Tampere University

Sanna Kallio

VASCULARIZATION IN ORGAN- AND BODY-ON-A-CHIP PLATFORMS

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ABSTRACT

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In tissue culture, soluble molecules such as gases, nutrients and waste are transported by diffusion. This limits the size of the tissue: if the dimensions of the tissue construct exceed the diffusion range, the inner parts of the tissue are deprived of nutrients. In the human body, long distance transport is covered by blood circulation. Vascularization of cultured tissue can help achieve adequate mass transport throughout tissue engineered constructs.

Organ- and body-on-a-chip platforms are cell culture devices based on microfluidics, *i.e.*, manipulation of small volumes of fluids. In comparison to traditional cell and tissue culture, the on-chip devices allow precise control of environmental parameters, such as perfusion of medium, and require only small amounts of reagents. The development of vascular networks is largely dependent on blood flow. Therefore, organ-on-a-chip platforms with controlled flow conditions are especially suitable in studies involving vascularization.

This bachelor's thesis discusses methods to build vasculature in organ-on-a-chip platforms. One of the two main approaches is guided self-organization of endothelial cells into vascular networks, mimicking the angiogenic processes *in vivo*. In the other approach, a pre-patterned blood vessel scaffold is fabricated first and then seeded with cells. In addition to research on blood vessel development and function, vascularized organ-on-a-chip devices can be applied in improving *in vitro* organ models and connecting multiple models together as body-on-a-chip platforms. Because nearly every human tissue type includes vasculature, it can be considered an essential component in all future *in vitro* models of human biology.

Keywords: organ-on-a-chip, vascularization, microphysiological system

TIIVISTELMÄ

Sanna Kallio: Vaskularisaatio organ- ja body-on-a-chip-monikudosmallinnuksessa
Kandidaatintyö
Tampereen yliopisto
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Kudosviljelyssä liukoiset molekyylit, kuten ravinteet, kaasut ja metaboliajäte, kulkeutuvat diffuusion avulla. Tämä rajoittaa kudosviljelmän kokoa: diffuusio ei ulotu liian suuren kudoskappaleen sisäosiin, jolloin ne eivät saa tarvitsemiaan ravinteita. Elimistössä molekyylit kulkeutuvat pitkiä matkoja verenkierron välityksellä. Vaskularisaatio eli verisuonituksen lisääminen kudosviljelmään edesauttaa tehokasta ravinteiden ja kaasujen kuljetusta.

Kudos- ja monikudosmallinnuksella (engl. *organ-* ja *body-on-a-chip*) tarkoitetaan mikrofluidistiikkaan perustuvia solukasvatuslaitteistoja. Verrattuna perinteiseen soluviljelyyn niiden etuja ovat kasvatusolosuhteiden, kuten nestevirtauksen, tarkka säätely sekä pieni reagenssien kulutus. Veren virtaus ja sen aiheuttamat voimat säätelevät verisuoniston kehitystä, minkä vuoksi organ-on-a-chip-laitteistot soveltuvat hyvin verisuonitettujen kudosmallien kasvattamiseen.

Kandidaatintyö selvittää ja kuvaa keinoja, joilla verisuonitusta voidaan rakentaa organ-on-a-chip-malleihin. Pääasiallisia lähestymistapoja on kaksi: endoteelisoluja voidaan ohjata järjestäytymään itse verkostoksi mukaillen luonnollista angiogeneesiä, tai solut voidaan istuttaa valmiiksi muotoiltuun verisuonistoa muistuttavaan tukirakenteeseen. Verisuoniston kehityksen ja toiminnan tutkimisen lisäksi verisuonitettuja organ-on-a-chip-laitteistoja voidaan hyödyntää kudosmallien elinkelpoisuuden ja toiminnallisuuden parantamiseen sekä useiden elinmallien yhdistämiseen. Koska lähes jokaisessa ihmisen kudostyypissä on verisuonitusta, vaskularisaatiota olisi hyvä hyödyntää yhä useammissa *in vitro* -kudosmalleissa.

Avainsanat: organ-on-a-chip, vaskularisaatio, monikudosmallinnus

CONTENTS

LIST OF SYMBOLS AND ABBREVIATIONS

2D	Two-dimensional
3D	Three-dimensional
cAMP	Cyclic adenosine monophosphate
CD31	Cluster of differentiation 31
EC	Endothelial cell
ECM	Extracellular matrix
EU ORCHID	An European Union-funded project on organ-on-chip development (2017–2019)
FBF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
GelMA	Gelatin methacrylate
HIF	Hypoxia-inducible (transcription) factor
HUVEC	Human umbilical vein endothelial cell
iPSC	Induced pluripotent stem cell
LIFT	Laser-induced forward transfer
µCCA	Microscale cell culture analog
MEA	Multielectrode array, microelectrode array
MPS	Microphysiological system
OOC	Organ-on-a-chip/organ-on-chip
PDMS	Polydimethylsiloxane
POMaC	Poly(octamethylene maleate (anhydride) citrate)
PS	Polystyrene
SEBS	Styrene-ethylene/butylene-styrene (block copolymer)
TEER	Transendothelial/transepithelial electrical resistance
TGF-β1	Transforming growth factor beta 1
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell

1. INTRODUCTION

Bioscientific research relies on cell culture and animal models. However, native tissues are structurally and functionally very complex and this complexity cannot be sufficiently recapitulated in traditional *in vitro* models. Three-dimensional tissue structures can be grown, but their size is limited due to challenges in achieving adequate perfusion. The models also lack tissue-tissue interaction on the systemic level. *In vivo* experiments in animal models have therefore been necessary, but the phylogenetic distance between humans and laboratory animals diminishes the predictive value animal experiments can provide on human biology. They also present ethical issues and are expensive to conduct.

Better approaches are needed. The most prominent example of this is drug development: on average, developing and bringing a new drug to market typically takes 9 years and costs $1.5 billion, but in a 2014 study, only 1 of 10 drug candidates in Phase I clinical trials were found to reach regulatory approval to enter the market in the United States [1]–[3]. The main reasons for failure are lack of efficacy or safety [4]. Being able to predict drug failure in as early phase as possible would cut the costs significantly and new treatments would enter the market earlier.

Organ-on-a-chip refers to microfluidic cell culture technology. It combines advances in microfabrication technologies to tissue engineering knowledge in order to create miniaturized organ models. The devices have advantages such as the possibility to strictly control aspects of the microenvironment, including medium perfusion and spatial arrangement of different cellular components in a tissue structure. Human cells can be used, which improves the predictive value of the models. Integrated sensors and automation make the devices well suited to rapid screening applications. Combining multiple organ-on-a-chip models into one system has the benefit of modelling systemic effects: this type of cell culture platform is typically referred to as a body-on-a-chip or a multi-organ microphysiological system.

The controlled perfusion in organ-on-a-chip systems makes them a good platform to study vascularization, as flow is an especially important factor in blood vessel formation [5]. For example, shear stress caused by the flowing blood induces sprouting of new vessels [6]. Vascularized organ-on-a-chip constructs can shed light on processes ex-

panding and remodelling vascular networks, which could be utilized in building vascularized tissues for therapeutical purposes. Most importantly, adding vasculature to an organ-on-a-chip improves the organ models. It can also be used to connect different organ compartments in a body-on-a-chip system.

The aim of this thesis is to provide a literature review on vascularization in organ- and body-on-a-chip platforms. The thesis consists of two main chapters. Chapter 2 gives an overview of organ-on-a-chip devices. After going through basic terminology, the aspects to consider when connecting multiple tissue types on the same platform are discussed. Section 2.3 describes the common materials and fabrication methods used when manufacturing the devices. The means of creating perfusion and performing measurements are presented in Sections 2.4–5. The chapter ends with discussion of how the technology can be transferred to broader use outside the academia.

Chapter 3 discusses vascularization and begins by shortly explaining the basics of two- and three-dimensional cell culture. The biology of blood vessel formation is then covered. Section 3.3 describes how vasculature can be engineered, and Section 3.4 explores combining these techniques with organ-on-a-chip platforms. The three main applications presented are vascular networks-on-a-chip, vascularized organ-on-a-chip models and connecting multiple organs-on-a-chip by vascular structures. The thesis concludes in Chapter 4, which wraps up the advantages and current challenges of vascularized organ-on-a-chip platforms.

2. BACKGROUND

2.1 Organ-on-a-chip concept

Organ-on-a-chip (OOC) is a microfluidic, biomimetic cell culture device, in which cells are seeded into micrometer-scale chambers, with a synthetic culture medium flowing through the device in integrated circuits. The goal is to model a minimal functional unit of a specified organ or tissue type, *e.g.*, the alveolar-capillary interface in the lung, and study its functions [7]. Before the term organ-on-a-chip was coined in 2010, similar miniaturized cell culture concepts were called microphysiological systems (MPS) or microscale cell culture analogs (μCCA) [7]. The term is derived from the earlier "lab-on-a-chip", which refers to microfluidic analysis devices developed in the 1980s.

Previously, cells have been cultured either in suspension, on a flat surface to which they can adhere (2D cell culture), or with three-dimensional scaffolding material (3D cell culture). Especially 2D cultures are simple to establish with plenty of existing knowledge and standardized protocols. They allow easy sampling, are widely used in expansion and differentiation cultures, and are a popular choice for high-throughput studies; however, their simplicity results in some limitations. 3D cultures provide more possibilities for establishing a native-like tissue structure, which in turn adds complexity in design and maintenance.

Compared to traditional cell culture methods, the microfluidic approach allows for perfusion of medium and better control of spatiotemporal parameters, such as chemical gradients and mechanical strain. Microscale implicates only small amounts of reagents and cells are needed, lowering the costs associated with them. Primary cells from small tissue samples can be used, which improves the model accuracy in comparison to using animal cells or immortalized cell lines. Process automation and sensor equipment can be integrated, making the use of the devices easier, aiding in data acquisition, and paving the way for more high-throughput applications.

There is yet no absolute consensus on the definition of organ-on-a-chip [8]. A suggestion by the EU ORCHID consortium is presented in Figure 1 [9]. Based on these properties, organ-on-a-chip devices can be tailored to better resemble real-life cell microenvironments and provide physiologically more relevant models for disease-related research or drug development. In addition to creating more mature tissue models, organ-

on-a-chip devices are used for observing some biological events in a new, more detailed way, which has not been possible with earlier technology [10].

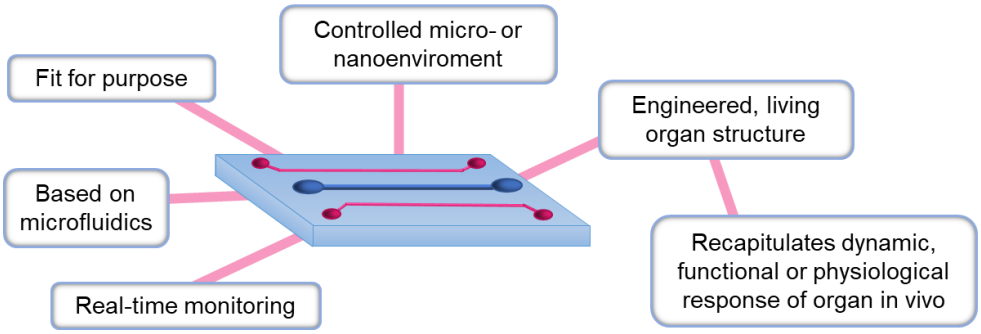

Figure 1. *As defined by EU ORCHID consortium* [9]*, an organ-on-a-chip model includes key features summarized in the figure.*

2.2 Connecting multiple tissues

2.2.1 Establishing connections

The human body is a dynamic system composed of multiple interconnected tissue types. This is evident also when culturing cells *in vitro*, as correct differentiation and functionality require intercellular stimuli in addition to environmental cues. While single cell or tissue type culture devices can give insight into cellular or tissue-level functions, modelling organs or even the whole body as a physiological system requires the integration of multiple tissue types in one culture system.

