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IMAGING AND ASSESSMENT OF MICROVASCULATURE-ON-CHIP

Bachelor's Thesis
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ABSTRACT

Emily Sihvola: Imaging and assessment of microvasculature-on-chip
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This bachelor's thesis is a literature review on the imaging and assessment of microvasculature-on-chip using various imaging techniques. Microvasculature plays a vital role in human body organs, and its dysfunctions have been linked to many diseases. Although animal models have traditionally been used for microvascular research, recent advances in fabrication strategies have led to the development of microfluidic devices, known as microvasculature-on-chip, that enable the in vitro modelling of the microvasculature. These devices offer several advantages over traditional animal models, including improved control over experimental conditions, reduced cost, and the ability to use human cells and tissues.

This literature review provides a comprehensive overview of the available imaging techniques for microvasculature-on-chip, including their advantages, limitations, and applications. Recently, advanced imaging techniques, such as fluorescence microscopy, selective plane illumination microscopy (SPIM), and photoacoustic tomography (PAT), have been applied to visualize microvasculature-on-chip. These imaging techniques provide high spatial resolution, deeper penetration depth, and practical information such as blood flow and oxygenation. However, analysing and quantifying the microvasculature images obtained from these techniques require sophisticated algorithms and software.

The thesis concludes that the combination of microfluidic devices with various imaging techniques provides an excellent platform to study microvascular networks in vitro, which can lead to important insights into the dynamics and behaviour of the microvasculature in various pathological conditions. Fluorescence microscopy, SPIM, and PAT have been discussed as the most commonly used imaging techniques for microvasculature-on-chip. Each imaging method has its unique advantages and limitations, and the most suitable imaging method depends on the specific research question and the desired spatial and temporal resolution. The thesis also highlights the challenges posed by the small size and complex architecture of microfluidic devices, the artifacts introduced by the materials used to construct these devices, and the high cost of equipment and expertise required for accurate and reliable microvasculature assessment.

Keywords: microvasculature-on-chip, imaging, assessment, fluorescence microscopy, SPIM, PAT

The originality of this thesis has been checked using the Turnitin OriginalityCheck service.

TIIVISTELMÄ

Emily Sihvola: Mikrovaskulaarisaation kuvantaminen ja arviointi in vitro kudosmalleissa
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Ihmisen elimissä olevaa mikrovaskulaarisaatiota mallinnetaan in vitro kudosmalleilla. Nämä kudosmallit edesauttavat tutkijoita ymmärtämään tautien kehittymistä, sillä mikrovaskulaarisaatio muuttuu monissa sairauksissa, esimerkiksi syövässä ja diabeteksessa. Perinteisesti tutkimuksissa on käytetty eläinmalleja tutkiessa mikrovaskulaarisaatiota. Valmistusmenetelmien kehittyminen kuitenkin johti mikrofluidisten alustojen ja samalla in vitro kudosmallien luontiin. In vitro kudosmalleilla on useita etuja perinteisiin eläinmalleihin verrattuna, muun muassa parempi kontrolli kokeellisiin olosuhteisiin, edullisemmat kustannukset sekä kyky käyttää ihmisen soluja, että kudoksia.

Tutkiessa mikrovaskulaarisaatiota in vitro kudosmalleilla vaaditaan prosessien dokumentointiin kuvantamismenetelmiä, joilla on korkea resoluutio. Tämän seurauksena kandidaatintyön tarkoituksena oli selvittää kolme yhteensopivinta kuvantamismenetelmää ja syventyä tutkimusartikkeleihin, joissa käytettiin näitä kolmea kuvantamismenetelmää. Kirjallisuuskatsauksessa käsitellään mikrovaskulaarisaation kuvantamista ja arviointia in vitro kudosmalleilla eri kuvantamismenetelmillä. Jokaisella kuvantamismenetelmällä on etunsa ja rajoitteensa, mitkä vaikuttavat kuvantamismenetelmän valintaan. Kolme yhteensopivinta kuvantamismenetelmää mikrovaskulaarisaation visualisointiin resoluution, tunkeutumissyvyyden sekä kuvattavien kohteiden perusteella ovat fluoresenssimikroskopia, valikoiva tasovalaistusmikroskopia (SPIM) ja fotoakustinen tomografia (PAT). Tekniikoista saatujen mikrovaskulaarisaatiokuvien lisäksi, näiden analysointi ja kvantifiointi edellyttää pitkälle kehitettyjä algoritmeja ja ohjelmistoja.

Tuloksien perusteella voidaan todeta, että in vitro kudosmallien ja erilaisten kuvantamismenetelmien yhdistäminen tarjoaa lupaavan alustan mikroverisuoniverkostojen tutkimiseen in vitro, mikä voi johtaa merkittäviin tietoihin mikrovaskulaarisaation dynamiikasta ja käyttäytymisestä erilaisissa patologisissa olosuhteissa. Fluoresenssimikroskopiaa, valikoivaa tasovalaistusmikroskopiaa ja fotoakustista tomografiaa on pidetty yleisimpänä mikrovaskulaarisaation kuvantamistekniikkana. Kuitenkin sopivimman kuvantamismenetelmän valinta riippuu erityisestä tutkimuskysymyksestä sekä halutusta spatiaalisesta ja ajallisesta resoluutiosta.

Lisäksi kandidaatintyössä tuodaan esiin in vitro kudosmallien pienen koon ja monimutkaisen rakenteen aiheuttamat haasteet, näiden kudosmallien rakentamiseen käytettyjen materiaalien tuomat häiriöt sekä tarkan ja luotettavan mikrovaskulaarisaation arvioinnin vaatimat korkeat laitteisto- ja osaamiskustannukset.

Avainsanat: mikrovaskulaarisaatio, in vitro kudosmalli, kuvantaminen,
fluoresenssimikroskopia, valikoiva tasovalaistusmikroskopia, fotoakustista tomografia,

Tämän julkaisun alkuperäisyys on tarkastettu Turnitin OriginalityCheck –ohjelmalla.

CONTENTS

LIST OF SYMBOLS AND ABBREVIATIONS

in vitro	Process performed outside living organisms
in vivo	Process conducted inside the living organisms
SPIM	Selective plane illumination microscopy
PAT	Photoacoustic tomography
PDMS	Polydimethylsiloxane
3D	Three-dimensional
EC	Endothelial cell
ATP	Adenosine triphosphate
NP	Nanoparticle
EGM	Endothelial growth medium
HUVEC	Human umbilical vein endothelial cell
NHLF	Normal human lung fibroblast
MRI	Magnetic resonance imaging
µ-CT	Microcomputed tomography
OCT	Optical coherence tomography
LDPI	Laser Doppler perfusion imaging
LSCI	Laser speckle contrast imaging
HIS	Hyperspectral imaging system
NIBIB	National Institute of Biomedical Imaging and Bioengineering
ECM	Extracellular matrix
SDCM	Spinning disk confocal microscopy
2D	Two-dimensional
DSLM	Digitally scanned laser light-sheet fluorescence microscopy

1. INTRODUCTION

The microvasculature plays a crucial role in the physiological functions of various organs in the human body, including the heart, brain, liver, and kidneys. Dysfunctions of the microvasculature have been linked to many diseases, including cancer, hypertension, and diabetes. According to a study conducted by Ritchie et al., the majority of deaths worldwide are caused by diseases related to the cardiovascular system, which highlights the importance of studying microvasculature (Ritchie, Spooner and Roser, 2018).

Traditionally, the primary emphasis in modern medicine has been on larger vessels, such as arteries and veins. However, the study of microvascular research has become increasingly important in recent years. The traditional approach to microvascular research has relied heavily on animal models, such as rats and zebrafish (Moses *et al.*, 2021). While these models provide valuable insights into microvascular physiology, their use can be limited by cost, ethical considerations, and the differences between animal and human physiology.

