo, A. G. (2018). Deciphering microvascular changes after myocardial infarction through 3D fully automated image analysis. Scientific Reports, 8(1):1854.

#### **Peer-reviewed conferences articles**

Gkontra, P., Żak, M. M., Norton, K.-A., Santos, A., Popel, A. S., and Arroyo, A. G. (2015). A 3D Fractal-Based Approach towards Understanding Changes in the Infarcted Heart Microvasculature. In Navab, N., Hornegger, J., Wells, W. M., and Frangi, A. F., editors, Medical Image Computing and Computer-Assisted Intervention - MICCAI 2015, Lecture Notes in Computer Science, vol 9351. Springer, Cham.

#### REFERENCES

- [1] A., I. P. (2006). Handbook of cardiac anatomy, physiology, and devices. *Journal of Cardiac Surgery*, 21(3):327–327.
- [2] Algranati, D., Kassab, G. S., and Lanir, Y. (2011). Why is the subendocardium more vulnerable to ischemia? A new paradigm. *Am. J. Physiol. Heart Circ. Physiol.*, 300(3):H1090– 1100.
- [3] Allan, C., Burel, J. M., Moore, J., Blackburn, C., Linkert, M., Loynton, S., Macdonald, D., Moore, W. J., Neves, C., Patterson, A., Porter, M., Tarkowska, A., Loranger, B., Avondo, J., Lagerstedt, I., Lianas, L., Leo, S., Hands, K., Hay, R. T., Patwardhan, A., Best, C., Kleywegt, G. J., Zanetti, G., and Swedlow, J. R. (2012). OMERO: flexible, model-driven data management for experimental biology. *Nat. Methods*, 9(3):245–253.
- [4] Anderson, B. G. and Anderson, W. D. (1980). Microvasculature of the canine heart demonstrated by scanning electron microscopy. *Am. J. Anat.*, 158(2):217–227.
- [5] Anversa, P., Loud, A. V., Levicky, V., and Guideri, G. (1985). Left ventricular failure induced by myocardial infarction. II. Tissue morphometry. *Am. J. Physiol.*, 248(6 Pt 2):H883–889.
- [6] Anversa, P., Ricci, R., and Olivetti, G. (1986). Quantitative structural analysis of the myocardium during physiologic growth and induced cardiac hypertrophy: a review. J Am Coll Cardiol., 7(5):1140–1149.
- [7] Auriault, J. L. (2011). Heterogeneous periodic and random media. are the equivalent macroscopic descriptions similar? *International Journal of Engineering Science*, 49(8):806 – 808.
- [8] authors listed, N. (2012). The quest for quantitative microscopy. Nat. Methods, 9(7):627.
- [9] Barr, A. H. (1981). Superquadrics and angle-preserving transformations. *IEEE Comput Graph Appl*, 1(1):11–23.
- [10] Bassingthwaighte, J. B., Yipintsoi, T., and Harvey, R. B. (1974). Microvasculature of the dog left ventricular myocardium. *Microvasc Res*, 7:229–249.
- [11] Bates, R., Risser, L., Irving, B., Papież, B. W., Kannan, P., Kersemans, V., and Schnabel, J. A. (2015). *Filling Large Discontinuities in 3D Vascular Networks Using Skeleton- and Intensity-Based Information*. Springer International Publishing.

- [12] Batra, S., Rakusan, K., and Campbell, S. E. (1991). Geometry of capillary networks in hypertrophied rat heart. *Microvasc Res*, 41:29—-40.
- [13] Bear, J. (1972). Dynamics of fluids in porous media. Dover's publications.
- [14] Berntson, G. M. and Stoll, P. (1997). Correcting for finite spatial scales of self-similarity when calculating fractal dimensions of real-world structures. *Proceedings of the Royal Society B: Biological Sciences*, 264(1387):1531–1537.
- [15] Bezerra, H. G., Costa, M. A., Guagliumi, G., Rollins, A. M., and Simon, D. I. (2009). Intracoronary optical coherence tomography: a comprehensive review clinical and research applications. *JACC Cardiovasc Interv*, 2(11):1035–1046.
- [16] Binek, A., Fernandez-Jimenez, R., Jorge, I., Camafeita, E., Lopez, J. A., Bagwan, N., Galan-Arriola, C., Pun, A., Aguero, J., Fuster, V., Ibanez, B., and Vazquez, J. (2017). Proteomic footprint of myocardial ischemia/reperfusion injury: Longitudinal study of the at-risk and remote regions in the pig model. *Sci Rep*, 7(1):12343.
- [17] Bjornsson, C. S., Lin, G., Al-Kofahi, Y., Narayanaswamy, A., Smith, K. L., Shain, W., and Roysam, B. (2008). Associative image analysis: a method for automated quantification of 3D multi-parameter images of brain tissue. J. Neurosci. Methods, 170(1):165–178.
- [18] Blinder, P., Tsai, P. S., Kaufhold, J. P., Knutsen, P. M., Suhl, H., and Kleinfeld, D. (2013). The cortical angiome: an interconnected vascular network with noncolumnar patterns of blood flow. *Nat. Neurosci.*, 16(7):889–897.
- [19] Block, A., von Bloh, W., and Schellnhuber, H. (1990). Efficient box-counting determination of generalized fractal dimensions. *Physical Review*. A, 42:1869–1874.
- [20] Braunwald, E. (2015). The war against heart failure: the Lancet lecture. *Lancet*, 385(9970):812–824.
- [21] Brown, M. D., Davies, M. K., and Hudlicka, O. (2005). Angiogenesis in ischaemic and hypertrophic hearts induced by long-term bradycardia. *Angiogenesis*, 8(3):253–262.
- [22] Brown, R. (1965). The pattern of the microcirculatory bed in the ventricular myocardium of domestic mammals. *Am J Anat*, 116:355–374.
- [23] Buades, A., Coll, B., and Morel, J. M. (2004). On image denoising methods. Technical report, TECHNICAL NOTE, CMLA (CENTRE DE MATHEMATIQUES ET DE LEURS APPLICATIONS.
- [24] Buades, A., Coll, B., and Morel, J.-M. (2005). A non-local algorithm for image denoising. *CVPR*, 2:60–65.
- [25] Burton, A. C. (1954). Relation of structure to function of the tissues of the wall of blood vessels. *Physiol. Rev.*, 34(4):619–642.
- [26] Burton, R. A., Plank, G., Schneider, J. E., Grau, V., Ahammer, H., Keeling, S. L., Lee, J., Smith, N. P., Gavaghan, D., Trayanova, N., and Kohl, P. (2006). Three-dimensional models of individual cardiac histoanatomy: tools and challenges. *Ann. N. Y. Acad. Sci.*, 1080:301–319.