In physiological conditions, many cell-cell and organ-organ interactions are mediated by blood. It circulates through the body, carrying nutrients, waste and signalling factors. Blood suits the needs of all cell and tissue types in the body, whereas cell culture media are often optimized to support a narrow range of tissue types in specific circumstances. This poses a challenge for (multi-)organ-on-a-chip device design. In addition to cell type-specific needs, the medium has to have oxygen transport properties similar to blood as the rate of oxygen delivery affects the metabolic rate of cells [11].

Unless a universal blood surrogate is developed, the need for both organ-specific and common circulating medium will remain. Differentiation is the stage of cell culture where the organ-specific medium formulation is the most critical. Organ compartments could first be cultured separately with their own media and later connected using a common maintenance medium when they are mature enough. If the device design does not allow for such flexibility, relevant medium components can be provided to

each cell type separately. For example, Zhang *et al*. cultured four different cell types in a compartmentalized microfluidic device and found that adding a growth factor, TGF-β1, to the common medium enhanced lung cell functions but inhibited liver cell functions. The problem was solved by providing TGF-β1 only to the lung compartment by controlled-release gelatin microspheres, limiting its access to the other compartments by a micropillar array. [12] The composition of the common medium could also be simplified in cases where cells are able to support each other in co-culture or in an engineered culture environment [13].

There are different ways to establish tissue-tissue interconnection in microfluidic devices. First of all, cells can be co-cultured in the same chamber. In this case, transportation of signalling molecules does not require controlled fluid flow but diffusion, as the cell clusters are close to each other; this is called static culture. When tissues are cultured in different compartments or devices, they can be directly coupled *via* fluidic circuits or tubing, through which medium is perfused. Unidirectional single-pass perfusion of medium allows cell-cell communication in downstream direction only, whereas recirculating flow resembles physiological blood circulation and is better applicable in models that involve metabolism. [14] Connection of tissue culture modules, *i.e*., establishing interaction between tissue types, can also be achieved by transferring samples of organ efflux in a sequential manner, for example *via* automated liquid handling systems [15], [16]. This type of indirect fluidic coupling enables adjusting media composition between organs and can be useful in optimization phases before physical coupling of organ modules, or in cases where integrated tubing poses challenges.

In perfused systems, the tissue compartments are often separated from the medium flow by a layer of endothelial cells (ECs) or a selective membrane barrier. The former is a type of chip vascularization, as the EC layer resembles the interface between tissues and vasculature in the body and can recapitulate vascular endothelial functions. This will be discussed in more detail in Chapter 3. Cell and membrane barriers shield the stromal cells from the physical flow but allow for exchange of soluble molecules. They also prevent extensive mixing of organ-specific and circulating media while perfusion is still maintained between tissue modules [14].

Establishing tissue-tissue connections is an important design aspect, as it in great part determines the flexibility, ease of use, and scalability of the model. As an example of a multi-organ microfluidic model that emphasizes flexibility, the µOrgano system by Loskill *et al*. utilizes specific adjustable connection blocks that create a fluidic channel between the in- and outlet of two separate chips (Fig. 2) [17]. This kind of approach has several advantages: Individual organs-on-a-chip can be first cultured using tissue-

specific protocols and later connected to the system when they have reached maturity. Defective chips can be disconnected and replaced. This way the failure of one organ chip does not result in failure of the whole multi-organ system. Multiple replicates of organ chips can be used to increase their relative level of contribution in the system if needed. [18]

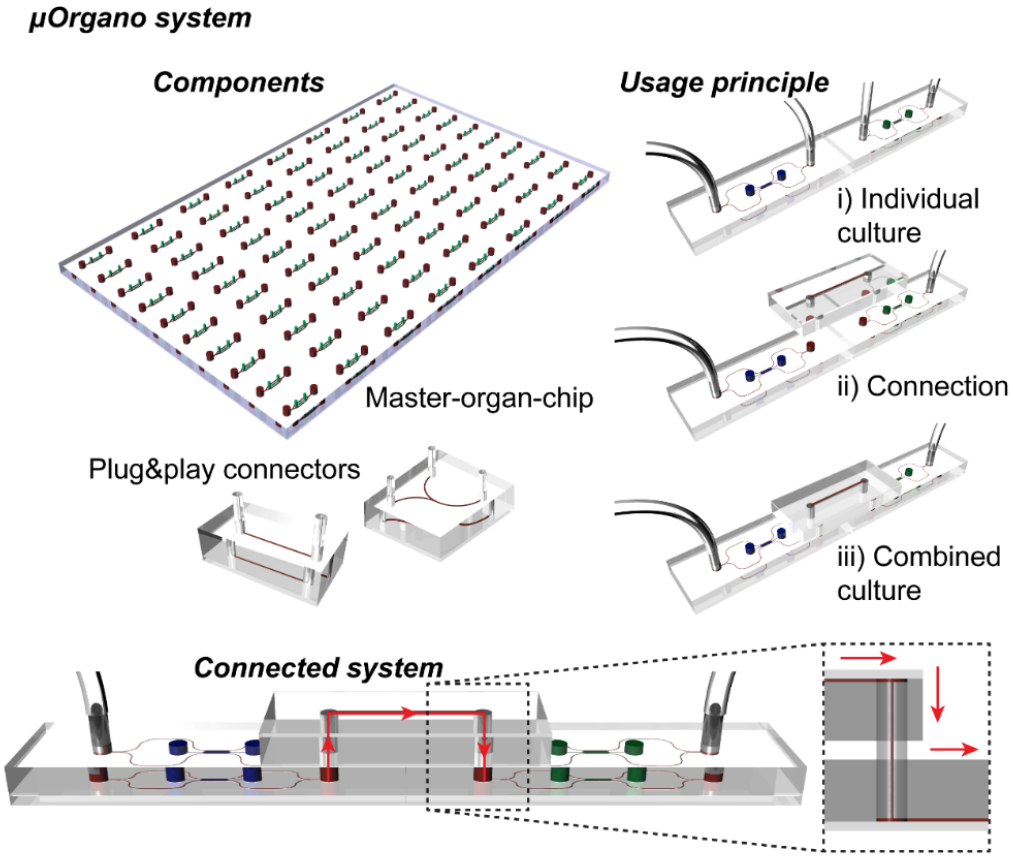

Figure 2. *The µOrgano system consists of a matrix of simple organ chips, which can be cultured separately or connected on demand using fluidic connector blocks. Figure modified from* [17].

Connecting multiple organs-on-a-chip into a functional system has several physiology-related questions worth considering. In addition to the medium requirements discussed earlier, each organ has its own optimal level for physico-chemical parameters such as flow and oxygenation. No model includes all the organ systems found in a human body, but how to compensate for the contributions of the organs missing? Most multi-organ-on-a-chip models focus on transport of biochemical molecules *via* circulating fluid, but research on transport of living cells, such as immune and cancer cells, is more scarce [19].

2.2.2 Scaling

Defined by Moraes *et al*., scaling means “reducing the relative size of each organ compartment to maintain appropriate functionality of the whole system” [20]. Scaling of an organ chip is done not only relative to the organ *in vivo*, but also between different organs in a multi-organ system [18]. Its importance is seen, for example, in drug metabolism studies: if a liver module is insufficiently scaled in relation to another organ module, a metabolite might be produced in such a low level that its effects do not manifest in other organ modules even though they would *in vivo* [21].

The earliest efforts to scale cell culture systems relative to humans used the principles of allometric scaling, in which scaling coefficients for different organs are determined from real-size organisms. However, scaling relationships are not perfectly conserved when organs are miniaturized, as cell functionality, *e.g*., metabolic rate, changes with the environment [20]. For example, Wikswo *et al*. show that allometric scaling would produce a microscale brain with twice the mass of the whole “micro-human” construct [22]. Allometric scaling can provide a good starting point but is largely replaced by functional scaling. It is based on organ properties such as blood flow or metabolic rates [14]. First, it should be decided which organ functions are the most important considering the model at hand, and then scale the organs in a way that these functions and their respective parameters fall in the physiological range [22].

Some aspects to consider in scaling include the surface area of tissue interfaces, blood residence time in the organ, and physical restrictions of tissue architecture: cells cannot be miniaturized. For example, tissues comprising many organized layers of different cell types *in vivo* cannot be recapitulated with a construct consisting of only a few cell layers without losing some functionality. [20], [22] Rare cell types or events might be omitted due to miniaturization. Solutions might include modelling a smaller part of the organ, or using multiple replicates of the same organ chip to achieve a more appropriate level of function [18]. *In vivo*, cells inhabit an environment with limited resources, so sometimes engineered solutions, such as limiting the oxygen carrying capacity of medium, are needed in order to maintain the physiological rate of metabolism [20].

2.3 Chip manufacturing

2.3.1 Design

The design requirements for organ-on-a-chip devices largely depend on whether they are designed for commercial or research purposes. In commercial applications, the production costs and the device being simple to use are important, whereas in re-

search, performance and suitability for rapid prototyping are preferred [23]. It should be noted, however, that in research, multiple parallel samples of the same experiments are needed, and the design should be reliably reproducible. A chip can be designed with features specifically tailored to meet the needs of one tissue type, or as a generic platform which can support many different tissue types.

A typical organ-on-a-chip device is a flat piece of transparent polymer, with microscale channels and integrated systems for controlling and monitoring, such as pumps, sensors, and imaging solutions. Media channels and cell culture chambers are often separated from each other using membranes or micropillar arrays, although there are barrier-free devices as well. For example, barrier-free methods to restrict hydrogels and liquids include phaseguides, which utilize changes in geometry or materials with different wettability properties to create energetically advantageous areas for the materials to stay in or advance along [24], as cited in [25]. In addition to completely closed microfluidic circuits, open-top approaches are being developed. They provide direct access to organ chambers and facilitate handling, although with the downside of contamination risk [26].

While designing microfluidic cell culture systems, one must consider what kind of cellular processes they aim to study, and which other activities result from the processes, to determine which parameters should be monitored. For practical reasons, the device should be portable, allow high-throughput sampling, and have external dimensions similar enough to standard cell culture vessels. Controlling and measuring equipment used with organs-on-a-chip often are external devices, increasing the complexity and the footprint of the system. Fortunately, integrated equipment become more common following the advances in microsystem technology in general.

2.3.2 Materials

Designing an organ-on-a-chip device often means balancing between material properties and ease of fabrication, as cell culture and device engineering have different requirements for the material. The device can be made from a single material, but a more flexible approach could be combining different materials for different parts of the chip and fabricating them separately. For example, the bulk of the device can be made of cheaper materials suitable for high-throughput manufacturing, and the parts that are in contact with cells can be made of materials tailored for the needs of the specific cell culture application.

For a long time, the material of choice for cell culture-ware was polystyrene (PS), an inert and transparent thermoplastic. There is a lot of existing knowledge about the ef-

fect of polystyrene on cells, which makes it an attractive material for organ-on-a-chip device fabrication. Polystyrene can be plasma treated in order to make its surface hydrophilic, enhancing cell adhesion; the surface modification is stable and device shelf life long, as PS does not exhibit hydrophobic recovery [27]. Polystyrene is not elastic, which limits its usability in applications such as culturing contractile tissue. Its stiffness is not comparable to native extracellular matrix (ECM) [28]. Another drawback is that rapid prototyping of PS devices is challenging. It is usually processed with methods more suitable for high-volume production, such as injection molding and hot embossing. Polystyrene is a cost-effective choice in commercial applications, in which a fixed design is manufactured in large quantities.

Polydimethylsiloxane (PDMS) is a popular material in microfluidic devices due to its ease of use and suitability for rapid, iterative prototyping. It can be easily micropatterned using stamping and lithographic methods, although those fabrication methods hamper high-volume production [27]. PDMS can easily be bonded with other surfaces, forming closed channels [27]. It is optically transparent. From a biological point of view, PDMS is elastomeric, non-cytotoxic and gas permeable, and its intrinsically hydrophobic surface can be modified to hydrophilic, although the modification reverses quickly due to hydrophobic recovery, limiting the shelf life of the devices [29], [30].