Recent advances in biofabrication strategies and engineered in vitro systems have provided promising tools for studying microvasculature (Pradhan *et al.*, 2020). Among these, microfluidic devices, also known as microvasculature-on-chip, have emerged as a powerful platform for the in vitro modelling of microvasculature (Santra, 2020, pp. 213–214). These devices enable researchers to mimic the complex three-dimensional structure of the microvasculature and the fluidic environment in vivo, allowing for the investigation of the physiological and pathological processes of the microvasculature. In addition, these devices offer several advantages over traditional animal models, such as improved control over experimental conditions, reduced cost, and the ability to use human cells and tissues (Pradhan *et al.*, 2020).

Imaging and assessing microvasculature-on-chip are critical to understanding microvascular physiology, transport, and function in vitro. Traditional imaging techniques, such as confocal and bright-field microscopy, have been widely used to visualise the microvasculature in vitro. However, these techniques have limitations, such as low spatial resolution, limited penetration depth, and lack of practical information.

Recently, advanced imaging techniques, such as fluorescence microscopy, selective plane illumination microscopy (SPIM), and photoacoustic tomography (PAT), have been applied to visualise the microvasculature-on-chip (Moses *et al.*, 2021). These imaging techniques provide high spatial resolution, deeper penetration depth, and practical information, such as blood flow and oxygenation. However, analysing and quantifying the microvasculature images obtained from these techniques require sophisticated algorithms and software.

Therefore, this bachelor's thesis is a literature review that aims to find the most suitable imaging methods for studying microvascularity in vitro and to describe the relevance of microvessels in the human body. The review will provide a comprehensive overview of the available imaging techniques for microvasculature-on-chip, including their advantages, limitations, and applications. The relevance of microvessels in different physiological and pathological conditions will also be discussed.

2. THE BLOOD-VASCULAR SYSTEM

The primary function of the blood vascular system, also known as the circulatory system, is to transport blood all over the body. The blood vascular system comprises two circulatory systems: systemic and pulmonary circulation. Systemic circulation transports oxygen and nutrient-rich blood all over the body. In contrast, pulmonary circulation transports oxygen-poor blood to the lungs for gas exchange, and afterwards, the oxygenated blood returns to the systemic circulation through the heart. (Betts *et al.*, 2013, p. 827)

In addition to transporting oxygen and nutrients to the cells, the circulatory system removes metabolic byproducts from the cells and maintains homeostasis. Homeostasis is a condition in which the body functions correctly and is stable. Its maintenance involves controlling the body's temperature and adjusting the oxygen and nutrient levels in different physiological states. Examples of typical metabolic byproducts are ammonia and urea, which result from protein metabolism. (Pappano, 2019, pp. 1–9)

The blood circulatory system comprises a heart, blood vessels and blood. First, the heart is an organ which pumps blood to blood vessels. The heart contains four chambers, two atria and two ventricles, made of myocardial muscle and is powered by electrical impulses. Second, blood is fluid that passes through the circulatory system. Blood consists of plasma and cells, such as platelets, red blood, and white blood cells. Lastly, blood vessels are tube-shaped structures which carry blood through the tissues. The primary types of blood vessels are introduced in the paragraphs below. (Betts *et al.*, 2013, pp. 783–974)

2.1 Types of blood vessels

The blood vessels can be divided into two levels based on the sizes of the vessels. The macrovessels, such as arteries and veins, are on the millimetre scale. On the other hand, microvessels, such as arterioles, capillaries, and venules, are on the micrometre scale. (Pappano, 2019, pp. 1–9) However, blood vessels are usually divided into five main types based on structure and function. These main types of blood vessels are arteries, veins, arterioles, venules, and capillaries. In addition to the main types, there are

metarterioles, a combination of an arteriole and a capillary. (Betts *et al.*, 2013, pp. 892–896)

Although the diverse kinds of blood vessels have various structures, they also have the same general characteristics. For instance, all blood vessels have a lumen, representing the space inside the blood vessel where the blood flows. However, the lumen size varies between the vessels because they help maintain blood pressure. For comparison, veins have the most significant lumen and capillaries the smallest. Besides the size, the shape of the lumen varies due to the vessel's diameter and the wall's thickness. For example, the lumens of arteries occur rounded because of thicker vessel walls and smaller diameters, and venous lumens occur flattened. (Betts *et al.*, 2013, pp. 889–891)

Another similarity between the blood vessel types, except capillaries, is the structures of vessel walls. However, all blood vessels contain endothelium, a single-layered line inside the blood vessel. The walls of arteries, arterioles, veins, and venules have the same three tissue layers, known as tunics. The innermost wall layer is called the tunica intima. The next layer is called the tunica media, and the outer layer is the tunica externa. However, the form and thickness of each layer differ according to the type of blood vessels. (Betts *et al.*, 2013, pp. 891–892)

Table 1 illustrates the differences between blood vessel types based on different parameters, such as the level of the vasculature, function, layers of the wall, valves, vessel wall thickness, muscle and elastic fibres, the flow rate of blood, blood pressure and diameter of the lumen. The numerical wall thickness and diameter values are from the book *Cardiovascular Physiology* (Pappano, 2019, pp. 1–9). On the other hand, numerical values for the blood flow rate and blood pressure are estimated from the book *Anatomy and Physiology* (Betts *et al.*, 2013, p. 906).

Table 1. *Different blood vessels*

VESSELS	ARTERIES	VEINS	ARTERIOLES	VENULES	CAPILLARIES
LEVEL OF VASCULATURE	Macro	Macro	Micro	Micro	Micro
FUNCTION	Transfers blood from the heart	Transfers blood to the heart	Transfers blood from arteries to capillaries	Transfers blood from capillaries to veins	The material exchange between the blood and tissues
WALL LAYERS	Three: Tunica intima, Tunica media, Tunica Externa	Three: Tunica intima, Tunica media, Tunica Externa	Three: Tunica intima, Tunica media, Tunica Externa	Three: Tunica intima, Tunica media, Tunica Externa	One: Tunica intima
VALVES	No	Yes	No	No	No
WALL THICKNESS	1,0 mm	0,5 mm	6,0 μm	1,0 μm	0,5 μm
MUSCLE AND ELASTIC FIBRES	Elastic tissue, smooth muscle, and fibrous tissue	Elastic tissue, smooth muscle, and fibrous tissue	Smooth muscle	Fibrous tissue	No
THE FLOW RATE OF THE BLOOD	22-33 cm/s	3 cm/s	6 cm/s	2 cm/s	2 cm/s
AVERAGE BLOOD PRESSURE	90–100 mmHg	10 mmHg	70 mmHg	20 mmHg	35 mmHg
DIAMETER	4 mm	5 mm	30 μm	20 μm	8 μm

2.2 Anatomy and Physiology of the Microvasculature

Microvasculature comprises arterioles, venules, metarterioles and capillaries. Firstly, the arteriole is a small, micrometre-sale artery that connects to a capillary. Secondly, capillaries are microscopic blood vessels in which perfusion occurs. Perfusion represents a process which supplies blood to all tissues in the body. Thirdly, a venule is a tiny vein that connects to capillaries and transports to more prominent veins. Lastly, a metarteriole combines a capillary and an arteriole that connects to arteriole, capillaries, and venules. (Betts *et al.*, 2013, pp. 892–896)

The microvessels mentioned above create a system known as microcirculation, or a capillary bed, illustrated in *Figure 1*. The capillary bed is the functional unit of microcirculation and can exchange water, oxygen, nutrients, metabolic byproducts and carbon dioxide between blood and tissues via passive diffusion. Passive diffusion is a phenomenon that allows molecules to move from higher concentrations to lower concentrations without additional energy. (Betts *et al.*, 2013, pp. 894–895)

The capillary bed comprises 10 to 100 capillaries, which receive the blood from the metarteriole. On the other hand, the metarteriole gets blood from the arteriole. The precapillary sphincter regulates blood flow with circular smooth muscle cells around the junction of the metarteriole and capillary. Consequently, the flow rate of blood is low in capillaries. (Betts *et al.*, 2013, pp. 894–895)