- [27] Cahill, T. J., Choudhury, R. P., and Riley, P. R. (2017). Heart regeneration and repair after myocardial infarction: translational opportunities for novel therapeutics. *Nat Rev Drug Discov*, 16(10):699–717.
- [28] Camici, P. G., d'Amati, G., and Rimoldi, O. (2015). Coronary microvascular dysfunction: mechanisms and functional assessment. *Nat Rev Cardiol.*, 12(1):48–62.
- [29] Camici, P. G., Gropler, R. J., Jones, T., L'Abbate, A., Maseri, A., Melin, J. A., Merlet, P., Parodi, O., Schelbert, H. R., Schwaiger, M., and Wijns, W. (1996). The impact of myocardial blood flow quantitation with PET on the understanding of cardiac diseases. *Eur. Heart J.*, 17(1):25–34.
- [30] Campbell, D. J., Somaratne, J. B., Jenkins, A. J., Prior, D. L., Yii, M., Kenny, J. F., Newcomb, A. E., Kelly, D. J., and Black, M. J. (2013). Reduced microvascular density in non-ischemic myocardium of patients with recent non-st-segment-elevation myocardial infarction. *Int J Cardiol.*, 167(3):1027–1037.
- [31] Captur, G., Karperien, A. L., Hughes, A. D., Francis, D. P., and Moon, J. C. (2016). The fractal heart - embracing mathematics in the cardiology clinic. *Nat Rev Cardiol.*, 14(1):56–64.
- [32] Carmeliet, P. (2005). Angiogenesis in life, disease and medicine. *Nature*, 438(7070):932–936.
- [33] Carmeliet, P. and Jain, R. K. (2011). Molecular mechanisms and clinical applications of angiogenesis. *Nature*, 473(7347):298–307.
- [34] Carpenter, A. E., Jones, T. R., Lamprecht, M. R., Clarke, C., Kang, I. H., Friman, O., Guertin, D. A., Chang, J. H., Lindquist, R. A., Moffat, J., Golland, P., and Sabatini, D. M. (2006). CellProfiler: image analysis software for identifying and quantifying cell phenotypes. *Genome Biol.*, 7(10):R100.
- [35] Cassot, F., Lauwers, F., Fouard, C., Prohaska, S., and Lauwers-Cances, V. (2006). A novel three-dimensional computer-assisted method for a quantitative study of microvascular networks of the human cerebral cortex. *Microcirculation*, 13(1):1–18.
- [36] Chareonthaitawee, P., Kaufmann, P. A., Rimoldi, O., and Camici, P. G. (2001). Heterogeneity of resting and hyperemic myocardial blood flow in healthy humans. *Cardiovasc. Res.*, 50(1):151–161.
- [37] Chenouard, N., Smal, I., de Chaumont, F., Ma?ka, M., Sbalzarini, I. F., Gong, Y., Cardinale, J., Carthel, C., Coraluppi, S., Winter, M., Cohen, A. R., Godinez, W. J., Rohr, K., Kalaidzidis, Y., Liang, L., Duncan, J., Shen, H., Xu, Y., Magnusson, K. E., Jalden, J., Blau, H. M., Paul-Gilloteaux, P., Roudot, P., Kervrann, C., Waharte, F., Tinevez, J. Y., Shorte, S. L., Willemse, J., Celler, K., van Wezel, G. P., Dan, H. W., Tsai, Y. S., Ortiz de Solorzano, C., Olivo-Marin, J. C., and Meijering, E. (2014). Objective comparison of particle tracking methods. *Nat. Methods*, 11(3):281–289.
- [38] Cleaver, O. and Melton, D. A. (2003). Endothelial signaling during development. *Nat. Med.*, 9(6):661–668. [DOI:10.1038/nm0603-661] [PubMed:12778164].

- [39] Clegg, L. E. and Mac Gabhann, F. (2015). Systems biology of the microvasculature. *Integr. Biol.*, 7:498–512.
- [40] Cleutjens, J. P., Blankesteijn, W. M., Daemen, M. J., and Smits, J. F. (1999). The infarcted myocardium: simply dead tissue, or a lively target for therapeutic interventions. *Cardiovasc Res.*, 44(2):232–241.
- [41] Cobb, F. R., Bache, R. J., Rivas, F., and Greenfield, J. C. (1976). Local effects of acute cellular injury on regional myocardial blood flow. J. Clin. Invest., 57(5):1359–1368.
- [42] Combs, C. A. and Shroff, H. (2017). Fluorescence Microscopy: A Concise Guide to Current Imaging Methods. *Curr Protoc Neurosci*, 79:1–2.
- [43] de Chaumont, F., Dallongeville, S., Chenouard, N., Herve, N., Pop, S., Provoost, T., Meas-Yedid, V., Pankajakshan, P., Lecomte, T., Le Montagner, Y., Lagache, T., Dufour, A., and Olivo-Marin, J. C. (2012). Icy: an open bioimage informatics platform for extended reproducible research. *Nat. Methods*, 9(7):690–696.
- [44] Díez-Sánchez, A., Martin-Padura, I., Sánchez-Alvarez, M., Catalá-Montoro, M., Osteso, M., Palacios, I., Albacete-Albacete, L., Acin-Perez, R., Jiménez-Carretero, D., Gkontra, P., Pavón, D., Montoya, M., Enriquez, J., Bjorkoy, G., and Del Pozo, M. (2018). Caveolin-1 expression in Cancer-Associated Fibroblasts determines functional proangiogenic signaling through organelle homeostasis to support ordered tumor growth. *In preparation*.
- [45] Di Carli, M. F., Prcevski, P., Singh, T. P., Janisse, J., Ager, J., Muzik, O., and Vander Heide, R. (2000). Myocardial blood flow, function, and metabolism in repetitive stunning. J. Nucl. Med., 41(7):1227–1234.
- [46] Dickie, R., Bachoo, R. M., Rupnick, M. A., Dallabrida, S. M., Deloid, G. M., Lai, J., Depinho, R. A., and Rogers, R. A. (2006). Three-dimensional visualization of microvessel architecture of whole-mount tissue by confocal microscopy. *Microvasc. Res.*, 72(1-2):20– 26.
- [47] Dietz, C. and Berthold, M. R. (2016). KNIME for Open-Source Bioimage Analysis: A Tutorial. Adv Anat Embryol Cell Biol, 219:179–197.
- [48] Djonov, V., Baum, O., and Burri, P. (2003). Vascular remodeling by intussusceptive angiogenesis. *Cell Tissue Res.*, 314:107–117.
- [49] Dobaczewski, M., Akrivakis, S., Nasser, K., Michael, L. H., Entman, M. L., and Frangogiannis, N. G. (2004). Vascular mural cells in healing canine myocardial infarcts. *Journal of Histochemistry & Cytochemistry*, 52(8):1019–1029.
- [50] Doblas, S., He, T., Saunders, D., Pearson, J., Hoyle, J., Smith, N., Lerner, M., and Towner, R. A. (2010). Glioma morphology and tumor-induced vascular alterations revealed in seven rodent glioma models by in vivo magnetic resonance imaging and angiography. *J Magn Reson Imaging*, 32(2):267–275.
- [51] Dunn, K. W., Kamocka, M. M., and McDonald, J. H. (2011). A practical guide to evaluating colocalization in biological microscopy. *Am. J. Physiol.*, *Cell Physiol.*, 300(4):C723–742.

- [52] Dweck, M. R., Doris, M. K., Motwani, M., Adamson, P. D., Slomka, P., Dey, D., Fayad, Z. A., Newby, D. E., and Berman, D. (2016). Imaging of coronary atherosclerosis evolution towards new treatment strategies. *Nat Rev Cardiol*, 13(9):533–548.
- [53] Dwyer, E. M., Dell, R. B., and Cannon, P. J. (1973). Regional myocardial blood flow in patients with residual anterior and inferior transmural infarction. *Circulation*, 48(5):924–935.
- [54] Ehling, J., Theek, B., Gremse, F., Baetke, S., Mockel, D., Maynard, J., Ricketts, S. A., Grull, H., Neeman, M., Knuechel, R., Lederle, W., Kiessling, F., and Lammers, T. (2014). Micro-CT imaging of tumor angiogenesis: quantitative measures describing micromorphology and vascularization. *Am. J. Pathol.*, 184(2):431–441.
- [55] Eissing, N., Heger, L., Baranska, A., Cesnjevar, R., Buttner-Herold, M., Soder, S., Hartmann, A., Heidkamp, G. F., and Dudziak, D. (2014). Easy performance of 6-color confocal immunofluorescence with 4-laser line microscopes. *Immunol. Lett.*, 161(1):1–5.
- [56] El-Bouri, W. K. and Payne, S. J. (2015). Multi-scale homogenization of blood flow in 3-dimensional human cerebral microvascular networks. *Journal of Theoretical Biology*, 380:40 – 47.
- [57] El-Bouri, W. K. and Payne, S. J. (2018). Investigating the effects of a penetrating vessel occlusion with a multi-scale microvasculature model of the human cerebral cortex. *Neuroimage*, 172:94–106.
- [58] Eliceiri, K. W., Berthold, M. R., Goldberg, I. G., Ibanez, L., a. M. B. S., Martone, M. E., Murphy, R. F., Peng, H., Plant, A. L., Roysam, B., Stuurman, N., Swedlow, J. R., Tomancak, P., and Carpenter, A. E. (2012). Biological imaging software tools. *Nature Methods*, 9:697–710.
- [59] Ezekowitz, J. A., Kaul, P., Bakal, J. A., Armstrong, P. W., Welsh, R. C., and McAlister, F. A. (2009). Declining in-hospital mortality and increasing heart failure incidence in elderly patients with first myocardial infarction. J. Am. Coll. Cardiol., 53(1):13–20.
- [60] Factor, S. M., Okun, E. M., Minase, T., and Kirk, E. S. (1982). The microcirculation of the human heart: end-capillary loops with discrete perfusion fields. *Circulation*, 66(6):1241–1248.
- [61] Fahraeus, R. (1929). The suspension stability of the blood. *Physiol Rev*, 9:241–274.
- [62] Fahraeus, R. (1931). The suspension stability of the blood. American Journal of Physiology, 96:562–568.
- [63] Fan, H., Ma, L., Fan, B., Wu, J., Yang, Z., and Wang, L. (2014). Role of pdgfrb/pi3k/akt signaling pathway in pdgf-bb induced myocardial fibrosis in rats. *Am J Transl Res*, 6(6):714–723.
- [64] Fang, Q., Sakadźić, S., Ruvinskaya, L., Devor, A., Dale, A. M., and Boas, D. A. (2008). Oxygen advection and diffusion in a three- dimensional vascular anatomical network. *Opt Express*, 16(22):17530–17541.