Elastic deformation properties of PDMS allow the fabrication of stretchable structures for applications such as lung-on-a-chip devices [7]. However, elasticity is an unfavourable characteristic in experiments that require control over shear stress, such as endothelium models [29]. Stiffness of PDMS can be increased by adjusting curing parameters [29]. Gas permeability is often desired in designs that involve closed channels, as oxygen and CO_2 can exchange freely. In contrast, if gas concentrations need to be strictly balanced, a PDMS device must be kept in an incubator or connected to other instrumentation for gas control. Gas permeability allows bubble formation and medium evaporation, which is especially detrimental in microfluidic devices with high surface-to-volume ratio [19].

The largest drawback of PDMS is that it absorbs small hydrophobic molecules [31]. Therefore, mass transport cannot be accurately modelled in PDMS devices. Experiments involving drug testing or cell signalling are especially vulnerable for unwanted absorption. Absorption can be prevented with different coatings, although they might negatively affect cell attachment. To solve this issue, new non-absorbent elastomeric materials are being developed, such as polyurethane elastomers and hybrid thermoplastic elastomers, *e.g.*, styrene-ethylene/butylene-styrene (SEBS) block copolymer,

which maintain the flexibility and good processability of PDMS but exhibit less small molecule absorption than PDMS [19], [28].

Hydrogels, 3D networks of hydrophilic polymer chains in aqueous medium, are often used in chips as the material in contact with cells or as the scaffold into which the cells are embedded. They are highly biocompatible and structurally close to ECM. Another advantage is that natural or naturally derived hydrogels can be combined with synthetic polymers to tailor their processability, load-bearing properties and rate of degradation [32]. For example, gelatin methacrylate (GelMA) is a functionalized natural hydrogel that has been made photopolymerizable by adding methacrylate groups [33]. Due to their softness, hydrogels are usually not used as the bulk material of organ-on-a-chip devices. Hydrogel structures are often fabricated with 3D bioprinting techniques, and their optical properties allow microscopy.

2.3.3 Fabrication methods

The choice of material determines the fabrication methods available for producing an organ-on-a-chip device. Fabrication methods differ in complexity and possibility for high-volume production. As previously discussed, different parts of the devices can be fabricated separately to optimize the ease of processability, cost and cell culture performance. For rapid prototyping, it might be useful to design a standardized housing for the chip, into which different inserts can easily be fitted.

Devices made of thermoplastics, such as polystyrene, are commonly fabricated by injection molding or hot embossing. Both methods require materials that endure high temperatures or pressures. They also cause surface roughness on the mold, which affects the optical properties of the final device and might require polishing as an extra step. [29]

In injection molding, the melted material is injected into a mold using a screw-like plunger (Fig. 3A). It is suitable for high-volume production, as it has high reproducibility. The main drawback is expensive instrumentation setup. Injection molding requires expensive steel molds, the fabrication of which also limits rapid prototyping possibilities. When established, the metal molds can be used millions of times [34]. Other materials for molds are developed, *e.g.*, epoxy, which can be worked with soft lithography but is not as durable as steel [29]. In addition to thermoplastics, injection molding is suitable for elastomers and glasses.

Hot embossing utilizes heat and compression to soften and press the substrate, such as thermoplastic polymers or glasses, against a mold (Fig 3B). The machinery is sim-

pler than in injection molding, therefore hot embossing is easier to set up and more flexible for low-volume production. The main drawback considering microfluidic applications is that ports for integrating automation and fluid flow equipment need to be drilled afterwards, which adds manual work [29].

Soft lithography is used in fabricating stamps and molds out of elastomeric materials, *i.e.*, replica molding. The process is illustrated in Figure 3C. The resulting structure can then be bonded to other components such as a glass slide, creating closed channels. Soft lithography is quite rapid and affordable, but requires manual work, making it better for prototyping than for high-volume production. Its advantages are that it can be used to create microfluidic channels, to create different surface topographies, even in nanometer scale, and to pattern the surfaces with molecules or cells. PDMS devices are often fabricated using soft lithography. [30]

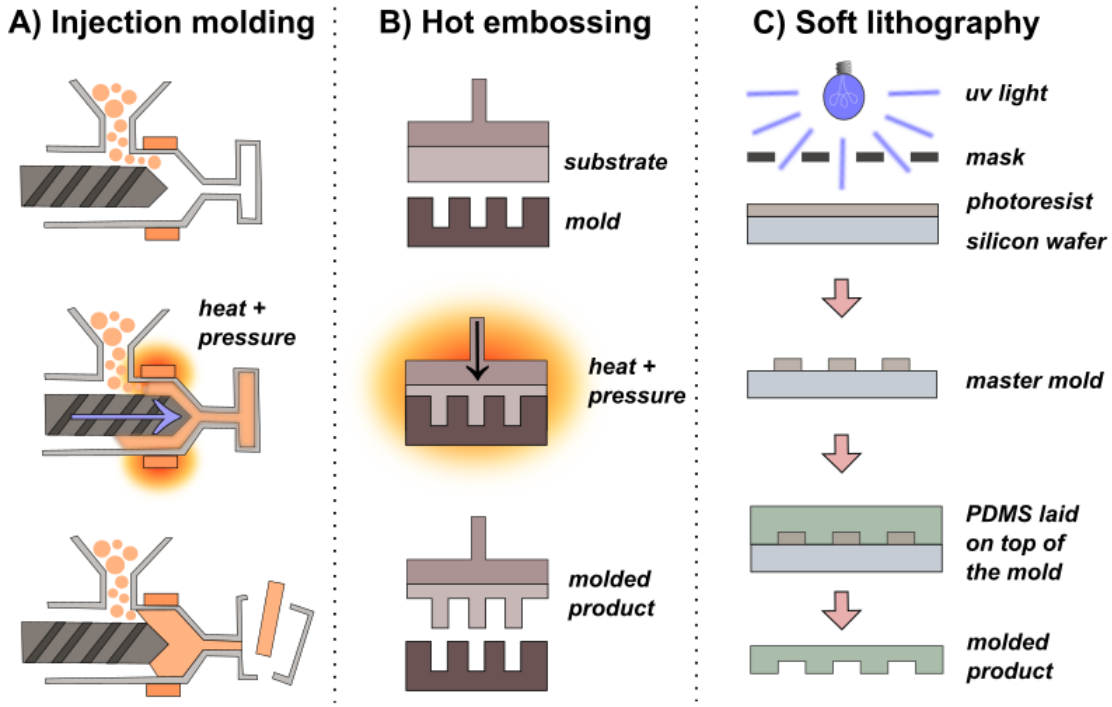

Figure 3. *A) In injection molding, substrate material granules are heated, and the melted mass is moved and compressed into a mold using a screw. B) In hot embossing, the substrate is pressed into the mold using heat and compression. C) In soft lithography, a master mold is first created on a silicon plate by photolithography: a photoresist laid on top of the plate is exposed to UV light through a mask with the desired geometry. After uncured photoresist is washed away, elastomer, such as PDMS, is poured over the master, cured, and peeled off.*

In 3D printing, material is added layer by layer according to a three-dimensional design made with a computer. 3D printing can be used for fabricating the gasket and the channels of microfluidic devices; for example, polycaprolactone and silicone have been used as materials in extrusion-printed chips [35], [36]. In addition, it is well suitable for patterning of biomaterials and cells, and the rest of this chapter will focus on application of 3D bioprinting in organ-on-a-chip devices.

Biofabrication refers to automated manufacturing technologies, such as 3D printing, that use cells and bioactive materials to produce biologically functional products. After the initial investment in instrumentation, biofabrication is suitable for rapid prototyping and some techniques are also easily scalable for mass production. Detailed, complex geometries can be achieved, and computer-made designs are rather easy to modify.

In biological applications, hydrogels are used as the material, ink, that is printed. Cells can be embedded in the bioink as well, allowing their precise spatial patterning. However, the printing process might be too harsh for some cells, and encapsulation of cells decreases cell-cell interactions [32]. Bioprinting results in higher cell densities than post-fabrication cell seeding [37].

A good bioink retains the printed structure and exhibits suitable shear thinning properties: at given shear rate, the material flows easily through the nozzle and then rapidly regains its gel character after exiting the nozzle. The faster the bioink solidifies, the better the resolution of the final construct [38]. Cross-linking is achieved by light or chemicals. A bioink should have correct biological and mechanical properties according to tissue type. In addition to cells and structural ECM proteins, bioactive molecules such as growth factors can be included in the bioink. Materials with low stiffness enable matrix remodelling by cells in the printed construct, whereas stiffer materials often are easier to print.

3D bioprinting allows for combining multiple materials. The materials can be mixed together and printed at the same time, or printed as separate layers using coaxial nozzles [38]. Different bioprinting techniques can be integrated for a synergistic effect. Four commonly used 3D bioprinting methods are presented in Table 1 and Figure 4. 3D bioprinting in vascular tissue engineering applications will be discussed further in Chapter 3.3.3.

Table 1. *Four commonly used methods for 3D bioprinting.*

Method	Premise	Material requirements	Advantages	Disadvantages
Extrusion-based 3D printing	A continuous, cylindrical (bio)ink filament is pushed through a nozzle using a screw or a piston.	Rapid gelation after extrusion [32], [38]	• Coaxial nozzles can be used to produce hollow or multi-layer fibers [37] • High speed [32] • High cell density [32]	• Resolution > 100 µm [38] • Solidification can impair cell viability [32]
Inkjet/drop-based 3D printing	Ink falls as droplets from the nozzle onto a substrate; position is determined by thermal or piezoelectric actuators, which control drop formation as the printer head moves.	Low viscosity, rapid gelation [32], [38]	• Resolution 20–100 µm [38] • Multiple bioink types can be printed in the same construct [38]	• Nozzle clogging [37] • Possible damage to cells [37]
Laser-assisted 3D printing, laser-induced forward transfer (LIFT)	Cells are placed on a donor slide (often a metal film), detached by a laser pulse, and transferred to a substrate.	Hydrogels, cells	• Cell-level resolution [39] • No nozzle clogging [37]	• Slow [37] • Low scalability [37] • Laser-induced cell damage [37] • Limited ability to work in z-dimension [39]
Stereolithography	A photosensitive prepolymer solution is solidified layer by layer using light; "free-form 3D printing".	Photosensitive polymers	• Processing time depends only on the thickness of the printed construct [38] • Resolution 20-100 µm [40]	• Possible cytotoxicity by photoinitiator chemical or laser light [37] • High cost [37] • Photocurable inks are usually not biodegradable [32]

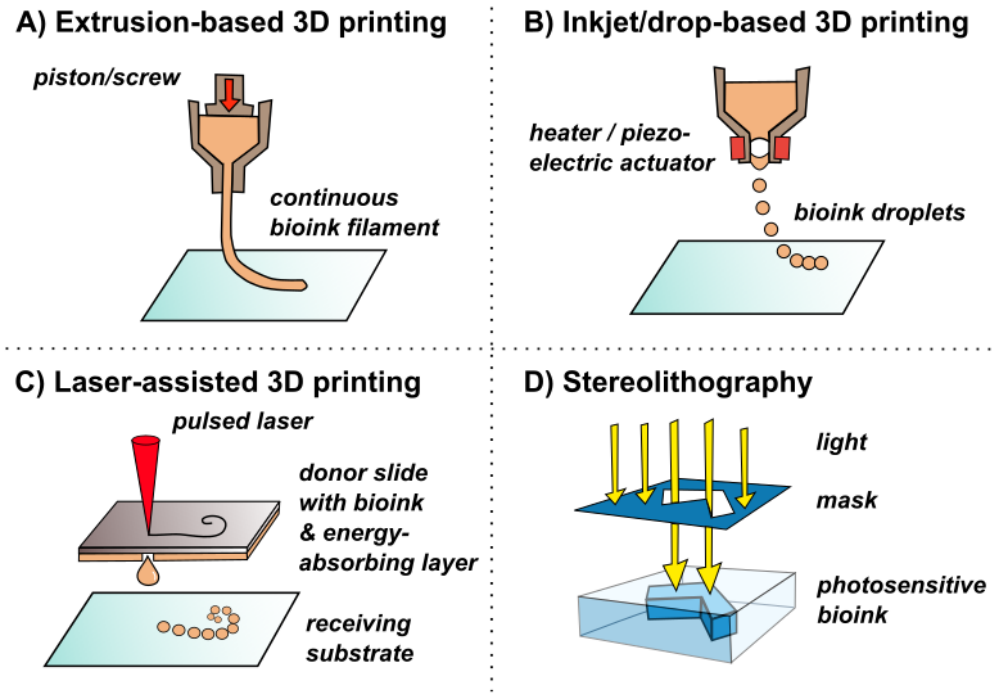

Figure 4. *Four commonly used 3D bioprinting methods: A) extrusion- and B) inkjet-based 3D printing, C) laser-assisted 3D printing and D) stereolithography. The working principles of each of the methods are described in Table 1 above.*

2.4 Perfusion and flow

Perfusion is the passage of fluid in an organ system, for example the delivery of blood to a tissue. In the organ-on-a-chip context, the term ‘perfusion’ can also be used to refer to transferring of fluid between organ compartments or creating flow inside micro-channels. This chapter discusses methods to create fluid flow in an organ-on-a-chip device and the importance of proper flow rate on tissues.