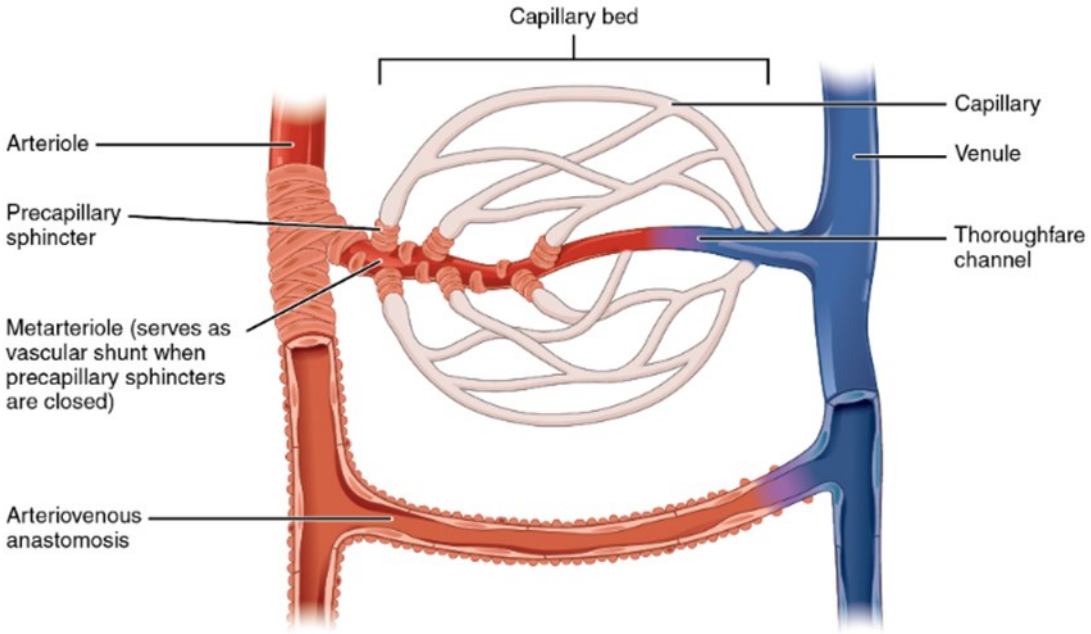

Figure 1. *Capillary bed. An arteriole carries the blood to a capillary bed, where a precapillary sphincter regulates the blood flow. A thoroughfare channel connects a me-*

tarteriole and a venule. An arteriovenous anastomosis directly connects the arteriole to the venule. (Betts et al.*, 2013, p. 895)*

2.2.1 Arterioles

Arterioles are micrometre-sized arteries that transport blood to capillaries. Structurally, arterioles have the same wall layers, known as tunics, as arteries. However, the thickness of tunics is much thinner. To illustrate, the middle layer, called tunica media, has only one or two layers of smooth muscle cells. On the other hand, tunica intima, the inner layer, and tunica externa, the outer layer of the arteriole, have the same form as the large arteries. (Betts *et al.*, 2013, p. 893)

The primary function of arterioles is to regulate blood pressure by resisting blood circulation. This is allowed due to smooth muscle contractions and the diameter of the lumen. The specific diameter of the lumen of arterioles is regulated with neural and chemical signals. In addition, the primary mechanisms for blood flow circulation in arterioles are vasodilation and vasoconstriction. In this context, vasodilation stands for the widening of arterioles due to the relaxation of the walls of the blood vessel. Similarly, vasoconstriction represents an arteriole's narrowing caused by the tunica media's smooth muscle cells. (Betts *et al.*, 2013, p. 893)

2.2.2 Venules

In the same way, as arterioles are small arteries, venules are tiny veins. The tunics in venules contain endothelium, the middle layer, and the outer layer. The middle layer, tunica media, consists of muscle cells and elastic fibres. Correspondingly, thin tunica externa consists of fibres of connective tissue. (Betts *et al.*, 2013, p. 895)

The main differences between venules and veins are that venules do not contain valves or vasa vasorum, blood vessels inside the large blood vessel. Vasa vasorum are the blood vessels that provide oxygen and nutrients to the blood vessels. On the other hand, valves prevent the backflow of blood. (Betts *et al.*, 2013, pp. 890–895)

In addition to structural differences, venules have contrasting functions compared to veins. Besides capillaries, venules enable diapedesis, a process where white blood cells penetrate through the walls of venules and transfer into the tissues. On the contrary, venules transport blood from capillary beds to veins. (Betts *et al.*, 2013, p. 895)

2.2.3 Capillaries

Capillaries are the type of microvessels where perfusion occurs. During perfusion, substances are exchanged between the blood and interstitial fluid surrounding the cells. This blood flow in capillaries could be steady, but it is irregular and pulsating, also known as vasomotion. Vasomotion is controlled by chemical signals resulting from changes in internal conditions. These measured levels are lactic acid, oxygen, carbon dioxide and hydrogen ion. For instance, during exercising, the capillary beds in skeletal muscles are open due to decreased oxygen levels and increased lactic acid, carbon dioxide and hydrogen ion levels. (Betts *et al.*, 2013, pp. 893–895)

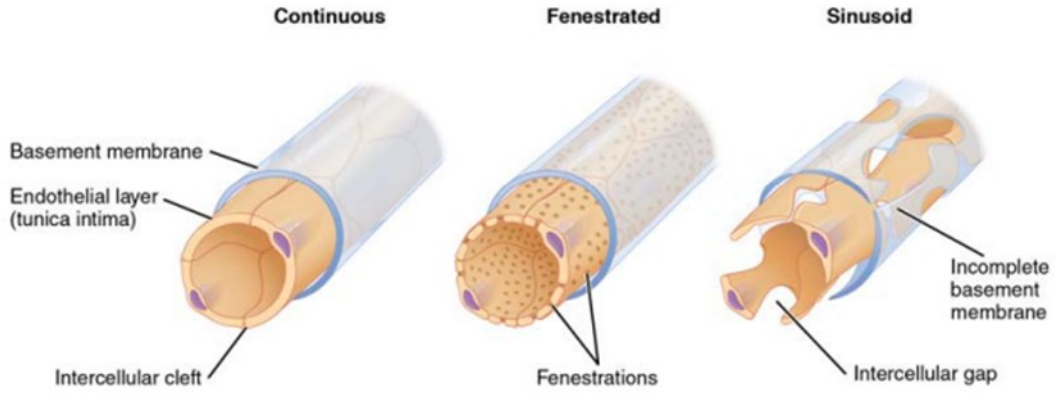

Figure 2. *The types of capillaries and their wall structure (Betts* et al.*, 2013, p. 894)*

Capillaries can be divided into three types, which are continuous, fenestrated and sinusoid capillaries. The types mentioned above differ from each other based on penetrating power. As illustrated in Figure 2, continuous capillaries are the most common and have the tightest wall structure that only passes through water, small molecules, and leucocytes. Next, fenestrated capillaries have pores in their walls and pass through larger molecules, such as nutrients, in the small intestine. The last type of capillaries is sinusoid capillaries, which are the rarest and have the most significant intercellular gaps due to the permeability of large proteins and cells. For example, the liver and bone marrow sinusoids. (Betts et al., 2013, pp. 893–894)

3. MICROVASCULATURE-ON-CHIP

To image and assess microvascularity in vitro, one must understand how microvasculature-on-chips are fabricated and their properties. These functions and mechanisms will affect the selection of the most suitable imaging methods. This chapter introduces and describes the principles of microvasculature-on-chip and the effects caused by fabrication methods and structures of the microvasculature-on-chip.

Microvasculature-on-chip is a system that combines microfluidics, tissue engineering, microfabrication technologies and biomaterials. During the development process, the microenvironment in the chip is regulated with different fluid flows, which represent microfluidics, growth factors and cells, which represent tissue engineering and biomaterials. Depending on the used biomaterial, the mechanical properties of the microvasculature-on-chip can be controlled. (Santra, 2020, p. 214) In addition, microfabrication technologies represent the methods of fabrication of microvasculature-on-chip.