- [65] Feher, A. and Sinusas, A. J. (2017). Quantitative Assessment of Coronary Microvascular Function: Dynamic Single-Photon Emission Computed Tomography, Positron Emission Tomography, Ultrasound, Computed Tomography, and Magnetic Resonance Imaging. *Circ Cardiovasc Imaging*, 10(8).
- [66] Fehrenbach, J., Weiss, P., and Lorenzo, C. (2012). Variational algorithms to remove stationary noise: applications to microscopy imaging. *IEEE Trans Image Process*, 21(10):4420–4430.
- [67] Feng, D., Marshburn, D., Jen, D., Weinberg, R. J., Taylor, R. M., and Burette, A. (2007). Stepping into the third dimension. *J. Neurosci.*, 27(47):12757–12760.
- [68] Fernández-Jiménez, R., Fernández-Friera, L., Sánchez-González, J., and nez, I. (2014). Animal models of tissue characterization of area at risk, edema and fibrosis. *Curr Cardiovasc Imaging Rep*, 7(9259).
- [69] Figueiredo, G., Brockmann, C., Boll, H., Heilmann, M., Schambach, S. J., Fiebig, T., Kramer, M., Groden, C., and Brockmann, M. A. (2012). Comparison of digital subtraction angiography, micro-computed tomography angiography and magnetic resonance angiography in the assessment of the cerebrovascular system in live mice. *Clin Neuroradiol*, 22(1):21–28.
- [70] Finegold, J. A., Asaria, P., and Francis, D. P. (2013). Mortality from ischaemic heart disease by country, region, and age: Statistics from world health organisation and united nations. *International Journal of Cardiology*, 168(2):934—-945.
- [71] Folarin, A. A., Konerding, M. A., Timonen, J., Nagl, S., and Pedley, R. B. (2010). Three-dimensional analysis of tumour vascular corrosion casts using stereoimaging and micro-computed tomography. *Microvasc. Res.*, 80(1):89–98.
- [72] Frangi, A., Niessen, W., Vincken, K., and Viergever, M. (1998). Multiscale vessel enhancement filtering. In *Medical Image Computing and Computer Assisted Intervention* (*MICCAI*), *Lecture Notes in Computer Science (LNCS)*, volume 1496, pages 130–137. Springer.
- [73] Frangogiannis, N. G. (2008). The immune system and cardiac repair. *Pharmacological Research*, 58(2):88–111.
- [74] Frangogiannis, N. G. (2014). The inflammatory response in myocardial injury, repair, and remodelling. *Nat Rev Cardiol*, 11(5):255–265.
- [75] Fry, B. C., Lee, J., Smith, N. P., and Secomb, T. W. (2012). Estimation of blood flow rates in large microvascular networks. *Microcirculation*, 19(6):530–538.
- [76] Fuster, V. and Kelly, B. (2010). Promoting Cardiovascular Health in the Developing World: A Critical Challenge to Achieve Global Health. Institute of Medicine (US) Committee on Preventing the Global Epidemic of Cardiovascular Disease: Meeting the Challenges in Developing Countries. National Academies Press (US), Washington (DC).

- [77] Gheorghiade, M., Sopko, G., De Luca, L., Velazquez, E. J., Parker, J. D., Binkley, P. F., Sadowski, Z., Golba, K. S., Prior, D. L., Rouleau, J. L., and Bonow, R. O. (2006). Navigating the crossroads of coronary artery disease and heart failure. *Circulation*, 114(11):1202–1213.
- [78] Gkontra, P., Norton, K.-A., Žak, M. M., Clemente, C., Agüero, J., Ibáñez, B., Santos, A., Popel, A. S., and Arroyo, A. G. (2018). Deciphering microvascular changes after myocardial infarction through 3d fully automated image analysis. *Scientific Reports*, 8(1):1854.
- [79] Gkontra, P., Żak, M. M., Norton, K.-A., Santos, A., Popel, A. S., and Arroyo, A. G. (2015). A 3d fractal-based approach towards understanding changes in the infarcted heart microvasculature. In Navab, N., Hornegger, J., Wells, W. M., and Frangi, A. F., editors, *Medical Image Computing and Computer-Assisted Intervention MICCAI 2015*, pages 173–180, Cham. Springer International Publishing.
- [80] Goldman, D. and Popel, A. S. (1999). Computational modeling of oxygen transport from complex capillary networks. Relation to the microcirculation physiome. *Adv. Exp. Med. Biol.*, 471:555–563.
- [81] Goldsmith, E. C., Bradshaw, A. D., Zile, M. R., and Spinale, F. G. (2014). Myocardial fibroblast-matrix interactions and potential therapeutic targets. *J. Mol. Cell. Cardiol.*, 70:92–99.
- [82] Gompper, G. and Fedosov, D. A. (2016). Modeling microcirculatory blood flow: current state and future perspectives. *Wiley Interdiscip Rev Syst Biol Med*, 8(2):157–168.
- [83] Gonzalez-Tendero, A., Zhang, C., Balicevic, V., Cardenes, R., Loncaric, S., Butakoff, C., Paun, B., Bonnin, A., Garcia-Canadilla, P., Munoz-Moreno, E., Gratacos, E., Crispi, F., and Bijnens, B. (2017). Whole heart detailed and quantitative anatomy, myofibre structure and vasculature from X-ray phase-contrast synchrotron radiation-based micro computed tomography. *Eur Heart J Cardiovasc Imaging*, 18(7):732–741.
- [84] Gould, D., Vadakkan, T., Poché, R., and Dickinson, M. (2011). Multifractal and lacunarity analysis of microvascular morphology and remodeling. *Microcirculation*, 18(2):136–151.
- [85] G.S., K. (2010). Geometrical Features of the Vascular System. Guccione J., Kassab G., Ratcliffe M. (eds) Computational Cardiovascular Mechanics, Springer, Boston, MA.
- [86] Habib, A., Lachman, N., Christensen, K. N., and Asirvatham, S. J. (2009). The anatomy of the coronary sinus venous system for the cardiac electrophysiologist. *Europace*, 11 Suppl 5:15–21.
- [87] Hamilton, N. (2009). Quantification and its applications in fluorescent microscopy imaging. *Traffic*, 10(8):951–961.
- [88] Hausenloy, D. J. (2013). Cardioprotection techniques: preconditioning, postconditioning and remote conditioning (basic science). *Curr. Pharm. Des.*, 19(25):4544–4563.