Perfusion culture means that the cell culture medium is replaced continuously, providing exchange of nutrients and gases, and removal of metabolic waste. This keeps the culture conditions stable and characterized, and helps maintain cell viability and normal physiological function [39]. Continuous perfusion is comparable to blood circulation in the body; this is especially highlighted in body-on-a-chip platforms, in which signalling molecules and metabolites travel downstream with the medium to the next connected organ compartment. In the other alternative, static culture, medium is exchanged in a batch-wise manner. This causes fluctuation in solute concentrations, and the distribution of molecules is mediated by diffusion only.

The profile of fluid flow varies between organ systems. Fluid flow exerts mechanical force on cells, including shear stress and hydrostatic pressure. Shear stress provides an *in vivo* -like environment for endothelial cells but can be detrimental for other cell

types. Therefore, in organ-on-a-chip devices, the parenchymal cell compartment is often shielded from flow, as described in Chapter 2.2.

Perfusion can be achieved with different methods. The simplest is hydrostatic pressure, also known as gravity-based flow. Adding different volumes of medium in opposite ends of a microfluidic channel creates a flow. Plate rockers can be used for maintaining the flow for longer periods. This simple approach enables open-well design and multiplexing [26]. The drawback of these methods is that they cannot establish continuous flow; instead, the flow starts at discrete timepoints depending on medium change or plate rocker cycle.

Pumps provide a controlled fluid flow. External pumps are connected to the chip *via* tubing. External pumps are less expensive than integrated ones, but they are bulky and increase risk for leakage. Tubing and liquid reservoirs add dead volume in the circuit, which increases the medium volumes that are needed and contradicts the goal of miniaturization. [40] In addition, materials of tubing and reservoirs have to be considered: for example, silicone is typically used as tubing material but it can absorb small molecules [41].

Peristaltic micropumps can be integrated on-chip. As such, they do not increase the system footprint and are simpler to handle. Other advantages include the possibility to use small medium volumes and to integrate multiple fluid circuits in the same device. However, they are the most expensive alternative when compared to gravity-based flow or external pumps. [40] Devices with pumps are closed systems and need to include bubble traps. Gas bubbles in culture medium can block channels disrupting fluid flow, and they can damage cells especially when bursting [27].

In addition to transporting materials and producing shear stress, perfusion methods are used to establish and maintain biomolecular gradients. For example, hydrostatic pressure enhances and expedites diffusion of biomolecules through a hydrogel region. A gradient requires a source and a sink: Fresh biomolecules can be added *via* medium inlet, or they can be produced by cells of an upstream organ module. In the case of biochemical factors, cells act as the sink, or the biomolecules can be carried away with effluent medium. The geometry of the channel between source and sink defines the concentration profile [42].

When establishing perfusion in a cell culture system, the flow rate has to be in a physiologically relevant range. In addition to the level of shear force, flow affects the residence time of medium in organ compartments. Cells may be exposed to unphysiological amounts of biochemical factors transported along the medium if the flow rate is not

optimized. Flow analysis is often based on computational simulation, and device design and experimental parameters are adjusted accordingly. It should be noted that a small change in channel diameter or shape has a significant effect on fluid flow rate, as fluidic resistance of the channel is inversely proportional to the fourth power of its radius [8]. The task is further complicated by the fact that the geometries inside the chip can change over time as cells migrate and proliferate, or as the hydrogel swells or shrinks.

Miniaturization adds a few special considerations concerning fluid dynamics. Some physical forces that are not noteworthy in our everyday macroscale life have a significant effect in microscale. Large area-to-volume ratio amplifies the effects of surface properties, such as capillary effect, and diffusion of molecules is fast due to short distances travelled [23], [43]. Small medium volume gives a more physiological cell-to-liquid ratio compared to non-microfluidic cell culture systems which helps avoid substantial dilution of soluble factors [29], [44]. When the medium volume is small, water evaporation has a larger effect on concentrations and flow; a humified environment is required if using permeable materials to prevent evaporation [27], [29].

2.5 Measurements and sampling

One of the advantages of organ-on-a-chip devices is that they can provide information on multiple parameters instead of focusing on high-throughput measuring of a single parameter [44]. In addition to collecting data to answer the research questions, monitoring different parameters in on-chip platforms is required for feedback-based control of the microenvironment and validation of the organ model. Automated in-line monitoring maximizes the amount of data gathered and minimizes the need for manual handling. In on-chip devices, in-line measurements are typically done by different optical methods and electrical or electrochemical sensors, including biosensors. A sensor consists of a sensing element, a signal transducer and a detector [45].

Electrical sensors are used for measuring electric activity of cells, barrier function, strain and cell morphology [46]. Many of them can be integrated in the chip as thin films or wires. Especially thin-film electrodes have a high signal-to-noise ratio and can be arranged into precise patterns *via* photolithography, but might complicate fabrication processes [18], [45]. Multi- or microelectrode arrays (MEA) measure changes in the extracellular field potential caused by de- and repolarization of electrically active cells, such as neurons and cardiomyocytes [45]. Barrier integrity can be assessed with transendothelial/transepithelial electrical resistance (TEER), which measures resistance between electrodes placed on both sides of a barrier tissue cultured on a semipermeable membrane [45], [47]. Strain gauges are based on a conductive element placed onto

the cell culture substrate. When the substrate deforms due to, *e.g.*, cardiac contraction, the resistance in the gauge changes. [45]

Electrochemical sensors selectively interact with a target biomarker, which produces an electrical signal proportional to the concentration of the molecule of interest. The sensor can be based on detecting current, potential or impedance [48]. The recognition element is coated with an enzyme, antibody, or aptamer, which defines the sensor's specificity and selectivity. The signal can be transduced into an electrical or optical readout, and multiple sensors can be integrated in a single chip. [48]–[50] Electrochemical sensors can be used for a variety of applications: to monitor cell viability, to identify different cell types, to quantify soluble proteins and other metabolites, and to monitor gas concentrations and pH [21], [49]. For example, enzyme-based glucose sensors make use of glucose oxidase. Its reaction with glucose produces hydrogen peroxide and can be detected amperometrically [49]. Inherent challenges of electrochemical sensors include the possible consumption of the analyte by the sensor and degradation or biofouling of the sensor element in long-term use [50].

Optical measurement methods include microscopy and techniques based on fluorescence or photoluminescence quenching. Microscopy allows for visual inspection of cells, their physical appearance, and their function, such as beating of cardiomyocytes. Optical methods can be used to localize fluorescent markers, to measure local oxygen concentrations, and to study cell barrier permeability [21], [49]. They mostly require transparent materials. Especially immunofluorescence imaging is widely used in organ-on-a-chip devices to assess expression of a specific protein. Unfortunately, fluorescent labelling is a terminal assay and a qualitative measurement [19]. Optical sensors detect changes in some optical property, such as absorption or luminescence. A luminescence sensor can be implemented by placing luminescent indicator dye for a specified target analyte inside the chip, for example as a pattern on the substrate surface. When excited by a light source, luminescent molecules emit a photon, which is then detected using an external detector. In microfluidic devices, luminescence sensors are used for measuring oxygen concentration. [45]

Depending on the chip design, sampling can be performed continuously in-line, or the samples can be physically extracted. Built-in sensors provide real-time quantitative data on cellular level and are non-terminal assays. They are often irreversibly enclosed into the device structure during fabrication. Effluent medium contains the proteins secreted by the cells and can be collected for biochemical assays, but volumes might be too small for some analyses [45]. Cells can also be extracted by detaching them and collecting the medium, or the device can be cut open to access the cells [51].

External off-chip measuring instrumentation might make the physical handling of the system complicated, and manual sampling requires more work and has the risk of disturbing or contaminating the culture. Many challenges concerning measurements and sampling are related to the small size of the devices. In addition to physically fitting all the required sensors into the chip, one has to consider the possible interference between sensor modalities [21]. Optical measurements are based on the spatial organization of the biomarker and using other sensor types can free up “optical bandwidth”. For electrical sensors, precise placement is important. In the future, sensors might be modularized and connected to the system when needed [18].

2.6 From academia to industry

All *in vitro* models should be validated to prove their physiological relevance. It is necessary to understand organ functionality both in health and disease, and the differences in biological functions between the model and native tissue. This way, the limitations of the model can be assessed. Model validation ensures its predictive value, but current validation protocols for *in vitro* models are limited. Suggestions include developing a panel of model drugs to be tested with every device, *in vivo* vs *in vitro* gene expression analysis, and comparing changes in defined clinical biomarkers between *in vitro* models and clinical studies [44], [52]. The customized nature of organs-on-a-chip adds an additional layer of complexity in comparing the devices to each other. In the heavily regulated field of biosciences, further standardization of on-chip devices is required to facilitate the process of gaining regulatory acceptance. In addition to defining acceptable performance criteria, standards could provide common terminology, promoting communication between different parties [8].

Biological material always exhibits variability and ideally all cells of an organ-on-a-chip would be derived from the same source. This is rarely possible due to availability and functionality issues. The experiments should also be reproducible. When it comes to standardization of cell culture, immortalized cell lines are the most straightforward option. They have unlimited expansion capacity, but usually do not exhibit correct phenotype due to genetic alteration during the immortalization process and their cancer background. From physiological point of view, mature primary cells would be the best option resembling cells in *in vivo* conditions. Unfortunately, they are difficult to obtain in high numbers, cannot be expanded in culture, and their functionality quickly deteriorates *ex vivo*. Induced pluripotent stem cells (iPSC) are a promising alternative: they are easy to obtain and, in theory, allow for creating multiple tissue types from a single donor. However, the robustness of differentiation protocols and the maturity of resulting

cells varies between tissue types, and standardized characterization methods throughout the differentiation processes are needed.

As with all model development, it depends on the application which characteristics are indispensable. Choice of cell source will probably be altered between different devices. Immortalized cell lines could work fine in models used for high-throughput experiments where a certain, tight set of characteristics is of importance. Mature primary cells can be used in basic research where it is necessary to model the native tissue as closely as possible. If the differentiation issues are overcome, iPSC-based organ-on-a-chip devices will be the best option in applications of personalized medicine. On-chip platforms might also shed new light on stem cell differentiation protocols [53].

For organ-on-a-chip models to become widely used beyond academy lab bench, they need to be accessible to the end users, providing additional value without added inconvenience or high translational costs. Many existing biological assays rely on multichannel pipetting or automated liquid handling. Thus, it would be easier to adopt the chip models if they were compatible with the standard instrumentation the laboratories already have. For example, a platform with open multi-well design is more versatile than a closed design with custom dimensions. Defining standards especially for fluidic and electrical connections is necessary for interconnection of different devices [49]. Throughput should be high enough to allow for replicates and controls in each experiment.