On the other hand, the term microvasculature-on-chip comprises various kinds of microvasculature models. The main differences between the chips are the structure, the location of the modelled microvasculature in the body, fabrication methods and the studied process of the microvasculature. To illustrate, the size of one chip, which is fabricated by Barbato et al., is 210 *µm* wide, 50 *µm* high and 2,7 *cm* long. (Barbato *et al.*, 2021). Furthermore, Dávila et al. fabricated a microfluidic chip which models nanomedicine transport by combining PDMS, human endothelial cells and nanoparticles. This chip is made with stereolithography 3D printing of PDMS due to the semi-circular shape of the blood vessels. (Dávila, Cacheux and Rodríguez, 2021) The vascularisation technique regarding the microfluidic chip fabricated by Dávila et al. was EC lining-based method. On the contrary, modelling cancerous tumours require more natural microvasculature, which can be succeeded with vasculogenesis- and angiogenesis-based methods. During these methods, the ECs do not require guiding by other microstructures, and the self-assembly of ECs is possible. (Santra, 2020, pp. 216–221)

3.1 Principles of microvasculature-on-chip

The microvasculature-on-chip aims to mimic both the 3D structure and functions of microvessels outside the human body. These functions include perfusion and selective barriers in the same way as in vivo microvasculature. The selective barrier function represents a capillary wall between the blood and tissue. This function controls the permeability of molecules due to active or passive transport. For instance, diffusion is a passive transport method compared to active transportation, which transports molecules from low to high concentrations. Consequently, active transportation requires energy, known as ATP. (Santra, 2020, p. 214)

Furthermore, constructing a homeostatic environment for microvessels is essential to modelling diseases (Santra, 2020, pp. 214–215). One sign of the progression of diseases, like ischemic microvascular disease, is that blood flow in microvessels and oxygenation in tissues are decreased. Consequently, the homeostatic environment in tissues deteriorates, and oxygen deficiency causes damage to the tissue. (Moses *et al.*, 2021) For these reasons, it is essential to develop a microfluidic device in which the flow of artificial blood and interactions between nanoparticles (NPs) and the endothelium layer in the chip can be controlled and monitored using imaging methods (Dávila, Cacheux and Rodríguez, 2021).

Another factor that influences the microvasculature-on-chip device's development process, besides perfusion and diffusion, is artificial blood flow (Pradhan *et al.*, 2020). Artificial blood combines endothelial growth medium (EGM) and xanthan gum. Endothelial growth medium contains substances that endothelial cells require, such as growth factors and vitamins. In addition to EGM, xanthan gum is a molecule which thickens the artificial blood to control the viscosity. Consequently, the flow in the microfluidic device is due to pressure difference. (Dávila, Cacheux and Rodríguez, 2021)

On the other hand, mimicking in vivo physiological conditions in a microvessels-on-chip environment requires specific shear flow, viscosity, temperature, and pressure. Firstly, shear flow presents flow caused by the forces in the fluid. Secondly, viscosity represents the resistance of the fluid's movement, which is why thicker liquids have a more significant viscosity. Lastly, dropping pressure for specific periods regulates the microfluidic device's flow rate. (Dávila, Cacheux and Rodríguez, 2021)

3.2 Fabrication of microvasculature-on-chip

Structurally, 3D microvasculature-on-chip comprises microchannels. These microfluidic devices are made of hydrogels, polydimethylsiloxane (PDMS), or their combination.

The foundation consists of PDMS, and inside the PDMS membrane, the hydrogel is utilised (Ferrari *et al.*, 2023). These techniques and structures affect this chapter's decisions and imaging results.

PDMS is a silicon-based organic polymer fabricating microvasculature-on-chips due to material properties, such as optical clarity, chemical non-reactivity, and non-toxicity. These properties are necessary for the chips, which are used to model diseases and transportation of medicine. For instance, the chip must be optically transparent to image the desired processes from the chip. Transparency of the chip enables the use of microscopy, which is introduced and described in Chapter 4. In addition, a chemically inert device does not react with the substances inside the chip. As a consequence, PDMS does not affect the results of the research. (Pradhan *et al.*, 2020)

Another way to fabricate hollow and transparent microvasculature-on-chips is with hydrogels. Hydrogels are polymer-based 3D networks that absorb and contain extensive quantities of water (Narain, 2020, p. 203). There is a variety of different hydrogels that are either synthetic or natural polymers. Fabrication of microvasculature-on-chip most commonly utilises collagen and fibrin, which are natural hydrogels. In addition, these natural polymers are biocompatible and non-toxic, contributing to the proper functionality of the chip. Furthermore, hydrogels are utilised because of their flexibility. To illustrate, the mechanical properties of these hydrogels can be adjusted by changing the temperature or the pH of collagen or by changing the concentration of fibrinogen and thrombin in fibrin. (Santra, 2020, p. 228) However, collagen and fibrin are not transparent, which causes potential distractions in images.

In addition to fabrication materials, the fabrication techniques affect the decision of imaging method or the final image results. The most commonly used EC lining-based, vasculogenesis-based, angiogenesis-based, and hybrid approaches (Santra, 2020, p. 216). As mentioned above, microfluidic chips fabricated utilising vasculogenesis- and angiogenesis-based methods have a more natural microvascular structure compared to the EC lining-based method. On the other hand, the hybrid method combines vasculogenesis, angiogenesis, and EC lining techniques, resulting in microfluidic chip models whole capillary bed system. (Santra, 2020, pp. 216–225)

Consequently, hybrid chips contain additional structures and cells, such as human umbilical vein endothelial cells (HUVECs), normal human lung fibroblasts (NHLFs) and protein fibres (Santra, 2020, p. 225). These additional structures complicate the assessment of the images that are described in *Chapter 5*.

4. IMAGING METHODS FOR MICROVASCULATURE-ON-CHIP

This chapter introduces different imaging methods and describes the principles of the three most suitable methods for imaging microvasculature-on-chip. An imaging device forms a visual representation of an object, which is essential to assess the microvasculature-on-chip. As mentioned in *Chapter 3*, the size of microvasculature-on-chips is on the microscale, which is one of the critical requirements for selecting an imaging method. Consequently, the suitable imaging method must have a high resolution to form a sharp image of microvasculature-on-chip.

4.1 Comparing different imaging methods

Visualising microvasculature-on-chip is possible with various imaging methods depending on the subject studied and the wanted information. *Table 2* illustrates ten commonly used imaging methods which can be applied to image microvasculature-on-chips. These imaging methods are magnetic resonance imaging (MRI), microcomputed tomography (micro-CT), optical coherence tomography (OCT), laser Doppler perfusion imaging (LDPI), laser speckle contrast imaging (LSCI), capillaroscopy, photoacoustic tomography (PAT), a hyperspectral imaging system (HIS), fluorescence microscopy and selective plane illumination microscopy (SPIM).

The imaging methods mentioned above can be divided into seven categories based on their principles. First, the most significant category is microscopy-based imaging techniques, such as capillaroscopy, fluorescence microscopy and SPIM, which utilise microscopes to form images of objects. Second, laser-based imaging methods, such as LDPI and LSCI, utilise laser light to monitor perfusion and assess blood flow (Guven *et al.*, 2023). The other imaging methods are categorised based on the principles introduced in their name. To illustrate, MRI is categorised as magnetic resonance-based imaging and micro-CT as X-ray-based imaging (Cheriyedath, 2017).

In addition to the mentioned categories, the majority of the imaging methods can be classified as optical imaging techniques, such as optical coherence tomography (OCT), laser speckle contrast imaging (LSCI), laser Doppler perfusion imaging (LDPI), photoacoustic tomography (PAT), a hyperspectral imaging system (HIS), capillaroscopy, fluo-

rescence microscopy and selective plane illumination microscopy (SPIM). These optical imaging techniques utilise light and properties of photons to form images of selected objects, which is microvasculature-on-chip in this thesis. (NIBIB, 2020)

Table 2 is a summary table which illustrates mentioned imaging methods and parameters. The parameters in *Table 2* describe the clinical use, objects to image from microvasculature-on-chip, accuracies, resolutions, the use of contrast, the imaging depth and the restrictions of these imaging methods mentioned above. All of the values of these parameters are considered in the context of imaging the microvasculature-on-chip. Based on the solutions to these parameters, the three most suitable imaging methods are chosen, and operating principles are described in *Subchapters 3.2, 3.3* and *3.4*.