- [89] Hausenloy, D. J., Botker, H. E., Engstrom, T., Erlinge, D., Heusch, G., Ibanez, B., Kloner, R. A., Ovize, M., Yellon, D. M., and Garcia-Dorado, D. (2017). Targeting reperfusion injury in patients with ST-segment elevation myocardial infarction: trials and tribulations. *Eur. Heart J.*, 38(13):935–941.
- [90] Heinzer, S., Krucker, T., Stampanoni, M., Abela, R., Meyer, E. P., Schuler, A., Schneider, P., and Muller, R. (2006). Hierarchical microimaging for multiscale analysis of large vascular networks. *Neuroimage*, 32(2):626–636.
- [91] Heusch, G. and Gersh, B. J. (2017). The pathophysiology of acute myocardial infarction and strategies of protection beyond reperfusion: a continual challenge. *Eur. Heart J.*, 38(11):774–784.
- [92] Hollander, M. R., de Waard, G. A., Konijnenberg, L. S., Meijer-van Putten, R.M., a. v. d. B. C. E., Paauw, N., de Vries, H. E., van de Ven, P. M., Aman, J., Van Nieuw-Amerongen, G. P., Hordijk, P. L., Niessen, H. W., Horrevoets, A., and Van Royen, N. (2016). Dissecting the effects of ischemia and reperfusion on the coronary microcirculation in a rat model of acute myocardial infarction. *PLoS One.*, 11(7):e0157233.
- [93] Hood, L. and Flores, M. (2012). A personal view on systems medicine and the emergence of proactive P4 medicine: predictive, preventive, personalized and participatory. *N Biotechnol*, 29(6):613–624.
- [94] Hood, L., Heath, J. R., Phelps, M. E., and Lin, B. (2004). Systems biology and new technologies enable predictive and preventative medicine. *Science*, 306(5696):640–643.
- [95] Hundley, W. G., Bluemke, D. A., Finn, J. P., Flamm, S. D., Fogel, M. A., Friedrich, M. G., Ho, V. B., Jerosch-Herold, M., Kramer, C. M., Manning, W. J., Patel, M., Pohost, G. M., Stillman, A. E., White, R. D., Woodard, P. K., Harrington, R. A., Anderson, J. L., Bates, E. R., Bridges, C. R., Eisenberg, M. J., Ferrari, V. A., Grines, C. L., Hlatky, M. A., Jacobs, A. K., Kaul, S., Lichtenberg, R. C., Lindner, J. R., Moliterno, D. J., Mukherjee, D., Pohost, G. M., Rosenson, R. S., Schofield, R. S., Shubrooks, S. J., Stein, J. H., Tracy, C. M., Weitz, H. H., and Wesley, D. J. (2010). ACCF/ACR/AHA/NASCI/SCMR 2010 expert consensus document on cardiovascular magnetic resonance: a report of the American College of Cardiology Foundation Task Force on Expert Consensus Documents. J. Am. Coll. Cardiol., 55(23):2614–2662.
- [96] Hyde, E. R., Cookson, A. N., Lee, J., Michler, C., Goyal, A., Sochi, T., Chabiniok, R., Sinclair, M., Nordsletten, D. A., Spaan, J., van den Wijngaard, J. P. H. M., Siebes, M., and Smith, N. P. (2014). Multi-scale parameterisation of a myocardial perfusion model using whole-organ arterial networks. *Annals of Biomedical Engineering*, 42(4):797–811.
- [97] Hyde, E. R., Michler, C., Lee, J., Cookson, A. N., Chabiniok, R., Nordsletten, D. A., and Smith, N. P. (2013). Parameterisation of multi-scale continuum perfusion models from discrete vascular networks. *Medical & Biological Engineering & Computing*, 51(5):557–570.
- [98] Ibáñez, B., Heusch, G., Ovize, M., and Van de Werf, F. (2015). Evolving therapies for myocardial ischemia/reperfusion injury. *J. Am. Coll. Cardiol.*, 65(14):1454–1471.
- [99] Ismail, J. A., Poppa, V., Kemper, L. E., Scatena, M., Giachelli, C. M., Coffin, J. D., and Murry, C. E. (2003). Immunohistologic labeling of murine endothelium. *Cardiovascular Pathology*, 12(2):82–90.
- [100] Jackson, W. F. (2012). Microcirculation. In Joseph Hill & Eric Olson (Eds.) Muscle: Fundamental Biology and Mechanisms of Disease, (Chapter 89):1197–1206.
- [101] Kaneko, N., Matsuda, R., Toda, M., and Shimamoto, K. (2011). Three-dimensional reconstruction of the human capillary network and the intramyocardial micronecrosis. *Am. J. Physiol. Heart Circ. Physiol.*, 300(3):H754–761.
- [102] Kankaanpaa, P. (2014). Developing bioimage informatics : form microscopy to software solutions : with a2β1 integrin as a case study . *Doctoral Dissertation, University* of Turku.
- [103] Kankaanpaa, P., Paavolainen, L., Tiitta, S., Karjalainen, M., Paivarinne, J., Nieminen, J., Marjomaki, V., Heino, J., and White, D. J. (2012). BioImageXD: an open, general-purpose and high-throughput image-processing platform. *Nat. Methods*, 9(7):683–689.
- [104] Kassab, G. S. and Fung, Y. C. (1994). Topology and dimensions of pig coronary capillary network. Am. J. Physiol., 267(1):H319—H325.
- [105] Kassab, G. S., Le, K. N., and C., F. Y. (1999). A hemodynamic analysis of coronary capillary blood flow based on anatomic and distensibility data. *Am J Physiol.*, 277(6 Pt 2):H2158–H2166.
- [106] Kelch, I., Bogle, G., Sands, G., Phillips, A., LeGrice, I., and Dunbar, P. (2015). Organwide 3d-imaging and topological analysis of the continuous microvascular network in a murine lymph node. Adv Anat Embryol Cell Biol, 16:16534.
- [107] Kelly, J., Ewen, J., Bernard, S., Glenny, R., and Barlow, C. (2000). Regional blood flow measurements from fluorescent microsphere images using an imaging cryomicrotome. *Review of Scientific Instruments* 71, 228, 71:228–234.
- [108] Kerschnitzki, M., Kollmannsberger, P., Burghammer, M., Duda, G. N., Weinkamer, R., Wagermaier, W., and Fratzl, P. (2013). Architecture of the osteocyte network correlates with bone material quality. *J Bone Miner Res.*, 28(8):1837–1845.
- [109] Kherlopian, A. R., Song, T., Duan, Q., Neimark, M. A., Po, M. J., Gohagan, J. K., and Laine, A. F. (2008). A review of imaging techniques for systems biology. *BMC Syst Biol*, 2:74.
- [110] Kim, E., Stamatelos, S., Cebulla, J., Bhujwalla, Z. M., Popel, A. S., and Pathak, A. P. (2012). Multiscale imaging and computational modeling of blood flow in the tumor vasculature. *Ann Biomed Eng*, 40(11):2425–2441.
- [111] Kirbas, C. and Quek, F. (2004). A review of vessel extraction techniques and algorithms. *ACM Comput. Surv.*, 36(2):81–121.