To successfully transfer organs-on-a-chip from academia to industry, they have to be commercially viable. Basically, this means that the platforms should be produced with high yield and high reproducibility but low cost. Disposable single-use microfluidic chips are already on market, as well as some tissue-specific applications developed for the chips by their manufacturers. Many complex chips are custom-made manually with slow fabrication methods such as soft lithography, but 3D printing could help increase their production volumes. As an alternative to manufacturing off-the-shelf chips, a company might create their business model as a service, to which research institutes could outsource their chip design and experiments [54].

Even if platforms might be relatively similar, every organ-on-a-chip model employs a unique set of cells. Therefore, each new application requires time for experiment design and model validation. This might be the largest obstacle in widespread adoption of the technology. Communication with the end users is essential already in the early phases of product development.

3. VASCULARIZED TISSUES IN ORGAN- AND BODY-ON-A-CHIP PLATFORMS

3.1 2D and 3D cell cultures

Cell culture in controlled conditions allows studying specific biological aspects and phenomena by adjusting different parameters, which is not possible in complex *in vivo* microenvironment. Designing culture methods is balancing between recapitulating the microenvironment as closely as possible and keeping the system simple enough for the experiments to be feasible. Different approaches are referred to as 2D or 3D culture depending on whether they provide a two- or three-dimensional substrate for the cells to grow on.

The simplest and most conventional design is 2D cell culture. Cells adhere on the flat surface of a culture vessel, forming layers. Each cell has access to similar amount of medium components, driving homogeneous growth. *In vivo*, cells reside in three-dimensional microenvironment with multiple cell types. They interact with each other and adhere to extracellular matrix, ECM, in all spatial directions. This aspect is not replicated in 2D culture, which causes considerable differences in cellular responses. Approaches such as collagen treatment or micropatterning of the substrate or culturing cells between two layers of extracellular matrix (sandwich culture) can reduce the effect of substrate flatness.

Not all applications require three-dimensionality. 2D monolayer cell culture is well suited for expanding cells and for differentiation of iPSCs [55]. In the context of toxicology, 2D cell culture models have been useful in initial screening of new compounds due to their low cost and high throughput [10]. Some measurements, such as microelectrode arrays and cellular responses to mechanical forces, require 2D culture [26]. One of its most important benefits is the abundance of standardized protocols, making it easier to compare results obtained with different models in different laboratories [56] .

3D cell culture aims to mimic the structural *in vivo* microenvironment more closely than 2D culture. Cells are better able to maintain their *in vivo*-like shapes, interactions with adjacent cells and ECM, and native tissue architecture, obviating the need to adapt to 2D surface and the stress caused by it [57]. 3D cell models include approaches such

as polymer scaffolds, embedding cells in hydrogels, forming spheroids and organoids, and using different bioreactors. The main drawback of 3D models is that the methods are less standardized. They also require more complex laboratory instrumentation than 2D culture: for example, imaging of 3D tissue constructs is more challenging, as the structures are in different focal planes.

Nutrient and waste transport dynamics differ between 2D and 3D cell culture due to the spatial organization of cells and the composition of extracellular space. In both cases, as the construct size increases, diffusion is no longer effective enough for proper transport of gases and solutes. Tuning scaffold properties, such as pore size, is one approach to enhance mass transport [58]. *In vivo*, the transport is carried out by a fluid conveying system, the vasculature. The concept has been adopted in engineered cell and tissue constructs as well.

3.2 Vasculo- and angiogenesis

Vasculogenesis is the formation of new blood vessels from endothelial progenitor cells, whereas angiogenesis refers to remodelling and expansion of pre-existing vessels. Introducing vascularization in engineered tissue constructs is necessary to decrease the distance of diffusion, as cells need to be within 100–200 µm from a blood vessel [59], as cited in [60]. This limits the possible size of the tissue: larger distances lead to formation of a necrotic core. Blood vessels also act as morphogenic cues for organs around them [61]. In addition, being able to model vasculo- and angiogenesis is relevant in modelling diseases, such as cardiovascular defects and tumor formation. Most of the research on angiogenic processes has been done in transgenic animal models such as zebrafish and rodents, focusing on the effect of biochemical factors, whereas research on flow is more scarce [62], [63].

Vascular development produces a hierarchical structure. *In vivo*, vasculogenesis starts with the formation of primary vascular plexus from mesoderm-derived angioblasts. The angioblasts arrange into an elongated structure, a lumen forms, and the cells on the tube wall differentiate into endothelial cells (ECs). ECs recruit mesenchymal cells nearby and induce their differentiation into pericytes or vascular smooth muscle cells (VSMC), which wrap around the newly formed vessel. These cells have important functions in stabilizing the EC tubes, controlling the permeability of the endothelium, and regulating vascular tone and blood flow by their contraction ability [60].

The vascular network is further refined by angiogenic sprouting, remodelling, and pruning of the sprouts. Sprouting is brought about as chemical gradients attract ECs to ex-

tend outside the existing vessel; some of the ECs differentiate into tip cells, which migrate forward in the ECM, whereas other ECs differentiate into stalk cells and proliferate as the vessel is elongated. Sprouts in different parts of the network connect to each other in a process called anastomosis. These phases happen simultaneously and continue throughout the lifetime of the organism. Network refinement is affected by various hemodynamic and metabolic factors, mechanical forces transmitted by the ECM, and growth factors. [64]

Blood vessel composition varies with size and reflects its functions. Hemodynamic and genetic factors determine which of the capillaries continue their development into larger vessels and whether they will acquire arterial or venous identity, carrying oxygenated or deoxygenated blood [65]. Capillary vessel (diameter 5–20 µm) wall consists only of a single layer of endothelial cells and basal membrane which regulates endothelial function. Larger vessels (diameter up to 30 mm) are surrounded by vascular smooth muscle cells, pericytes, and connective tissue with elastic fibers. Diameter and curvature of the vessels affect flow dynamics, which in turn affects cells.

The structure of vasculature has to provide enough blood flow in all parts of the body or the tissue construct, all the while optimizing the energy needed to maintain the flow. Solute transport in blood is performed *via* two mechanisms: Diffusion due to thermal energy can affect short distances (20–200 µm) and is energetically inexpensive but requires a steep concentration gradient to be efficient. For long distances, an energy-requiring, pump-powered convection is required; the smaller the diameter of the vessel, the more energy convection requires. The vasculature has to find an energetically efficient balance between these two. [64] Thus, convection is utilized in larger arteries and veins, which branch into smaller arterioles and venules and then capillaries. The surface area of capillaries is large and facilitates diffusion of solutes into peripheral tissues.

Vascular endothelial cells are a barrier between blood and tissue and provide two-way selective transport of molecules. They are involved in release of different molecules, such as blood-clotting coagulants and cytokines, and also participate in vessel contraction with the smooth muscle layer. The endothelium and its microenvironment interact, affecting both the construction of vascular microenvironment and inducing specific EC characteristics. Signalling includes both biochemical and biophysical signals [63]. Endothelial cells are heterogenic in structure, function and expression of junctional proteins [47], [66], thus the endothelial structures formed by them differ; for example, different organ types have different needs regarding solute transport and the endothelium functions as a selective barrier [67]. Consequently, the blood-brain barrier is very

dense, whereas hepatic sinusoid endothelium is fenestrated. Vessel architecture differs in different organs but can also differ in different parts of the same organ.

Vascular formation is regulated by different environmental factors. Hypoxia, *i.e.*, low oxygen level, is an essential trigger of angiogenesis. When oxygen supply does not meet the demand, the cells secrete hypoxia-inducible transcription factor, HIF, which in turn upregulates VEGF (vascular endothelial growth factor) production. As a result, angiogenic sprouting is stimulated, until oxygen levels normalize [68]. Other important growth factors regulating angiogenesis include fibroblast growth factor (FBF) and angiopoietins.

Flow of blood is a particularly relevant aspect in vasculature and affects its function *via* quite a many mechanisms, mainly integrated and transduced by endothelial cells which line the inner wall of blood vessels [5]. Flow stretches and causes friction on the vessel wall by shear forces, hydrostatic pressure, and cyclic strain. Different vessels have their own characteristic shear stress and pressure levels; it has been found that disturbed flow leads to pathologies [69]. Keeping blood flow constant is achieved by regulating vascular tone, *i.e.*, the constriction status of vascular smooth muscle cells. The mechanical signal of shear stress is transmitted to the VSMCs by ECs *via* signalling such as nitric oxide production [70], [71]. Shear stress is also involved in the regulation of inflammation and thrombosis, induces sprouting, and helps stabilize vessels [6], [71], [72]. Another important flow type is interstitial flow, *i.e.*, flow of extravascular fluid through ECM. It is required for vasculogenesis, promotes angiogenic sprouting in the direction opposite to the flow, and regulates capillary morphogenesis [73]–[75].

3.3 Principles of engineering vasculature

3.3.1 Overview

Cell culture models of microvasculature have been applied in research of transport, barrier and secretion functions of vascular endothelium, and in understanding angiogenic processes [76]. From the clinical perspective, modelling angiogenesis is important for studying wound healing, tissue regeneration, vascular diseases and cancer [77]. In addition to these more specific applications, microvasculature is an appropriate component to include in any tissue model, as vascular-parenchymal interface is present in nearly every organ.

Blood vessel formation involves interaction of different cell types both spatially and temporally. Vascular network is a dynamic system and responds to different biophysical and biochemical cues in order to make sure blood flow meets the demand through-

out the tissue volume under different conditions. In assessing the organization and maturity of a vascular network, the functionality of the overall system is more important than the characteristics of individual vessels [60]. It should be considered that in addition to proper initial structure, enabling remodelling of it is important [10]. Some parameters to assess vessel maturity are listed in Table 2.

Table 2. *Assessment of vessel quality and maturity.*

Parameter	**Assessment method**
Number and length of branches Vessel diameter	Image analysis
Expression of tight junction and basement membrane proteins	Immunocytochemical staining
Barrier function and permeability	TEER, impedance measurements Tracking of molecule transport through barrier
Perfusability of vessel network	Fluorescently labelled microbeads
EC functionality	Thrombotic response [77]
EC polarity	Protein expression *via* immunocytochemical staining

According to Mandrycky *et al*., the most often used cell type in vascular modelling has been terminally differentiated ECs, lacking plasticity and proliferation potential that could be provided by iPSCs [63]. However, using iPSCs brings challenges such as the maturity of cells and maintenance of long-term experiments [78]. Heterogeneity of ECs was discussed in the previous chapter; in addition, stromal cells differ between tissue types and tissue-specific differentiation is important [78]. Pericytes are essential in formation and maturation of vasculature; without them, proper side branches are not formed [79]. Adding smooth muscle cells to the models is not yet widely in practice, as achieving the correct geometry is difficult [10].

Blood vessels exhibit apical-basal polarization of the endothelial cells and are circular in cross-section. These properties are challenging from the point of view of engineering vascularization *in vitro*; for example, some chip manufacturing techniques based on soft lithography produce shapes with rectangular cross-section [67]. Vessel structures with straight geometry are simple to produce, but curved and branched structures are more physiologically relevant. It should be noted that vessel geometry affects the possibility to mathematically model fluidic flow properties.

An ideal vascular model would consider hierarchical 3D vessel network with closely located parenchyma, suitable cell types and ECM, organ-specific function, and hemodynamics [63]. ECM has to allow remodelling due to vascular sprouting; materials often used include collagen type I and fibrin. Substrate patterning, growth factor gradients, hypoxia, and different flow profiles can be utilized to induce desired characteristics in the model.