Table 2. *Different imaging methods*

	MAGNETIC RESONANCE IMAGING (MRI)	**MICRO COMPUTED TOMOGRAPHY (MICRO-CT)**	**OPTICAL COHERENCE TOMOGRAPHY (OCT)**	**LASER DOPPLER PERFUSION IMAGING (LDPI)**	**LASER SPECKLE CONTRAST IMAGING (LSCI)**	**CAPILLAROSCOPY**	**PHOTOACOUSTIC TOMOGRAPHY (PAT)**	**HYPERSPECTRAL IMAGING METHODS (HIS)**	**FLUORESCENCE MICROSCOPY**	**SELECTIVE-PLANE ILLUMINATION MICROSCOPY (SPIM)**
CLINICAL USAGE	Clinically used	Clinically used	Clinically used	Clinically used	Clinically used	Clinically used	Preclinically used	Clinically used	Preclinically used	Preclinically used
IMAGING OBJECT IN THE CHIP	Blood vessels: vessel size, blood flow, vascular density	Blood vessels: vessel size, blood flow, vascular density	Blood vessels: vessel size, blood flow, vascular density Microfluidic channels: geometry of channels, flow rates, mechanics of the fluid	Blood vessels: vessel size, blood flow, vascular density	Blood vessels: vessel size, blood flow, vascular density	Morphological properties and architectures of capillaries	Blood vessels: vessel size, blood flow, vascular density	Extracellular matrix (ECM): structure, formation, remodelling	Cells: cell morphology, behaviour, cell communication Extracellular matrix (ECM): structure, formation, remodelling Particles: other nanoparticles (NPs)	Cells: cell morphology, behaviour, cell communication Extracellular matrix (ECM): structure, formation, remodelling Microfluidic channels: geometry of channels, flow rates, mechanics of the fluid Particles: nanoparticles (NPs)

ACCURACY	Limited by the quality of the MRI device, imaging parameters and quality of the microvasculature-on-chip	Limited by the dosage of radiation	Limited by the quality of the chip, accurate alignment and movement correction	Limited by the sensitivity to motion artifact	Limited by accurate alignment of the chip and movement correction	Limited by the quality of the chip	Limited by the quality of the chip, the sensitivity of acoustic attenuation and absorption in the microfluidic channels, accurate alignment and movement correction	Limited by the quality of the chip	Limited by the quality of the chip, the choice of fluorophores and labelling techniques	Limited by the quality of the chip
RESOLUTION	High 1–2 mm	High 5–150 µm	High 15–40 µm	Low temporal resolution	High spatial and temporal resolution	High spatial resolution dependent on the imaging mode	High Few micrometres to several micrometres, depending on the acoustic frequency	Spatial information: 0,1 mm/pixel at a distance of 50 cm Spectral resolution: 5 nm	High spatial resolution	High spatial resolution
CONTRAST	Gadolinium-based contrast agents Iron oxide NPs	X-ray attenuation contrasts: iodine or gold NPs	Used between scattering properties of tissues	Used between the reflectivity of the tissues and their environment	Used between speckled patterns of diffused light	Used between the reflectivity of the tissues and their environment	Used between the acoustic properties of the tissues Optical contrasts: gold NPs and fluorescent dyes	Used between the staining of the tissues Histological stains: haematoxylin and eosin	Requires a contrast: fluorescent tags	Fluorescently labelled proteins and dyes
DEPTH	High imaging depth 1 mm to 1 m	Up to a few centimetres	Up to a few millimetres	Up to a few millimetres	Up to a few millimetres	Up to a few hundred micrometres	Up to a few centimetres	Up to a few hundred micrometres	Up to a few hundred micrometres	Up to a few hundred micrometres
RESTRICTIONS AND LIMITATION	Low resolution compared to optical imaging techniques Spatial resolution decreases when the imaging depth increases MRI scanners can be expensive	The use of radiation The difficulty of visualising chancing microfluidic devices The specialised micro-CT scanner can be expensive.	Media opacities can interfere with the imaging Sensitive to motion artifacts, which affect the quality of the images.	Difficulties in differentiating arterial and venous flow	Sensitive to motion artifacts affecting the quality of images	Limited picture quality Limited use in imaging of microfluidic devices.	Limited by the acoustic properties, which affect the quality of images Sensitive to motion artifacts	Cost and complexity It may cause damage or distortion to the chip.	Limited by low spatial resolution because of the diffraction of light Potential photobleaching and phototoxicity	Shadows and stripes A specialised microscope is required, which can be time-consuming to set up.

Based on the answers in *Table 2,* each imaging method has advantages and disadvantages regarding microvasculature-on-chips imaging. However, this thesis focuses on three imaging methods. According to the imaging object row in *Table 2,* the most comprehensive imaging method is selective plane illumination microscopy (SPIM) since it can be utilised to visualise cells, ECM, microfluidic channels and nanoparticles from microvasculature-on-chips. In addition, accuracy in SPIM is only limited to the quality of the microfluidic device. Because of the high spatial resolution, SPIM is one of the most suitable imaging methods.

The second method is the most commonly used, fluorescence microscopy, due to desired properties, such as high spatial resolution and the possibility of using fluorescent labels. In addition, SPIM is a type of fluorescence microscopy that differs from the traditional fluorescence microscopy described in *subchapter 4.2*. (Huisken and Stainier, 2009)

The third imaging method is photoacoustic tomography (PAT) due to its imaging object, blood vessels. Neither fluorescence microscopy nor SPIM images blood vessels. In addition, PAT combines the advantages of optical and acoustic imaging techniques and provides high-resolution 3D images with high contrast (Xia, Yao and Wang, 2014). The principle of PAT is described in *subchapter 4.4*.

In conclusion, the three imaging methods chosen are fluorescence microscopy, selective plane illumination microscopy (SPIM) and photoacoustic tomography (PAT) due to their answers in *Table 2* and their suitability to image microvasculature-on-chip according to their imaging objects, resolution and imaging depth. In addition, the limitations of these imaging methods do not significantly reduce the resulting images.

4.2 Fluorescence Microscopy

Fluorescence microscopy is a type of optical microscopy that utilises fluorophores. Fluorophores represent molecules that can absorb photons with specific energy and emit photons with energy lower than absorbed. For instance, the most commonly utilised fluorophores in microscopy are fluorescent molecules. Consequently, emitted photons have longer wavelengths compared to absorbed energy, resulting in a phenomenon known as fluorescence. (Kubitscheck, 2017, pp. 86–87)

A fluorescence microscope has been constructed to use short-wavelength light to excite the fluorescence to form high-resolution real-time images with high image contrast. The high-energy light is emitted from a light source, such as a mercury lamp, that reflects through a two-coloured mirror toward the sample. This mirror is located at a 45° angle compared to incident light and reflects a certain length of photons to the sample allowing photons with lower energy to pass through. These passed-through photons are considered the fluorescence emission. Furthermore, the type of two-coloured mirror is chosen based on the application. To illustrate, the selected mirror varies depending on the fluorescent labels or probes used in microvasculature-on-chip imaging. (Kubitscheck, 2017, pp. 95–97)

In addition to the light source, sample and two-coloured mirror, an inverted fluorescence microscope contains various structures, such as an objective lens, collector lens,

and ocular lens, also known as the eyepiece, digital camera, halogen lamp, emission filter and excitation filter. This microscope with the eyepiece positioned below the sample does not disturb the sample. As a result, inverted fluorescence microscopes enable imaging of living cells and microfluidic devices, such as microvasculature-on-chips. (Kubitscheck, 2017, pp. 96–97)