## References

- [112] Kiyooka, T., Hiramatsu, O., Shigeto, F., Nakamoto, H., Tachibana, H., Yada, T., Ogasawara, Y., Kajiya, M., Morimoto, T., Morizane, Y., Mohri, S., Shimizu, J., Ohe, T., and Kajiya, F. (2005). Direct observation of epicardial coronary capillary hemodynamics during reactive hyperemia and during adenosine administration by intravital video microscopy. *Am. J. Physiol. Heart Circ. Physiol.*, 288(3):H1437–1443.
- [113] Klem, I. (2016). Expanding CT Application to Myocardial Tissue Characterization. *JACC Cardiovasc Imaging*, 9(7):846–848.
- [114] Kloner, R. A., Brown, D. A., Csete, M., Dai, W., Downey, J. M., Gottlieb, R. A., Hale, S. L., and Shi, J. (2017). New and revisited approaches to preserving the reperfused myocardium. *Nat Rev Cardiol*, 14(11):679–693.
- [115] Korn, C. and Augustin, H. G. (2015). Mechanisms of vessel pruning and regression. *Dev Cell.*, 34(1):5–17.
- [116] Koudstaal, S., Jansen Of Lorkeers, S. J., van Slochteren, F. J., van der Spoel, T. I., van de Hoef, T. P., Sluijter, J. P., Siebes, M., Doevendans, P. A., Piek, J. J., and Chamuleau, S. A. (2013). Assessment of coronary microvascular resistance in the chronic infarcted pig heart. *J Cell Mol Med.*, 17(9):1128–1135.
- [117] Krogh, A. (1919). The number and distribution of capillaries in muscles with calculations of the oxygen pressure head necessary for supplying the tissue. *J Physiol*, 52(6):409–415.
- [118] Kvilekval, K., Fedorov, D., Obara, B., Singh, A., and Manjunath, B. S. (2010). Bisque: a platform for bioimage analysis and management. *Bioinformatics*, 26(4):544–552.
- [119] LeCun, Y., Bengio, Y., and Hinton, G. (2015). Deep learning. *Nature*, 521(7553):436–444.
- [120] Lee, J., Beighley, P., Ritman, E., and Smith, N. (2007). Automatic segmentation of 3D micro-CT coronary vascular images. *Med Image Anal*, 11(6):630–647.
- [121] Lee, J., Niederer, S., Nordsletten, D., Le Grice, I., Smail, B., Kay, D., and Smith, N. (2009). Coupling contraction, excitation, ventricular and coronary blood flow across scale and physics in the heart. *Philosophical Transactions of the Royal Society of London A: Mathematical, Physical and Engineering Sciences*, 367(1896):2311–2331.
- [122] Lee, J. and Smith, N. P. (2012). The multi-scale modelling of coronary blood flow. Ann Biomed Eng, 40(11):2399–2413.
- [123] Lee, J. C., Pullan, A. J., and Smith, N. P. (2004). A computational model of microcirculatory network structure and transient coronary microcirculation. *Conf Proc IEEE Eng Med Biol Soc.*, 5:3808–3811.
- [124] Lee, S. C. and Bajcsy, P. (2006). Intensity correction of fluorescent confocal laser scanning microscope images by mean-weight filtering. J Microsc, 221(Pt 2):122–136.
- [125] Lee, T., Kashyap, R., and Chu, C. (1994). Building skeleton models via 3-d medial surface/axis thinning algorithms. *Computer Vision, Graphics, and Image Processing*, 56(6):462–478.

- [126] Lee, W. N., Pernot, M., Couade, M., Messas, E., Bruneval, P., Bel, A., Hagege, A. A., Fink, M., and Tanter, M. (2012). Mapping myocardial fiber orientation using echocardiography-based shear wave imaging. *IEEE Trans Med Imaging*, 31(3):554–562.
- [127] Legland, D., Kiêu, K., and Devaux, M.-F. (2007). Computation of minkowski measures on 2d and 3d binary images. *Image Anal. Stereol.*, 26:83–92.
- [128] LeGrice, I., Sands, G., Hooks, D., Gerneke, D., and Smaill, B. (2004). Microscopic imaging of extended tissue volumes. *Clin. Exp. Pharmacol. Physiol.*, 31(12):902–905.
- [129] Lesage, D., Angelini, E. D., Bloch, I., and Funka-Lea, G. (2009). A review of 3d vessel lumen segmentation techniques: Models, features and extraction schemes. *Medical Image Analysis*, 13(6):819 – 845. Includes Special Section on Computational Biomechanics for Medicine.
- [130] Lipowsky, H. H., Kovalcheck, S., and Zweifach, B. W. (1978). The distribution of blood rheological parameters in the microvasculature of cat mesentery. *Circ. Res.*, 43(5):738–749.
- [131] Lipowsky, H. H., Usami, S., and Chien, S. (1980). In vivo measurements of "apparent viscosity" and microvessel hematocrit in the mesentery of the cat. *Microvasc. Res.*, 19(3):297–319.
- [132] Liu, P., Sun, J., Zhao, J., Liu, X., Gu, X., Li, J., Xiao, T., and Xu, L. X. (2010). Microvascular imaging using synchrotron radiation. *J Synchrotron Radiat*, 17(4):517– 521.
- [133] Long, F., Zhou, J., and Peng, H. (2012). Visualization and analysis of 3D microscopic images. *PLoS Comput. Biol.*, 8(6):e1002519.
- [134] Lopes, R. and Betrouni, N. (2009). Fractal and multifractal analysis: a review. *Med Image Anal*, 13(4):634–649.
- [135] Lorthois, S. and Cassot, F. (2010). Fractal analysis of vascular networks: insights from morphogenesis. *J Theor Biol.*, 262(4):614–633.
- [136] Lorthois, S., Cassot, F., and Lauwers, F. (2011). Simulation study of brain blood flow regulation by intra-cortical arterioles in an anatomically accurate large human vascular network: Part i: Methodology and baseline flow. *NeuroImage*, 54(2):1031 – 1042.
- [137] Low, A. F., Tearney, G. J., Bouma, B. E., and Jang, I. K. (2006). Technology Insight: optical coherence tomography–current status and future development. *Nat Clin Pract Cardiovasc Med*, 3(3):154–162.
- [138] Ludwig, G. (1971). Capillary pattern of the myocardium. *Methods Achiev Exp Pathol*, 5:238–271.
- [139] Majesky, M. W., Dong, X. R., Hoglund, V., Mahoney, W. M., and Daum, G. (2011). The adventitia: a dynamic interface containing resident progenitor cells. *Arterioscler. Thromb. Vasc. Biol.*, 31(7):1530–1539.

- [140] Mandelbrot, B. B. (1982). *The fractal geometry of nature*. Freeman, San Francisco, CA.
- [141] Matsui, Y., Morimoto, J., and Uede, T. (2010). Role of matricellular proteins in cardiac tissue remodeling after myocardial infarction. *World J Biol Chem*, 1(5):69–80.
- [142] Matsumoto, T. and Kajiya, F. (2005). Coronary microcirculation: Physiology and mechanics. *Fluid Dynamics Research*, 37(1-2):60.
- [143] Mayerich, D., Abbott, L., and McCormick, B. (2008). Knife-edge scanning microscopy for imaging and reconstruction of three-dimensional anatomical structures of the mouse brain. *J Microsc*, 231(Pt 1):134–143.
- [144] Meijering, E., Carpenter, A. E., Peng, H., Hamprecht, F. A., and Olivo-Marin, J.-C. (2016). Imagining the future of bioimage analysis. *Nature Biotechnology*, 34:1250–1255.
- [145] Meijering, E., Dzyubachyk, O., and Smal, I. (2012). Methods for cell and particle tracking. *Meth. Enzymol.*, 504:183–200.
- [146] Melo, R. H. C. andConci, A. (2013). How succolarity could be used as another fractal measure in image analysis. *Telecommunication Systems*, 52(3):1643–1655.
- [147] Meyer, F. (1994). Topographic distance and watershed lines. *Signal Processing*, 38(1):113–125.
- [148] Mittal, N., Zhou, Y., Linares, C., Ung, S., Kaimovitz, B., Molloi, S., and Kassab, G. S. (2005). Analysis of blood flow in the entire coronary arterial tree. *Am. J. Physiol. Heart Circ. Physiol.*, 289(1):H439–446.
- [149] Momose, A., Takeda, T., and Itai, Y. (2000). Blood vessels: depiction at phase-contrast X-ray imaging without contrast agents in the mouse and rat-feasibility study. *Radiology*, 217(2):593–596.
- [150] Nehrhoff, I., Ripoll, J., Samaniego, R., Desco, M., and Gomez-Gaviro, M. V. (2017). Looking inside the heart: a see-through view of the vascular tree. *Biomed Opt Express*, 8(6):3110–3118.
- [151] Ntziachristos, V. (2010). Going deeper than microscopy: the optical imaging frontier in biology. *Nat. Methods*, 7(8):603–614.
- [152] Otsu, N. A. (1979). Threshold selection method from gray-level histograms. *IEEE Transactions on Systems, Man, and Cybernetics*, 9(1):62–66.
- [153] Pan, Q., Wang, R., Reglin, B., Fang, L., Pries, A. R., and Ning, G. (2014). Simulation of microcirculatory hemodynamics: estimation of boundary condition using particle swarm optimization. *Biomed Mater Eng*, 24(6):2341–2347.
- [154] Pawley, J. B. (2006). Handbook of Biological Confocal Microscopy. Springer.
- [155] Peng, H. (2008). Bioimage informatics: a new area of engineering biology. *Bioinfor-matics*, 24(17):1827–1836.