Simple models of vascular perfusion utilize techniques such as microfluidic geometry (*e.g.*, micropillars), semi-permeable 2D membranes and EC monolayers [78]. More advanced vascularization approaches can be divided in two categories: self-organizing and pre-patterning methods. In self-organizing approaches, hydrogel-embedded ECs form networks on their own, guided by physiological cues. Pre-patterning refers to first building a scaffold by 3D biofabrication methods and then adding the cells. [80] These two approaches are explained in the following Chapters 3.3.2 and 3.3.3.

3.3.2 Self-organizing vascular networks

Endothelial cells have inherent capability of assembling into vascular networks in the right conditions. Engineered self-organizing vascular networks can be formed *via* a process resembling vasculogenesis or angiogenesis. Culture conditions are optimized to stimulate the vascular developmental process – this includes surface micropatterning for mechanical guidance, gradients of growth factors and other biochemical signals, co-culture with supporting cells, and establishing interstitial flow [19]. Randomly distributed ECs embedded in ECM form an organized structure, and the self-assembled microvasculature can then be connected to a microfluidic circuit for perfusion, as demonstrated by Moya *et al*. [81]. Pericytes are required for vessel maturation [82].

The simplest self-organizing technique might be direct cell-in-gel culture, in which endothelial cells and supporting cells are embedded in 3D matrix. They organize into vessel structures, resembling vasculogenesis. Angiogenesis-resembling vessel formation is often done in combination with pre-patterning methods. For example, soft lithography can be used to create a hollow tubule inside a hydrogel. The tubule is then endothelialized, and the endothelial cells are guided to sprout into ECM by a growth factor gradient, just like in angiogenesis.

A common device design for self-organizing vasculature employs microfluidic channels for gel-embedded cells and medium, separated by microposts. Kim *et al*. developed a microfluidic model to mimic the sequential steps of angiogenesis. A PDMS device, fabricated by replica molding, consisted of a fibrin gel-filled vessel channel, flanked on both sides by medium channels, and a fibroblast channel (Fig. 5A). Human umbilical

vein endothelial cells (HUVECs) and human placental pericytes were seeded on the vessel channel wall. Biochemical factors secreted by the fibroblasts induced sprouting angiogenesis through the fibrin gel. Pericytes were found to promote vessel maturation and regulate the network morphology in comparison to EC-only culture. Dermal fibroblasts induced better EC-pericyte interaction compared to lung fibroblasts. [82] As the channel height was only 100 µm, the model cannot be considered fully three-dimensional.

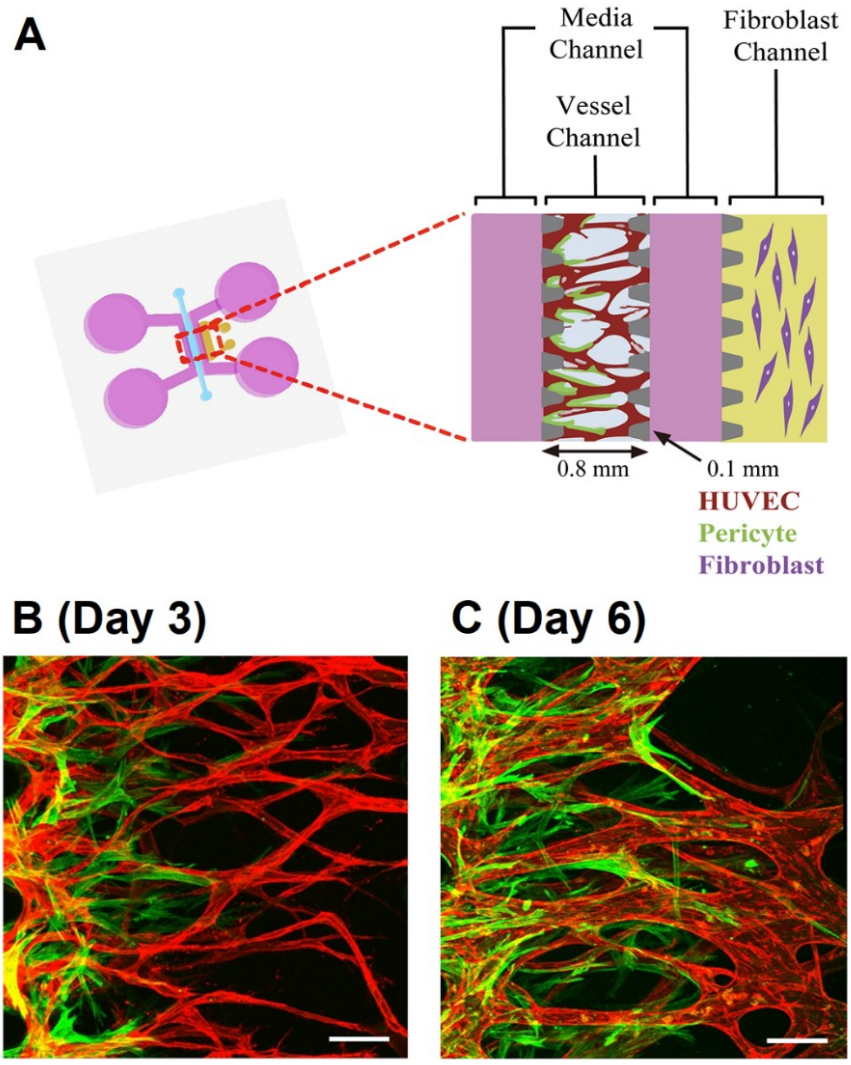

Figure 5. *A) A schematic of the microfluidic device. Endothelial cells and pericytes are seeded on the left side wall of the vessel channel, and fibroblasts induce EC sprouting through the fibrin gel. Pericytes later follow and cover the formed vessels. B), C) Confocal images show EC sprouting (red) at day 3 (B), and pericyte coverage (green) of the vascular network at day 6 (C). Scale bars: 100 µm. Figure modified from* [82].

The major advantage of self-organizing approaches is that the vessel networks produced exhibit similar morphology to those *in vivo,* due to close mimicking of physiological vascular development. They will continue to remodel throughout their lifetime and are geometrically more complex than those formed by pre-patterning techniques. Self-organizing of ECs depends greatly on ECM characteristics: cells must be able to degrade the material in order to migrate, and the mechanical properties such as stiffness influence cell behaviour. Well-established, natural biopolymers collagen and fibrin have most often been used as hydrogel scaffold. [78] Due to randomness of self-organization, fluid flow is difficult to analyze and control. The largest challenge might be the correct temporal and spatial patterning of multiple growth factors during the whole vessel formation process. One solution would be inducing cells to express angiogenic genes by environmental control: for example, oxygen gradient affects local VEGF expression. Cells then regulate secretion of angiogenic proteins themselves, similar to *in vivo* angiogenesis. [60]

3.3.3 Pre-patterning approaches

Pre-patterning, also known as templating, refers to creating an engineered structure of specified geometry to house cells. Cells can be embedded in the material or seeded into the construct after fabrication. Advanced pre-patterning approaches make use of biofabrication methods, such as 3D bioprinting, and allow for creating complex three-dimensional geometries using multiple different components in the same construct. Often, pre-patterning alone is not enough to produce a vascular network. It provides the initial structure, but remodelling of the vessels is evident, and some biochemical or mechanical cues might be needed to guide the remodelling process in the desired direction; this should be taken into account when choosing materials for the model [60].

Pre-patterning approaches are divided in two: additive and subtractive approaches. Additive approaches refer to methods in which material is added layer by layer to build the three-dimensional structure. In subtractive approaches, material is removed. Examples include laser patterning and the use of temporary structures as a template on which another material is cast, and which is then removed.

Seeding of pre-made channels with a confluent monolayer of ECs is challenging and requires either incubation for several days or more complex, dynamic seeding techniques, which might be difficult to use on microfluidic devices [83]. The approach can be used for larger vessels but not capillaries. The minimum diameter of a channel in which cells can be seeded is determined by the diameter of ECs: as the vessel diameter approaches the diameter of a single EC in suspension (10–15 μm), clogging occurs.

Linville *et al*. managed to seed channels of 20 µm diameter by the combination of a stiff collagen type I scaffold, reverse pressure (*i.e*., flow is in the direction opposite to seeding), and elevated cAMP levels. 1 mm long perfusable capillaries could be formed in 3 days. [84]

The simplest method to create a hollow tube is to insert a needle or other mechanical spacer in liquid hydrogel, let the hydrogel polymerize, and then remove the spacer [51], [85]. The mechanical spacer of choice can be coated with cells to remove the post-polymerization seeding step: Sadr *et al.* used oligopeptides to coat gold rods with HUVEC monolayers. The rods were then placed in gelatin methacrylate (GelMA) hydrogel, and the cells were detached from the rods by using a photoinitiator and UV, or by applying an electric potential. Hollow tubes with HUVECs lining the inner wall were created in the hydrogel when the rods were removed. [86] It is, however, difficult to create branched structures using mechanical spacer approaches.

Photoablation, *i.e*., laser patterning, is a method of subtractive pre-patterning that can be used in engineering vascular structures. Focalized pulsed lasers are used to create cavities in a hydrogel, and complex geometries can be achieved due to computer-aided operation. For example, Brandenberg and Lutolf fabricated a perfusable microfluidic network based on a photograph of a capillary bed (Fig. 6). They demonstrated that the network had predictable mass transport behavior and could maintain stable gradients for a week. [87] Three-dimensional computer tomography images of *in vivo* vasculature could also be used. Another benefit of photoablation is that the network can be modified during the experiment by adding new branches.

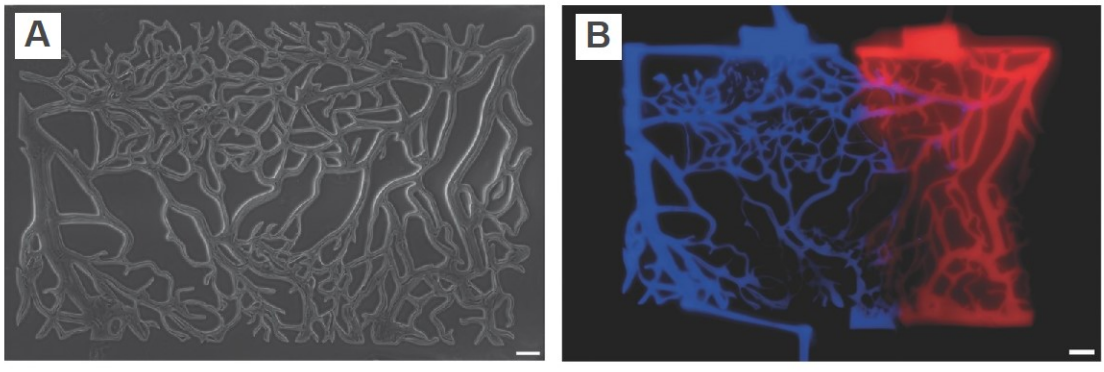

Figure 6. *A photograph of a capillary bed was used as a template for microfluidic network created by photoablation in a poly(ethylene glycol)-based hydrogel. Figure shows the structure before and after connecting it to a perfusion system (A, B; respectively). Scale bar: 100 µm. Figure modified from* [87].

The basic principle of 3D bioprinting was described in Chapter 2.3.3. In vascular applications, it can be used for direct printing of ECM, with or without cells embedded, into vessel structures. This allows for excellent spatial control of cell placement; however, the printing process might be too harsh for some cells. [78], [80], [88] In indirect printing, vessel structures are printed with a temporary mold and hydrogel is cast on top of it, creating a “negative” of the printed construct [88]. It is especially used when the ECM-mimicking material is difficult to print and modify [37]. Indirect printing is suitable for creating complex network geometries, but cell distribution is not controlled precisely. It also involves using multiple materials in multiple steps, which adds complexity in designing the structures.

Issues to consider when bioprinting vascular structures include material properties. The material cannot be too stiff, or EC sprouting will not be successful. In the other hand, the material has to be stiff enough to be able to support its own weight and keep the vessel lumen open. [78] Supporting baths with hydrogel pre-polymer can be used in some cases to help the printed filaments retain their structure [89]. 3D bioprinting methods that are especially applicable to printing vascular structures are coaxial nozzles and printing sacrificial templates, as they allow for printing hollow vessels and vessel networks.