After separating emitted light and fluorescence with filters, fluorescence is detected as bright-coloured areas on dark image background. Consequently, fluorescence microscopy enables imaging of dynamic interaction between artificial endothelium and nanoparticles and other parameters in the context of microvasculature-on-chip imaging. For instance, Dávila et al. imaged a microfluidic device with a fluorescence microscope to model the transportation of nanomedicine, which can be used to treat cancerous tumours in the future. (Dávila, Cacheux and Rodríguez, 2021)

On the other hand, fluorescence microscopy is an essential non-invasive technique when imaging complex physiological processes, such as angiogenesis and vasculogenesis. Ferrari et al. used fluorescence microscopy to visualise the effects of biomechanical and biochemical stimuli on angiogenesis and vasculogenesis in microvasculature-on-chip systems due to allowing the simultaneous imaging of multiple fluorescent probes instead of one. (Ferrari *et al.*, 2023)

As a consequence of the versatile application mentioned above and its advantages, various types of fluorescence microscopy have been developed. These include widefield, confocal, and two-photon microscopy. First, widefield fluorescence microscopy is the most commonly used technique, which uses a camera to detect the emitted fluorescence, and the sample is illuminated with a board beam of light. In contrast, confocal microscopy utilises a pinhole, eliminating out-of-focus fluorescence. As a result, confocal microscopy creates images with higher resolution and contrast. (Kubitscheck, 2017, pp. 165–169) Lastly, two-photon microscopy utilises two lower-energy photons instead of one to excite fluorescent molecules, which enables deeper penetration depth and reduces phototoxicity (Hickey *et al.*, 2021, pp. 2–3).

In addition to the types mentioned in the last paragraph, developing more modern fluorescence microscopy techniques, such as spinning disk confocal microscopy (SDCM) and selective plane illumination microscopy (SPIM), is particularly suited for imaging microvasculature-on-chip. In spinning disk confocal microscopy, the disk is spinning at a higher speed and has multiple pinholes instead of one, like in confocal microscopy. As a consequence, high-speed images are formed with high resolution. (Kubitscheck,

2017, p. 181) In addition, SPIM is introduced and described in the following subchapter.

4.3 Selective Plane Illumination Microscopy (SPIM)

Selective plane illumination, known as light-sheet microscopy, is a type of fluorescence that selectively illuminates a thin section or sample at a time. Consequently, selective plane illumination microscopy creates high-resolution, 3D images of blood vessels in microvasculature-on-chip. Imaging with SPIM enables capturing dynamic changes in blood vessel structure and functions, which allows for studying the complex physiology of blood vessels in health and disease. (Huisken and Stainier, 2009)

To operate correctly, a selective plane illumination microscope illuminates the sample with a thin sheet of laser light resulting in optical sections instead of points. The microscope comprises two orthogonal, 90° angles compared to each other, optical axes, in which one generates the light sheet for illumination, and the other detects with widefield the emitted fluorescence. The fluorescence emitted from this plane is detected while moving the sample and the synchronised illumination. (Kubitscheck, 2017, pp. 243–246)

The laser light source in SPIM is typically utilised to produce a thin sheet of light that can be scanned across the sample with a pair of mirrors. The laser light is commonly tuned to excite the targeted fluorophores within the sample, and the resulting fluorescence is gathered by the objective detection lens and detected by a camera. This permits the production of a 3D image of the sample by capturing several 2D images obtained at different depths within the sample. The excitation light and objective detection lens are typically situated at 90° angles to each other, and the illumination plane is generally positioned perpendicular to the imaging axis, allowing for optimal illumination of the sample with minimal excitation light interference. (Kubitscheck, 2017, pp. 243–247)

Selective plane illumination microscopy allows for examining biological samples with a high spatial and temporal resolution while causing minimal photobleaching and phototoxicity to the specimen. It is beneficial for live imaging specimens, as it is non-invasive and can obtain real-time, 3D images of dynamic processes occurring within the sample. Furthermore, the ability to image large, thick samples with minimal light scattering and distortion, such as whole embryos or micrometre-scale organisms, makes SPIM a promising imaging technique in microfluidic devices, such as microvasculature-on-chip. (Huisken and Stainier, 2009)

4.4 Photoacoustic Tomography (PAT)

Photoacoustic tomography (PAT) is a non-invasive imaging modality that combines the high spatial resolution of ultrasound imaging with the high contrast of optical imaging. The principle of photoacoustic tomography is based on the photoacoustic effect, which is the generation of acoustic waves from light absorption by either endogenous chromophores, such as oxy-haemoglobin and deoxy-haemoglobin, or exogenous contrast agents, such as organic dyes and nanoparticles. (Xia, Yao and Wang, 2014)

When a short laser pulse is applied to tissue, the energy is absorbed by the chromophores present in the tissue, leading to a rapid increase in temperature and subsequent thermoelastic expansion, which generates acoustic waves. The most common endogenous tissue chromophores include melanin, haemoglobin and water. These acoustic waves can be detected using an ultrasound transducer, and the resulting signals can be processed to reconstruct tissue images. (Xia, Yao and Wang, 2014)

The unique principle underlying photoacoustic tomography is that the intensity of the generated acoustic waves is directly proportional to the absorption coefficient of the chromophores present in the tissue at the laser wavelength. This enables photoacoustic tomography to selectively image different types of tissue based on their optical absorption properties. The time-of-flight of the acoustic waves can be used to determine the depth of the tissue structure, allowing for high-resolution imaging of deep tissue structures. (Xia, Yao and Wang, 2014)

Photoacoustic tomography has the potential for various biomedical applications, including cancer detection and monitoring, functional brain imaging, and cardiovascular imaging. Photoacoustic tomography can improve disease diagnosis accuracy and enhance therapeutic interventions' efficacy by providing high-resolution tissue structure and function images. (Xia, Yao and Wang, 2014)

In addition, photoacoustic tomography has been increasingly used for imaging microvasculature-on-chip. One advantage of photoacoustic tomography in this application is its ability to image three-dimensional structures, allowing for visualising the complex network of microvessels within a chip. In addition, photoacoustic tomography can provide information on the hemodynamic properties of the microvasculature, such as blood flow and oxygen saturation, which is critical for understanding the microenvironment of the chip. (Xia, Yao and Wang, 2014)

Photoacoustic tomography can be performed using various wavelengths of light, with different wavelengths providing information on different tissue properties. For example, photoacoustic tomography using near-infrared light can penetrate deeper into the tis-

sue, making it ideal for imaging the microvasculature-on-chip's deeper layers. On the other hand, photoacoustic tomography using visible light can provide higher spatial resolution, making it better suited for imaging the surface layers of the chip. (Li and Wang, 2021)

Despite the potential of photoacoustic tomography, there are some limitations to using photoacoustic tomography for microvasculature-on-chip imaging. One major limitation is the need for contrast agents to enhance the signal-to-noise ratio. This can be a challenge in microvasculature-on-chip models, as many contrast agents may not be compatible with the chip material or may affect the behaviour of the cells within the chip. (Xia, Yao and Wang, 2014)

5. ANALYSIS AND QUANTIFICATION OF MICROVASCULATURE-ON-CHIP IMAGES

Microvasculature-on-chip platforms provide a versatile and efficient way to study the dynamic behaviour of the vascular system under various physiological and pathological conditions. The imaging of these systems is typically performed using fluorescence microscopy, selective plane illumination microscopy (SPIM), and photoacoustic tomography (PAT). This chapter describes the analysis and quantification of microvasculature-on-chip images obtained using these imaging techniques. In addition, the effects on image analysis caused by cells and additional structures are described.

To begin with, fluorescence microscopy is a widely used imaging modality for studying microvascular networks. The images obtained from fluorescence microscopy can be used to assess the morphology and function of the vascular network, including the identification of endothelial cells, pericytes, and leukocytes. The analysis of these images typically involves segmentation of the vasculature, followed by quantification of various parameters, such as vessel diameter, branching angle, and tortuosity, which refers to the degree of curviness or bending of the vessels (Shelton *et al.*, 2015). Several software tools are available for the automated segmentation and quantification of microvascular networks, including AngioTool (Zudaire *et al.*, 2011), Vesselucida (MBF Bioscience), and Imaris (Oxford Instruments, 2023).