- [156] Peng, H., Bria, A., Zhou, Z., Iannello, G., and Long, F. (2014a). Extensible visualization and analysis for multidimensional images using Vaa3D. *Nat Protoc*, 9(1):193–208.
- [157] Peng, H., Ruan, Z., Long, F., Simpson, J. H., and Myers, E. W. (2010). V3D enables real-time 3D visualization and quantitative analysis of large-scale biological image data sets. *Nat. Biotechnol.*, 28(4):348–353.
- [158] Peng, H., Tang, J., Xiao, H., Bria, A., Zhou, J., Butler, V., Zhou, Z., Gonzalez-Bellido, P. T., Oh, S. W., Chen, J., Mitra, A., Tsien, R. W., Zeng, H., Ascoli, G. A., Iannello, G., Hawrylycz, M., Myers, E., and Long, F. (2014b). Virtual finger boosts three-dimensional imaging and microsurgery as well as terabyte volume image visualization and analysis. *Nat Commun*, 5:4342.
- [159] Petersen, J. W. and Pepine, C. J. (2015). Microvascular coronary dysfunction and ischemic heart disease: Where are we in 2014? *Trends Cardiovasc Med.*, 25(2):98–103.
- [160] Peyrounette, M., Davit, Y., Quintard, M., and Lorthois, S. (2018). Multiscale modelling of blood flow in cerebral microcirculation: Details at capillary scale control accuracy at the level of the cortex. *PLoS ONE*, 13(1):e0189474.
- [161] Pinto, A. R., Ilinykh, A., Ivey, M. J., Kuwabara, J. T., D'Antoni, M. L., Debuque, R., Chandran, A., Wang, L., Arora, K., Rosenthal, N. A., and Tallquist, M. D. (2016). Revisiting Cardiac Cellular Composition. *Circ. Res.*, 118(3):400–409.
- [162] Poiseuille, J. (1846). Recherches expérimentales sur le mouvement des liquides dans les tubes de très-petits diamêtres. *Mem. Acad. R. Sci. Inst. Fr.*, 9:433–544.
- [163] Popel, A. S. and Johnson, P. C. (2005). Microcirculation and hemorheology. Annu Rev Fluid Mech, 37:43–69.
- [164] Potente, M. and Makinen, T. (2017). Vascular heterogeneity and specialization in development and disease. *Nat. Rev. Mol. Cell Biol.*, 18(8):477–494.
- [165] Prabhu, S. D. and Frangogiannis, N. G. (2016). The Biological Basis for Cardiac Repair After Myocardial Infarction: From Inflammation to Fibrosis. *Circ. Res.*, 119(1):91– 112.
- [166] Pries, A. and Secomb, T. (2008a). *Blood Flow in Microvascular Networks*, pages 3–36. Elsevier Inc.
- [167] Pries, A. R., Neuhaus, D., and Gaehtgens, P. (1992). Blood viscosity in tube flow: dependence on diameter and hematocrit. *Am. J. Physiol.*, 263(6 Pt 2):H1770–1778.
- [168] Pries, A. R., Reglin, B., and Secomb, T. W. (2005). Remodeling of blood vessels: responses of diameter and wall thickness to hemodynamic and metabolic stimuli. *Hypertension*, 46(4):725–731.
- [169] Pries, A. R. and Secomb, T. W. (2003). Rheology of the microcirculation. *Clin. Hemorheol. Microcirc.*, 29(3-4):143–148.
- [170] Pries, A. R. and Secomb, T. W. (2005). Microvascular blood viscosity in vivo and the endothelial surface layer. *Am. J. Physiol. Heart Circ. Physiol.*, 289(6):H2657–2664.

- [171] Pries, A. R. and Secomb, T. W. (2008b). Blood flow in microvascular networks. Compr Physiol 2011, Supplement 9: Handbook of Physiology, The Cardiovascular System, Microcirculation, pages 3–36.
- [172] Pries, A. R. and Secomb, T. W. (2008c). Modeling structural adaptation of microcirculation. *Microcirculation.*, 15(8):753–764.
- [173] Pries, A. R. and Secomb, T. W. (2008d). Modeling structural adaptation of microcirculation. *Microcirculation.*, 15(8):753–764.
- [174] Pries, A. R. and Secomb, T. W. (2014). Making microvascular networks work: Angiogenesis, remodeling, and pruning. *Physiology (Bethesda)*, 29(6):446–455.
- [175] Pries, A. R., Secomb, T. W., Gaehtgens, P., and Gross, J. F. (1990a). Blood flow in microvascular networks. Experiments and simulation. *Circ. Res.*, 67(4):826–834.
- [176] Pries, A. R., Secomb, T. W., Gaehtgens, P., and Gross, J. F. (1990b). Blood flow in microvascular networks. Experiments and simulation. *Circ. Res.*, 67(4):826–834.
- [177] Pries, A. R., Secomb, T. W., Gessner, T., Sperandio, M., Gross, J., and Gaehtgens, P. (1994). Resistance to blood flow in microvessels in vivo. *Circ. Res.*, 75(5):904–915.
- [178] Pusztaszeri, M. P., Seelentag, W., and Bosman, F. (2006). Immunohistochemical expression of endothelial markers cd31, cd34, von willebrand factor, and fli-1 in normal human tissues. *Journal of Histochemistry & Cytochemistry*, 54(4):385–395.
- [179] Reed, G. W., Rossi, J. E., and Cannon, C. P. (2017). Acute myocardial infarction. *Lancet*, 389(10065):197–210.
- [180] Reichold, J., Stampanoni, M., Lena Keller, A., Buck, A., Jenny, P., and Weber, B. (2009). Vascular graph model to simulate the cerebral blood flow in realistic vascular networks. *J. Cereb. Blood Flow Metab.*, 29(8):1429–1443.
- [181] Ren, G., Michael, L. H., Entman, M. L., and Frangogiannis, N. G. (2002). Morphological characteristics of the microvasculature in healing myocardial infarcts. *J Histochem Cytochem.*, 50(1):71–79.
- [182] Risser, L., Plouraboue, F., and Descombes, X. (2008). Gap filling of 3-d microvascular networks by tensor voting. *IEEE Transactions on Medical Imaging*, 27(5):674–687.
- [183] Risser, L., Plouraboue, F., Steyer, A., Cloetens, P., Le Duc, G., and Fonta, C. (2007). From homogeneous to fractal normal and tumorous microvascular networks in the brain. *J. Cereb. Blood Flow Metab.*, 27(2):293–303.
- [184] Ritman, E. L. (2011). Current status of developments and applications of micro-CT. *Annu Rev Biomed Eng*, 13:531–552.
- [185] Ronneberger, O., Fischer, P., and Brox, T. (2015). U-net: Convolutional networks for biomedical image segmentation. In Navab, N., Hornegger, J., Wells, W. M., and Frangi, A. F., editors, *Medical Image Computing and Computer-Assisted Intervention – MICCAI* 2015, pages 234–241, Cham. Springer International Publishing.