Coaxial nozzles can be used for printing tubular structures that can later be filled with a cell-laden hydrogel, or printing multi-layer tissue constructs, in which the cells are embedded in the bioink [37], [90], [91]. Branched structures are yet to be achieved [32]. In a study by Gao *et al*., HUVECs were embedded in vascular tissue-derived decellularized ECM/alginate hybrid bioink. This HUVEC-laden bioink was printed through the shell part of coaxial nozzle, with the core being fugitive Pluronic-F127. Due to presence of Ca^{2+} ions, the alginate component cross-linked immediately after printing and no support matrix was needed. Medium was perfused through the construct and the Pluronic-F127 was dissolved in and washed away by it, leaving behind a lumen in the tubular structure. The principle is visualized in Figure 7. ECs formed a confluent monolayer on the vessel surface by day 7. [92]

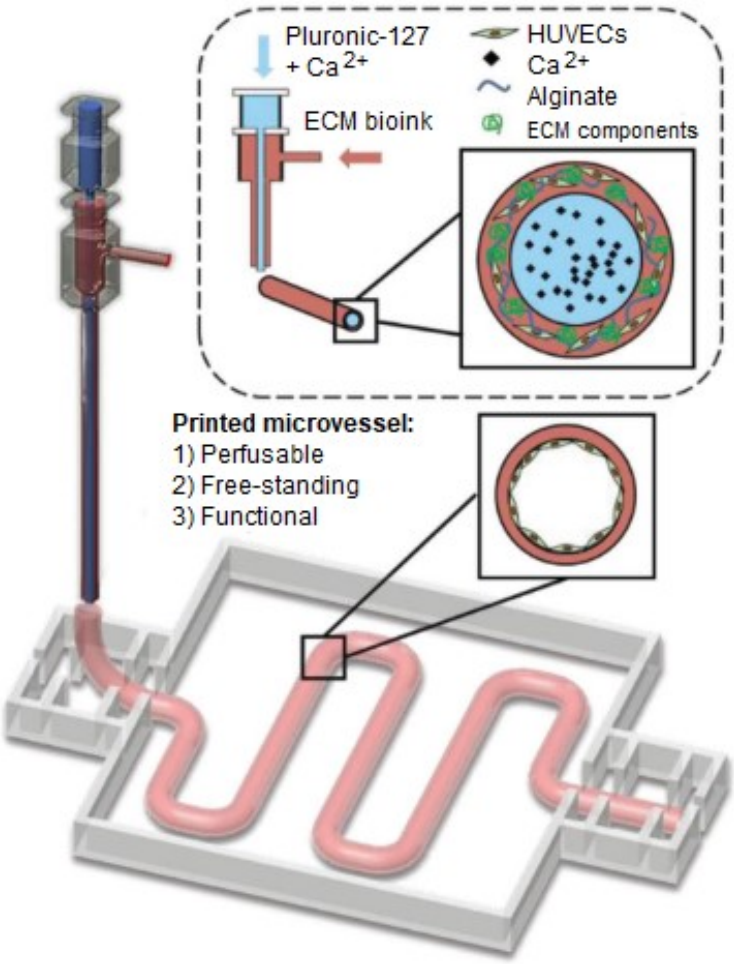

Figure 7. *Free-standing vessels with a diameter of 250–850 µm were 3D printed inside a custom-made perfusion chamber using a coaxial nozzle and Ca^{2+} cross-linked fugitive bioink. Figure modified from* [92].

In sacrificial templating, the branched structure is printed in temporary material and ECM hydrogel is cast around it. After ECM gelation, the temporary material is removed by, *e.g.*, temperature change or solubilization, leaving hollow channels in the ECM. The channels are then seeded with ECs. Another approach is to suspend ECs in the sacrificial template bioink; when the template is dissolved, ECs adhere to the internal vessel walls and form a lumen [47]. Sacrificial template materials that have been used include Pluronic-based bioinks, alginate, gelatin and carbohydrate glass [32], [78], [89], [93].

3.4 Vascularized organ- and body-on-a-chip platforms

3.4.1 Overview

As flow is an important factor in vascular function, microfluidic on-chip devices provide an excellent platform for modelling vasculature. Precise control of flow-related parameters allows maintaining flow in the physiological range in a mature model. The way different changes or disturbances in flow affect the vascular structures can be studied, and the effects can be exploited. For example, controlled shear forces have been utilized to induce maturation of vascular structures [94]. Spatial and temporal patterning of biomolecules is possible in on-chip devices; VEGF gradients have been used to guide angiogenesis [95].

Illustrating the importance of vascular compartments in organ-on-a-chip platforms, Lin, Guo and Zhang [10] did an analysis of organ-on-a-chip articles published between 2008 and 2017. The word ‘vascular’ was mentioned in almost 2/3 of the organ-on-a-chip publications they examined. It should be noted that the analysis does not specify if the vasculature in each study is established or if it is only mentioned as a target for future development.

Novak *et al*. define vascularized organs-on-a-chip as “microfluidic cell culture devices containing separate vascular and parenchymal compartments lined by living human organ-specific cells” [16]. According to Meng *et al*., chips involving vasculature can be categorized into 5 types: Microfluidic devices with a mechanically stretchable membrane; devices using a synthetic elastomer as a scaffold; devices consisting of hydrogels with microchannels; self-assembled microvasculature; and devices incorporating open-well design, in which pre-formed parenchymal tissue is added on a pre-formed vascular bed. [76]

Vasculature in chip models can be established for different aims:

1. exploration of vascular network formation and angiogenic processes,
2. providing mass transport in an organ model,
3. creating a vascular-parenchymal interface and studying its barrier functions, and
4. connecting organ compartments to each other using endothelialized, vessel-like structures.

A selection of examples is presented in the following subsections. They are chosen to cover the aims listed above and to illustrate some design choices that are common to vascular chips.

3.4.2 Vascular networks on chips

Vascular chip designs can explore the processes of vasculo- or angiogenesis, anastomosis, and the interconnection of capillary networks and larger vessels that have been built using different methods. Wang *et al*. co-cultured endothelial cells and normal human lung fibroblasts in fibrin gel inside a diamond-shaped tissue microchamber. Interstitial flow induced vasculogenesis and the formation of a capillary network. The chamber was flanked on both sides by laminin-coated microfluidic channels lined with endothelial cells, and small pores connected the channels to the tissue chamber. The design modelled the *in vivo* interconnected artery – capillary bed – vein network. [96]

Bidirectional anastomosis was achieved: endothelial cells would migrate from the tissue chamber into the lined microfluidic channels, and *vice versa*, facilitating the connection between the two vascular compartments (Fig. 8 B, D). Basal-to-apical transendothelial flow, VEGF gradient, and laminin coating of channels were found to be crucial factors in this. Intact network was formed by day 12, and the tightness of the interconnection was confirmed by perfusion of 15 µm diameter, fluorescent FITC-dextran particles (Fig. 8C). However, the average diameter of the capillaries was larger than those *in vivo*. [96]

The authors stated that the approach could be used to create vascularized organ- and body-on-a-chip devices. However, scaling up would be difficult due to the multi-step process of seeding the capillary bed chamber and EC-lined channels. Another limitation was the lack of smooth muscle cells and therefore the lack of vasoconstriction. [96]

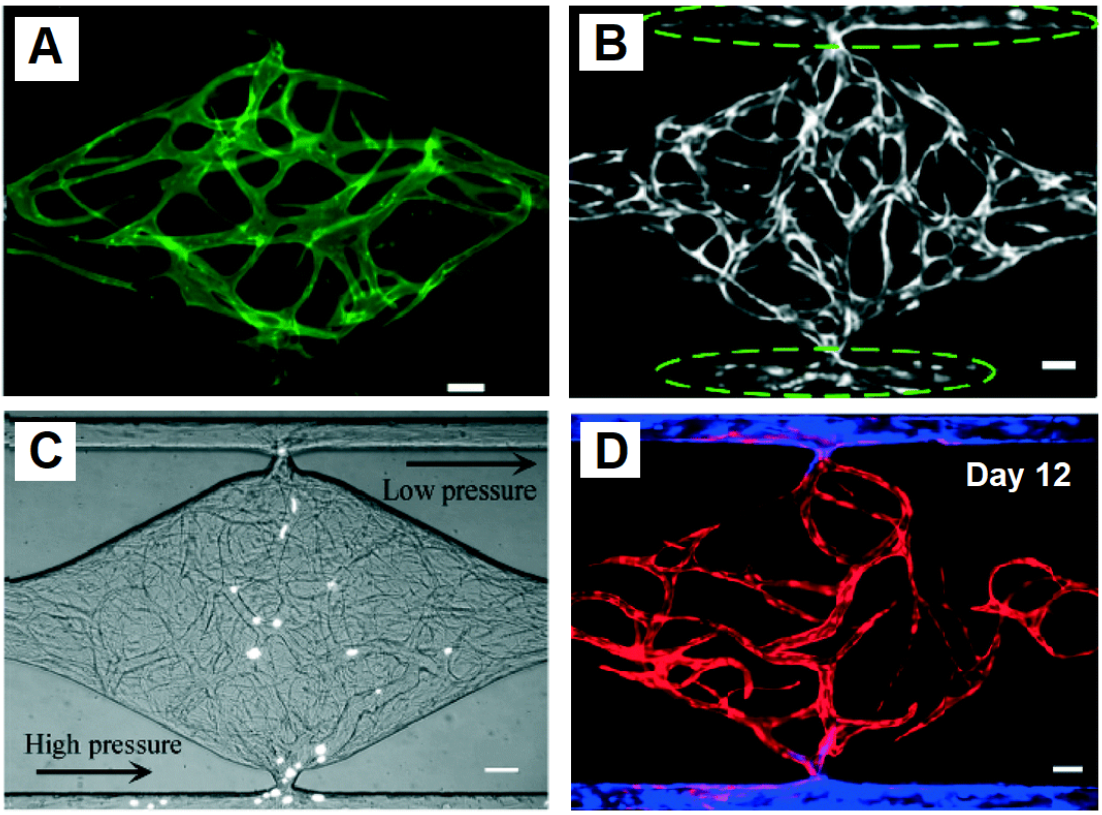

Figure 8. *Endothelial cells were co-cultured with lung fibroblasts in a fibrin gel-filled tissue chamber between two EC-lined channels. (A) CD31-immunostained capillary network was formed via vasculogenesis. (B) Endothelial cells migrated through the communication pores into the medium channels (marked with dashed ellipses). (C) 15 µm dextran particles (in white) were perfused through the network. (D) Red, mCherry-expressing ECs and blue fluorescent protein - expressing ECs were cultured in different compartments to visualize the bidirectional anastomosis. Scale bars: 100 µm. Figure modified from* [96].

3.4.3 Vascularized organs-on-a-chip

Adding vascularization to an organ-on-a-chip shifts the focus from vascular cells to the parenchyma and adds up on the number of aspects to consider. In many vascularized organ-on-a-chip models the endothelialized medium channel acts as the vasculature. Here, we focus on models in which blood vessels are grown into the parenchymal tissue compartment.

Both self-organising and pre-patterning approaches can be used. With self-organising vasculature, angiogenesis can proceed alongside organ development and maturation, as it would *in vivo*. Pre-patterning lacks this aspect, but adequate blood supply, in a desired vessel geometry, would be available through the whole parenchymal maturation stage. However, creating capillary-scale vessels (diameter approx. 10 µm) with templating methods is not yet feasible.

In their review article, Zhang *et al*. suggest that a good solution would be to first create a pre-patterned scaffold with large-diameter vessels, add the endothelial cells, and promote their sprouting into the surrounding tissue in order to obtain small-diameter vessels. They also present an approach of combining pre-vascularized organoids and engineered perfusable capillary beds; however, achieving functional anastomosis between the two constructs might be challenging. [80] Subjecting the organoids to high fluidic shear stress and using organ-specific endothelial cells have been used to aid in organoid vascularization [36], [97].