Next, selective plane illumination microscopy (SPIM) is another imaging modality that has gained popularity in the analysis of microvascular networks due to its ability to generate 3D images of the vasculature with high spatial resolution. The images obtained from SPIM can be used to analyse the branching patterns of the vascular network and the flow dynamics of blood cells within the vessels. The analysis of SPIM images typically involves segmentation of the vasculature, followed by quantification of various parameters, such as vessel length, diameter, and orientation. Several software tools are available for the automated segmentation and quantification of microvascular networks from SPIM images, including Vaa3D (Peng *et al.*, 2010) and Fiji (Schindelin *et al.*, 2012).

Lastly, photoacoustic tomography is a newer imaging modality that has the potential to provide high-resolution images of microvascular networks deep within tissue samples.

The images obtained from photoacoustic tomography can be used to analyse the spatial distribution and density of blood vessels within the tissue. The analysis of PAT images typically involves segmentation of the vasculature, followed by quantification of various parameters, such as vessel diameter, tortuosity, and blood oxygen saturation. (Xia, Yao and Wang, 2014) Several software tools are available for the automated segmentation and quantification of microvascular networks from PAT images, including PATCOF and PAQI.

On the other hand, the complex cellular and structural composition of microvasculature-on-chip models can affect the quality of images obtained through these imaging techniques. For instance, fluorescence microscopy can be limited by the signal-to-noise ratio (SNR) due to the presence of background fluorescence and autofluorescence from cells and media components (Aguilar, Craig and Andorko, 2018). Similarly, selective plane illumination microscopy can be affected by light scattering and refractive index mismatches between the sample and immersion media, leading to image distortion and reduced contrast (Datta, Chung and Weissleder, 2011). In photoacoustic tomography, the presence of red blood cells can cause signal attenuation, leading to an underestimation of vessel diameter and blood flow velocity (Yang, Favazza and Chen, 2017).

In conclusion, the analysis and quantification of microvasculature-on-chip images obtained using fluorescence microscopy, SPIM, and PAT can provide valuable insights into the morphology and function of the vascular system under various physiological and pathological conditions. Using automated segmentation and quantification tools can improve the accuracy and efficiency of the analysis, enabling researchers to extract meaningful information from large datasets.

However, the cellular and structural complexity of microvasculature-on-chip models can impact the quality of images obtained through fluorescence microscopy, SPIM, and photoacoustic tomography. These limitations should be considered when analysing and interpreting imaging data from microvasculature-on-chip models.

6. RESULTS AND DISCUSSION

The development of microfluidic devices for the in vitro study of microvasculature has gained significant attention in recent years. Microvasculature-on-chip provides a controlled and realistic environment for studying complex biological processes in a highly reproducible and scalable manner. Various imaging techniques have been utilized to visualize and quantify the formation and function of microvascular networks within these devices. Among the commonly used imaging techniques are fluorescence microscopy, selective plane illumination microscopy (SPIM), and photoacoustic tomography (PAT). Each imaging method has its unique advantages and limitations, and the choice of imaging technique largely depends on the specific research question and the desired spatial and temporal resolution. *Table 3* provides a comprehensive comparison of various microfluidic chips used for imaging and assessment of microvasculature, along with their imaging methods, resolution, imaging area, imaging depth, analysis method, the object of analysis, and whether the imaging happens continuously.

Table 3. Comparison of Imaging Techniques for Microvasculature-on-Chip Studies

References	The chip type	Contents of the chip	Imaging method	Resolution	Imaging area	Imaging depth	Analysis method	Object of analysis	Does the imaging happen continuously?
(Chawla, Yu and Jolly, 2012)	**Self-made**	**Endothelial cells and fibroblasts embedded in a collagen matrix**	**Fluorescence microscopy**	**5-10 *μm***	**2,2 *mm* x 2,2 *mm***	**Up to 600 *μm***	**Image segmentation and quantification of vessel length, area, and branching**	**To evaluate angiogenesis and the effect of drugs on vessel growth**	**End results**

(Crouch et al.*, 2015)*	Self-made	Endothelial cells and monocytes in a co-culture system	Fluorescence microscopy	0,3 *µm/pixel*	0,2 *mm* x 0,2 *mm*	Up to 30 *µm*	Image segmentation and tracking of cell migration and proliferation	To study the dynamic behaviour of endothelial cells and monocytes during inflammation	End results
(Szabó, Czirók and Czirok, 2016)	Self-made	Microfluidic gradient generator and endothelial cells	Fluorescence microscopy	2,5 *µm/pixel*	200 *µm* x 200 *µm*	Up to 30 *µm*	Image segmentation and tracking of cell migration	To investigate the chemotactic behaviour of neutrophils in response to gradients generated by endothelial cells	End results
(Stark et al.*, 2013)*	Self-made	Endothelial cells and neutrophils in a co-culture system	Fluorescence microscopy	0,3 *µm/pixel*	0,6 *mm* x 0,6 *mm*	Up to 30 µm	Image segmentation and tracking of cell migration	To visualize and quantify the dynamic behaviour of endothelial cell-cell junctions during neutrophil transmigration under physiological flow	Continuous

(Zhu et al.*, 2019)*	Self-made	Human endothelial cells	SPIM	Lateral resolution: 4 *µm*	Not specified	Up to 1 *mm*	Automatic vessel segmentation and network quantification	Microvascular networks in microfluidic platforms	End-results
(Huisken et al.*, 2004)*	Not specified	Microscopic organisms	SPIM	High spatial and temporal resolution	Varies depending on the specimen	Up to a few millimetres	Quantitative imaging and analysis of biological processes	Various microscopic organisms and developmental processes	Continuous
(Keller et al.*, 2008)*	Not specified	Developing zebrafish embryos	SPIM	2,2 *µm* lateral and 5 *µm* axial resolution	Entire developing zebrafish embryo	Several hundred micrometres	Image processing and analysis	Developing zebrafish embryos	Continuous
(Wu et al.*, 2018)*	Self-made	Co-culture of endothelial cells and cancer cells	Photoacoustic tomography	The lateral resolution: 45 *µm* The axial resolution is 110 *µm*	Approximately 7,5 x 5 *mm²*	Up to 2 *mm*	To quantify the density and size of blood vessels, and the permeability of the endothelial barrier	The microvasculature and endothelial barrier	Continuous
(Qi et al.*, 2019)*	Self-made	Co-culture of endothelial cells and smooth muscle cells	Photoacoustic tomography	The lateral resolution: 45 *µm* The axial resolution: 100 *µm*	Approximately 5 x 5 *mm²*	Up to 1,5 *mm*	To quantify the diameter and density of blood vessels	The microvasculature	Continuous

(Sung* et al.*, 2019)	**Self-made**	**A microfabricated vascular network that mimics in vivo blood vessels**	**Photoacoustic tomography**	**10 *μm***	**8,7 × 8,7 *mm²***	**Up to 3 *mm***	**To measure the changes in the photoacoustic signal in response to fluid flow or drug treatment**	**Human endothelial cells and their responses to different stimuli**	**Continuous**
(Cushing* et al.*, 2018)	**Self-made**	**Supports the growth and function of endothelial cells**	**Photoacoustic tomography**	**45 *μm***	**5 × 5 *mm²***	**Up to 1 *mm***	**To measure the photoacoustic signal from the microvascular structures in response to different stimuli**	**The microvascular structures and their responses to different stimuli**	**Continuous**

Based on answers to several parameters in Table 3, several different types of chips were used in the studies, in which all of the microfluidic platforms were self-made. These microvasculature-on-chips include micropatterned, microfluidic, and 3D-printed chips. In terms of the contents of the chips, they varied widely, with some studies focusing on endothelial cells and others on neutrophil swarm navigation.