- [186] Roysam, B., Lin, G., Abdul-Karim, M.-A., Al-Kofahi, ., Al-Kofahi, K., Shain, W., Szarowski, D., and Turner, J. (2006). Automated three-dimensional image analysis methods for confocal microscopy. In J., P., editor, *Handbook Of Biological Confocal Microscopy.*, chapter 15, pages 316–337. Springer, Boston, MA.
- [187] Russell, R. A., Adams, N. M., Stephens, D. A., Batty, E., Jensen, K., and Freemont, P. S. (2009). Segmentation of fluorescence microscopy images for quantitative analysis of cell nuclear architecture. *Biophys. J.*, 96(8):3379–3389.
- [188] Rutherford, S. L., Trew, M. L., Sands, G. B., LeGrice, I. J., and Smaill, B. H. (2012). High-resolution 3-dimensional reconstruction of the infarct border zone: impact of structural remodeling on electrical activation. *Circ. Res.*, 111(3):301–311.
- [189] Salerno, M., Sharif, B., Arheden, H., Kumar, A., Axel, L., Li, D., and Neubauer, S. (2017). Recent Advances in Cardiovascular Magnetic Resonance: Techniques and Applications. *Circ Cardiovasc Imaging*, 10(6).
- [190] Sands, G. B., Gerneke, D. A., Smaill, B. H., and Le Grice, I. J. (2006). Automated extended volume imaging of tissue using confocal and optical microscopy. *Conf Proc IEEE Eng Med Biol Soc*, 1:133–136.
- [191] Sarder, P. and Nehorai, A. (2006). Deconvolution methods for 3-d fluorescence microscopy images. *IEEE Signal Processing Magazine*, 23(3):32–45.
- [192] Satter, A. and Iqbal, G. M. (2016). Fundamentals of fluid flow through porous media. In *Reservoir Engineering*, pages 155–169. Elsevier.
- [193] Schaad, L., Hlushchuk, R., Barre, S., Gianni-Barrera, R., Haberthur, D., Banfi, A., and Djonov, V. (2017). Correlative Imaging of the Murine Hind Limb Vasculature and Muscle Tissue by MicroCT and Light Microscopy. *Sci Rep*, 7:41842.
- [194] Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J. Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P., and Cardona, A. (2012). Fiji: an open-source platform for biological-image analysis. *Nat. Methods*, 9(7):676–682.
- [195] Schneider, M., Hirsch, S., Weber, B., Székely, G., and Menze, B. H. (2014). TGIF: Topological Gap In-Fill for Vascular Networks, pages 89–96. Springer International Publishing.
- [196] Schuster, A., Sinclair, M., Zarinabad, N., Ishida, M., van den Wijngaard, J. P., Paul, M., van Horssen, P., Hussain, S. T., Perera, D., Schaeffter, T., Spaan, J. A., Siebes, M., Nagel, E., and Chiribiri, A. (2015). A quantitative high resolution voxel-wise assessment of myocardial blood flow from contrast-enhanced first-pass magnetic resonance perfusion imaging: microsphere validation in a magnetic resonance compatible free beating explanted pig heart model. *Eur Heart J Cardiovasc Imaging*, 16(10):1082–1092.
- [197] Secomb, T. W. (2008). Theoretical models for regulation of blood flow. *Microcirculation*, 15(8):765–775.
- [198] Secomb, T. W. (2016). Hemodynamics. Compr Physiol, 6(2):975–1003.

- [199] Secomb, T. W. (2017). Blood flow in the microcirculation. *Annual Review of Fluid Mechanics*, 49(1):443–461.
- [200] Secomb, T. W., Alberding, J. P., Hsu, R., Dewhirst, M. W., and Pries, A. R. (2013). Angiogenesis: an adaptive dynamic biological patterning problem. *PLoS Comput. Biol.*, 9(3):e1002983.
- [201] Secomb, T. W., Hsu, R., Park, E. Y. H., and Dewhirst, M. W. (2004). Green's function methods for analysis of oxygen delivery to tissue by microvascular networks. *Annals of Biomedical Engineering*, 32(11):1519–1529.
- [202] Secomb, T. W. and Pries, A. R. (2011). The microcirculation: physiology at the mesoscale. J. Physiol. (Lond.), 589(Pt 5):1047–1052.
- [203] Secomb, T. W. and Pries, A. R. (2013). Blood viscosity in microvessels: experiment and theory. *C R Phys*, 14(6):470–478.
- [204] Seidel, T., Edelmann, J. C., and Sachse, F. B. (2016). Analyzing remodeling of cardiac tissue: A comprehensive approach based on confocal microscopy and 3d reconstructions. *Ann Biomed Eng.*, 44(5):1436–1448.
- [205] Shipley, R. J. and Chapman, S. J. (2010). Multiscale modelling of fluid and drug transport in vascular tumours. *Bulletin of Mathematical Biology*, 72(6):1464–1491.
- [206] Sinclair, M. D., Lee, J., Cookson, A. N., Rivolo, S., Hyde, E. R., and Smith, N. P. (2015). Measurement and modeling of coronary blood flow. *Wiley Interdiscip Rev Syst Biol Med*, 7(6):335–356.
- [207] Smith, A. F., Secomb, T. W., Pries, A. R. and, S. N. P., and Shipley, R. J. (2014a). Structure-based algorithms for microvessel classification. *Microcirculation*, 22(2):99–108.
- [208] Smith, A. F., Shipley, R. J., Lee, J., Sands, G. B., LeGrice, I. J., and Smith, N. P. (2014b). Transmural variation and anisotropy of microvascular flow conductivity in the rat myocardium. *Annals of Biomedical Engineering*, 42(9):1966–1977.
- [209] Smith, K., Li, Y., Piccinini, F., Csucs, G., Balazs, C., Bevilacqua, A., and Horvath, P. (2015). CIDRE: an illumination-correction method for optical microscopy. *Nat. Methods*, 12(5):404–406.
- [210] Smith, R. M., Matiukas, A., Zemlin, C. W., and Pertsov, A. M. (2008). Nondestructive optical determination of fiber organization in intact myocardial wall. *Microsc. Res. Tech.*, 71(7):510–516.
- [211] Spaan, J., Kolyva, C., van den Wijngaard, J., ter Wee, R., van Horssen, P., Piek, J., and Siebes, M. (2008). Coronary structure and perfusion in health and disease. *Philos Trans A Math Phys Eng Sci*, 366(1878):3137–3153.
- [212] Spaan, J. A., ter Wee, R., van Teeffelen, J. W., Streekstra, G., Siebes, M., Kolyva, C., Vink, H., Fokkema, D. S., and VanBavel, E. (2005). Visualisation of intramural coronary vasculature by an imaging cryomicrotome suggests compartmentalisation of myocardial perfusion areas. *Med Biol Eng Comput*, 43(4):431–435.

- [213] Stamatelos, S., Kim, E., Pathak, A., and Popel, A. (2014). A bioimage informatics based reconstruction of breast tumor microvasculature with computational blood flow predictions. *Microvasc Res.*, 91:8–21.
- [214] Su, S. (2011). Modeling Blood Flow and Oxygen Transport in the Human Cerebral Cortex . *Doctoral Dissertation, University of Oxford*.
- [215] Szeliski, R. (2006). Image alignment and stitching: A tutorial. Found. Trends. Comput. Graph. Vis., 2(1):1–104.
- [216] T., M. (1971). Application of the scanning electron microscope to the study of the fine distribution of the blood vessels. *Arch Histol Jpn.*, 32(5):445–454.
- [217] Thygesen, K., Alpert, J. S., Jaffe, A. S., Simoons, M. L., Chaitman, B. R., White, H. D., Thygesen, K., Alpert, J. S., White, H. D., Jaffe, A. S., Katus, H. A., Apple, F. S., Lindahl, B., Morrow, D. A., Chaitman, B. A., Clemmensen, P. M., Johanson, P., Hod, H., Underwood, R., Bax, J. J., Bonow, R. O., Pinto, F., Gibbons, R. J., Fox, K. A., Atar, D., Newby, L. K., Galvani, M., Hamm, C. W., Uretsky, B. F., Steg, P. G., Wijns, W., Bassand, J. P., Menasche, P., Ravkilde, J., Ohman, E. M., Antman, E. M., Wallentin, L. C., Armstrong, P. W., Simoons, M. L., Januzzi, J. L., Nieminen, M. S., Gheorghiade, M., Filippatos, G., Luepker, R. V., Fortmann, S. P., Rosamond, W. D., Levy, D., Wood, D., Smith, S. C., Hu, D., Lopez-Sendon, J. L., Robertson, R. M., Weaver, D., Tendera, M., Bove, A. A., Parkhomenko, A. N., Vasilieva, E. J., Mendis, S., Bax, J., Baumgartner, H., Ceconi, C., Dean, V., Deaton, C., Fagard, R., Funck-Brentano, C., Hasdai, D., Hoes, A., Kirchhof, P., Knuuti, J., Kolh, P., McDonagh, T., Moulin, C., Popescu, B. A., Reiner, ., Sechtem, U., Sirnes, P. A., Tendera, M., Torbicki, A., Vahanian, A., Windecker, S., Morais, J., Aguiar, C., Almahmeed, W., Arnar, D. O., Barili, F., Bloch, K. D., Bolger, A. F., B?tker, H. E., Bozkurt, B., Bugiardini, R., Cannon, C., de Lemos, J., Eberli, F. R., Escobar, E., Hlatky, M., James, S., Kern, K. B., Moliterno, D. J., Mueller, C., Neskovic, A. N., Pieske, B. M., Schulman, S. P., Storey, R. F., Taubert, K. A., Vranckx, P., and Wagner, D. R. (2012). Third universal definition of myocardial infarction. Eur. Heart J., 33(20):2551-2567.
- [218] van den Borne, S. W., Diez, J., Blankesteijn, W. M., Verjans, J., Hofstra, L., and Narula, J. (2010). Myocardial remodeling after infarction: the role of myofibroblasts. *Nat Rev Cardiol.*, 7(1):30–37.
- [219] van den Wijngaard, J. P., Schwarz, J. C., van Horssen, P., van Lier, M. G., Dobbe, J. G., Spaan, J. A., and Siebes, M. (2013). 3D Imaging of vascular networks for biophysical modeling of perfusion distribution within the heart. *J Biomech*, 46(2):229–239.
- [220] van den Wijngaard, J. P., van Horssen, P., ter Wee, R., Coronel, R., de Bakker, J. M., de Jonge, N., Siebes, M., and Spaan, J. A. (2010). Organization and collateralization of a subendocardial plexus in end-stage human heart failure. *Am. J. Physiol. Heart Circ. Physiol.*, 298(1):H158–162.
- [221] van der Laan, A. M., Piek, J. J., and van Royen N. (2009). Targeting angiogenesis to restore the microcirculation after reperfused mi. *Nat Rev Cardiol.*, 6(8):515–523.

- [222] van Horssen, P., Siebes, M., Hoefer, I., Spaan, J. A., and van den Wijngaard, J. P. (2010). Improved detection of fluorescently labeled microspheres and vessel architecture with an imaging cryomicrotome. *Med Biol Eng Comput*, 48(8):735–744.
- [223] Velagaleti, R. S., Pencina, M. J., Murabito, J. M., Wang, T. J., Parikh, N. I., D'Agostino, R. B., Levy, D., Kannel, W. B., and Vasan, R. S. (2008). Long-term trends in the incidence of heart failure after myocardial infarction. *Circulation*, 118(20):2057–2062.
- [224] Vestweber, D. (2008). Ve-cadherin. the major endothelial adhesion molecule controlling cellular junctions and blood vessel formation. *Arterioscler Thromb Vasc Biol.*, 28(2):223–232.
- [225] Villa, A. D., Sammut, E., Nair, A., Rajani, R., Bonamini, R., and Chiribiri, A. (2016). Coronary artery anomalies overview: The normal and the abnormal. *World J Radiol*, 8(6):537–555.
- [226] Vracko, R. and Thorning, D. (1991). Contractile cells in rat myocardial scar tissue. *Lab Invest*, 65(2):214–227.
- [227] Wang, A., Yan, X., and Wei, Z. (2018). ImagePy: an open-source, Python-based and platform-independent software package for boimage analysis. *Bioinformatics*.
- [228] Watanabe, N., Akasaka, T., Toyota, E., Fujimoto, K., Kajita, T., Shigeto, F., Ogasawara, Y., and Yoshida, K. (2004). Three-dimensional microstructural abnormality of the coronary capillary network after myocardial reperfusion–comparison between 'reflow' and "noreflow'. *Circ J.*, 68(9):868–872.
- [229] Waters, S., Alastruey, J., Beard, D., Bovendeerd, P., Davies, P., Jayaraman, G., Jensen, O., Lee, J., Parker, K., Popel, A., Secomb, T., Siebes, M., Sherwin, S., Shipley, R., Smith, N., and van de Vosse, F. (2011). Theoretical models for coronary vascular biomechanics: progress and challenges. *Prog. Biophys. Mol. Biol.*, 104:49–76.
- [230] Wearn, J. T. (1928). The extent of the capillary bed of the heart. *J Exp Med*, 47:273–290.
- [231] Willems, I. E., Havenith, M. G., De Mey, J. G., and Daemen, M. J. (1994). The alpha-smooth muscle actin-positive cells in healing human myocardial scars. *Am J Pathol.*, 145(4):868–875.
- [232] Winter, K., Metz, L. H. W., Kuska, J. P., and Frerich, B. (2007). Characteristic quantities of microvascular structures in clsm volume datasets. *IEEE Transactions on Medical Imaging*, 26(8):1103–1114.
- [233] Wu, X., Kumar, V., Ross Quinlan, J., Ghosh, J., Yang, Q., Motoda, H., McLachlan, G. J., Ng, A., Liu, B., Yu, P. S., Zhou, Z.-H., Steinbach, M., Hand, D. J., and Steinberg, D. (2008). Top 10 algorithms in data mining. *Knowledge and Information Systems*, 14(1):1–37.
- [234] Zagorchev, L., Oses, P., Zhuang, Z. W., Moodie, K., Mulligan-Kehoe, M. J., Simons, M., and Couffinhal, T. (2010). Micro computed tomography for vascular exploration. J Angiogenes Res, 2:7.

- [235] Żak, M., Gkontra, P., Squadrito, M. L., Ferrarini, A., Mota, R. A., Rocha, S., Agüero, J., Vázquez, J., Palma, M., Ibáñez, B., and Arroyo, A. (2018). Combined VEGFA/S1P gene-cell therapy improves vascularization and limits adverse cardiac remodeling after myocardial infarction in mice. *Submitted*.
- [236] Zakrzewicz, A., W., S. T., and R., P. A. (2002). Angioadaptation: keeping the vascular system in shape. *News Physiol. Sci.*, 17:197–201.
- [237] Zeller-Plumhoff, B. (2017). An image-based computation of microvascular dysfunction. University of Southampton, Faculty of Engineering and the Environment, Doctoral Thesis.
- [238] Zhang, L., Wang, K., Zheng, F., Li, X., and Luo, S. (2014). 3D cardiac microvessels embolization imaging based on X-ray phase contrast imaging. *Biomed Eng Online*, 13:62.
- [239] Zhou, P. and Pu, W. T. (2016). Recounting Cardiac Cellular Composition. Circ. Res., 118(3):368–370.
- [240] Ziaeian, B. and Fonarow, G. C. (2016). Epidemiology and aetiology of heart failure. *Nat Rev Cardiol*, 13(6):368–378.
- [241] Zudaire, E., Gambardella, L., Kurcz, C., and Vermeren, S. (2011). A computational tool for quantitative analysis of vascular networks. *PLOS ONE*, 6(11):1–12.