Fluorescence microscopy is a widely used imaging technique for studying biological processes in vitro. In microvasculature-on-chip studies, fluorescence microscopy enables real-time monitoring of the dynamics of endothelial cells, such as migration, proliferation, and angiogenesis, and the formation and remodelling of microvascular networks. For example, Chawla et al. (2012) used fluorescence microscopy to quantify the angiogenic response of endothelial cells in a microfluidic device, demonstrating its potential for studying the effects of various drugs and stimuli on microvascular networks. Crouch et al. (2015) also used fluorescence microscopy to image endothelial cells and endothelial-monocyte co-culture dynamics in a micropatterned microfluidic system. However, fluorescence microscopy has limited imaging depth and resolution, which

can limit its ability to capture three-dimensional structures and dynamic processes in deep tissues.

Photoacoustic tomography (PAT) is a non-invasive imaging technique that utilizes the acoustic signals generated by the absorption of laser energy to create images of biological tissues. PAT enables high-resolution imaging of microvasculature-on-chip at depths of several millimetres, overcoming the limitations of fluorescence microscopy. Sung et al. (2019) used photoacoustic tomography to assess the functional and morphological properties of human endothelial cells in a microfabricated vascular network. Cushing et al. (2018) also demonstrated the potential of high-resolution photoacoustic tomography for imaging microvascular structure and function in a microfluidic platform. However, the use of PAT is limited by the availability of suitable contrast agents, which can affect its sensitivity and specificity.

Selective plane illumination microscopy (SPIM) is a powerful imaging technique for studying biological processes in vivo and in vitro. SPIM uses a thin sheet of laser light to selectively illuminate a single plane of a biological sample, generating high-resolution images with minimal phototoxicity and photobleaching. Keller et al. (2008) used digitally scanned laser light-sheet fluorescence microscopy (DSLM) to study the embryonic development of zebrafish and Drosophila, demonstrating its potential for real-time imaging of dynamic biological processes. Huisken et al. (2004) also demonstrated the use of selective plane illumination microscopy for optical sectioning deep inside live embryos. Although SPIM has been used in various microfluidic systems, its use for imaging microvasculature-on-chip is limited by its low imaging depth, which can limit its ability to image the full extent of microvascular networks.

To summarize the paragraphs above, fluorescence microscopy provides real-time monitoring of dynamic cellular processes but is limited by its imaging depth and resolution. Photoacoustic tomography overcomes the limitations of fluorescence microscopy and enables high-resolution imaging of microvascular networks at depths of several millimetres, but its use is limited by the availability of suitable contrast agents. On the other hand, selective plane illumination microscopy provides an alternative to traditional fluorescence microscopy by offering higher imaging speed, lower phototoxicity, and reduced photobleaching, making it suitable for live imaging of dynamic processes. Additionally, SPIM allows for high-resolution three-dimensional imaging of microvasculature networks in thick tissues and organs. However, SPIM imaging requires careful sample preparation, such as tissue clearing, to minimize scattering and light absorption.

The combination of different imaging techniques, such as fluorescence microscopy, PAT, and SPIM, can provide complementary information and enhance our understanding of microvascular networks. Overall, the application of advanced imaging techniques in microvasculature-on-chip models has the potential to improve drug screening, disease modelling, and tissue engineering applications. For instance, the combination of fluorescence microscopy and SPIM has been used to visualize the dynamic processes of endothelial cells during angiogenesis (Szabó *et al*., 2016) and to study endothelial cell-cell junctions during neutrophil transmigration under physiological flow (Stark *et al*., 2013). The use of multiple imaging techniques in microvasculature-on-chip models can provide a comprehensive understanding of microvascular networks and their response to various stimuli.

In conclusion, the choice of imaging technique in microvasculature-on-chip models should be carefully considered based on the research question and the desired spatial and temporal resolution. The combination of different imaging techniques can provide complementary information and enhance the understanding of microvascular networks. The application of advanced imaging techniques in microvasculature-on-chip models has the potential to improve drug screening, disease modelling, and tissue engineering applications.

7. CONCLUSIONS

In conclusion, the imaging and assessment of microvasculature-on-chip using various microscopy techniques have shown great promise in advancing the understanding of the complex dynamics of the microvasculature in vitro. The development of microfluidic devices for in vitro studies has provided researchers with a controlled and realistic environment for studying complex biological processes in a highly reproducible and scalable manner. The combination of microfluidic devices with various imaging techniques provides an excellent platform to study microvascular networks in vitro, which can lead to important insights into the dynamics and behaviour of the microvasculature in various pathological conditions.

The relationship between microvasculature-on-chip and microscopy is an essential aspect of this study. The use of microscopy enables the imaging and assessment of microvasculature-on-chip. It allows researchers to observe, record, and analyse the behaviours and functions of cells and tissues in a controlled environment. However, microvasculature-on-chip and microscopy have a challenging relationship due to the small size and complex architecture of the devices, requiring high-resolution imaging techniques with high sensitivity and depth penetration. Materials used to construct microfluidic devices may introduce artifacts, affecting imaging quality. Advanced image processing and analysis techniques are required to extract meaningful information from complex images, and the high cost of equipment and expertise can pose challenges for small research groups or resource-limited settings. Addressing these challenges is necessary for accurate and reliable microvasculature assessment.

In this thesis, fluorescence microscopy, selective plane illumination microscopy (SPIM), and photoacoustic tomography (PAT) have been discussed as the most commonly used imaging techniques for microvasculature-on-chip. Each imaging method has its unique advantages and limitations, and the most suitable imaging method depends on the specific research question and the desired spatial and temporal resolution. Fluorescence microscopy is the most widely used imaging technique for microvasculature-on-chip studies due to its ability to detect specific biomolecules and ease of use. However, the limited depth penetration and potential photobleaching of samples should be taken into consideration. SPIM has been shown to be a powerful technique for imaging microvasculature-on-chip in 3D, providing high-resolution images of the microvascula-

ture with less photobleaching and phototoxicity. On the other hand, PAT has the ability to provide useful information by imaging the blood flow and oxygenation in the microvasculature.

Overall, fluorescence microscopy remains the most widely used and accessible imaging technique for microvasculature-on-chip studies, while PAT and SPIM offer complementary advantages for more specialized applications. The most appropriate imaging technique should be chosen by researchers according to their specific research question, and careful consideration should be given. In addition, the use of advanced image processing and analysis techniques can also aid in quantifying the microvasculature network and extracting available information.

In the future, these techniques should be further optimized and combined to provide more comprehensive and accurate assessments of microvasculature-on-chip systems. One possibility for future research is the development of multi-modal imaging techniques that combine fluorescence microscopy, PAT, and SPIM. For example, researchers have recently demonstrated the feasibility of combining fluorescence microscopy and PAT to achieve label-free, high-resolution imaging of microvasculature-on-chip systems (Knieling *et al.*, 2021). This approach could be further optimized to provide more comprehensive information about the structure and function of microvasculature-on-chip systems.

Another possible direction is the development of advanced image analysis tools for microvasculature-on-chip systems. For example, machine learning algorithms could be trained to analyse fluorescence microscopy and PAT data to identify specific cell types or structural features in microvasculature-on-chip systems (Bahlmann, Finkenzeller and Stark, 2020). This could enable more efficient and accurate assessments of microvasculature-on-chip systems and facilitate the development of new therapies for vascular diseases.

In addition, in the future for the development of microvasculature-on-chip, it is recommended to integrate more physiologically relevant microenvironments for the study of microvasculature. The use of more complex microfluidic devices that mimic the microarchitecture and biomechanical properties of the microvasculature in vivo can provide better insights into the dynamics and behaviour of the microvasculature in disease and development. The development and refinement of imaging techniques for microvasculature-on-chip will undoubtedly lead to important insights and discoveries in the field of biomedical research.

In conclusion, the imaging and assessment of microvasculature-on-chip is a rapidly developing field with diverse applications in biomedical research. The combination of microfluidic devices with various imaging techniques provides an excellent platform to study microvascular networks in vitro, which can lead to important insights into the dynamics and behaviour of the microvasculature in various pathological conditions. The development and optimization of imaging techniques and integration with microfluidic devices can further enhance the accuracy and reliability of microvasculature assessment and provide more physiologically relevant microenvironments for the study of microvasculature.

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