As an example, a full-thickness skin construct with vasculature was developed by Jones *et al*. [98]. The PDMS device consisted of a flat, round compartment for vascular tissue culture and circumferential medium channels on both sides of the tissue compartment. The tissue compartment had a round opening in its ceiling, making up an open well for dermal tissue. The round design was found to allow for the growth of more homogeneous vasculature in comparison to a rectangular one.

HUVECs in fibrin gel, with or without pericytes, were first loaded in the central tissue culture compartment. After 4 days, human dermal fibroblasts from an immortalized cell line were embedded in collagen gel and added in the well on top of the chip. After polymerization of the gel, human cell line-derived keratinocyte suspension was injected in the well in order to create the epidermal compartment. The design is visualized in Figure 9. The chips were cultured for 7–14 days, after which the tissue was harvested for histological staining.

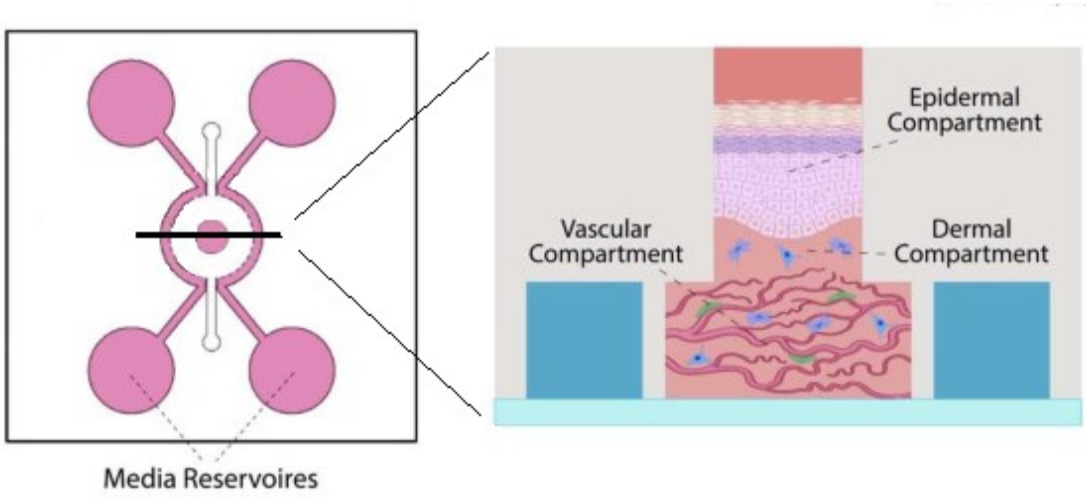

Figure 9. *A schematic describing the round chip design that was used in a vascularized skin-on-a-chip. Dermal fibroblasts and keratinocytes were cultured on top of a pre-formed vascular network. Modified from* [98].

The structures of the skin constructs and vascular networks were compared between three experimental setups: HUVECs with pericytes, HUVECs only, and no vasculature as a control. *Ex vivo* skin samples were also used to assess the physiological relevancy of the skin constructs. The microvasculature extended into the dermal compartment and was found to enhance the stratification and differentiation in the epidermal compartment. Including pericytes in the culture increased the thickness of the epidermis. [98]

3.4.4 Building bodies-on-a-chip *via* vascularization

Connecting multiple organs-on-a-chip to each other creates a microfluidic multi-organ-on-a-chip system, also known as body-on-a-chip. As vasculature connects organs in the human body, engineered vasculature would be an excellent solution in multi-organ-on-a-chip systems as well. The general aspects of connecting tissues were discussed in Chapter 2.2. This chapter describes two examples of using endothelialized vessel structures as the means of organ-organ connection instead of conventional tubing or non-functionalized microchannels.

A vascular compartment can pass through the organ culture compartment, in a way similar to a blood vessel inside tissue. The same vessel-like structure can then continue to the next organ. Lai *et al*. created the InVADE platform, short for “integrated vasculature for assessing dynamic events” [99]. They utilized the AngioChip scaffold, previously developed by the same group, which is a perfusable microchannel scaffold supporting the assembly of parenchymal cells on its biodegradable elastomer matrix surface [100]. Ten hepatic and ten cardiac or tumor tissue culture chambers were placed in an open 96-well plate platform. Endothelialized, tubular AngioChip scaffold, with a luminal diameter of 100 µm and patterned with 15 µm microholes, was suspended across each organ-specific parenchymal tissue chamber. Each scaffold was connected to an inlet and outlet well, linking the organ models in series. The device is illustrated in Figure 10.

With the platform, the team could replicate cancer invasion-metastasis cascade for the first time: cancer cells could escape the tumor chip and invade the liver chip placed downstream through the common vascular scaffold. [99] The scaffold is made of poly(octamethylene maleate (anhydride) citrate) (POMaC), a biodegradable elastomer, which is stable enough to prevent remodelling and movement, such as cardiac contraction, in parenchymal space from blocking the vessel [44].

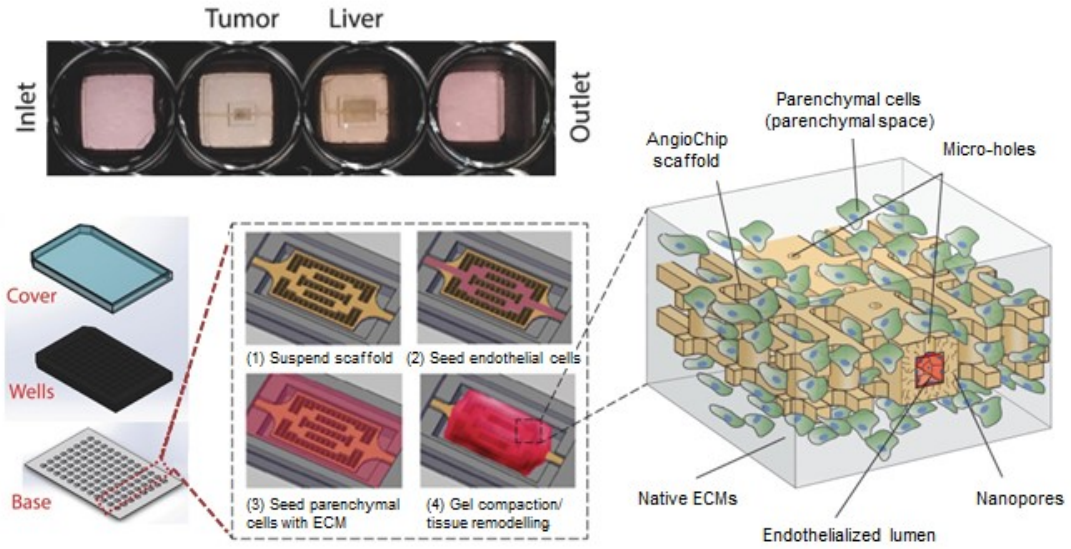

Figure 10. *In the InVADE platform, ten tumor-liver models fit on a 96-well plate. A vascular scaffold is placed across each tissue culture chamber and seeded with endothelial cells. Parenchymal cells embedded in fibrin gel are added in the chamber, creating tissue around the vascular scaffold. Adapted from* [99], [100].

To replace conventional plastic tubing with a more biomimetic way of interconnecting organs-on-a-chip, Zhang *et al.* developed a templating method to produce hollow, elastomeric PDMS tubes with different diameters and wall thicknesses [101]. The tubes were endothelialized and used as a blood vessel mimic between organ compartments (Fig. 11). First, PDMS pre-polymer was loaded into a plastic tube, and a metal rod was used to create the lumen. After the curing process, the plastic tube and rod were removed, and the inner walls of the PDMS tube were coated with fibronectin and seeded with HUVECs. The tubes were then connected to a peristaltic pump for perfusion culture, and intact endothelium was formed in 7 days. The endothelialized tubes were fully perfusable, expressed the junction biomarker CD31, and responded to a panel of drugs in a correct, dose- and time-dependent manner. Endothelium stayed stable over 14 days, as indicated by secretion of endothelin-1. With length up to 20 cm, the tubes were used to connect separate liver, heart, and lung compartments *via* a common medium circulation (Fig. 11D).

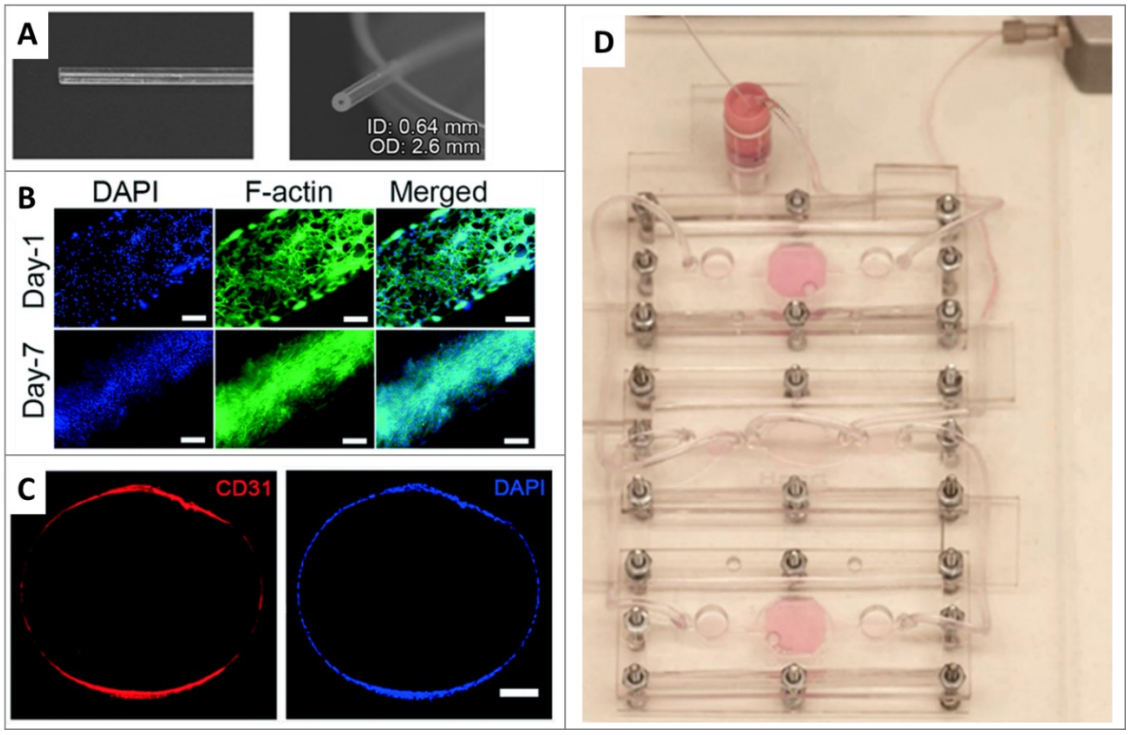

Figure 11. *(A) A PDMS tube with inner diameter of 0.64 mm and outer diameter of 2.6 mm, before endothelialization. (B) Fluorescence micrographs of a similar tube with 0.64 mm inner diameter. DAPI staining (blue) was performed to visualize the nuclei and F-actin (green) to visualize cytoskeletons. Scale bar: 200 µm. (C) Confocal fluorescence micrographs of a cross-section of an endothelialized tube. CD31 (red) shows a junction biomarker and DAPI (blue) the nuclei. Scale bar: 100 µm. (D) Three organs-on-a-chip interconnected using the endothelialized tubes. Modified from* [101].

The method allows for adjusting the diameters and wall thicknesses of the tubes and the modulus of PDMS used. Arteries and veins with different diameters and hardened, diseased vessels can therefore be modelled. However, very thin tubes were found to be difficult to handle and break easily during production, so they were not used in the endothelialization tests. [101] The tubes also encourage modular chip designs. The same method was later complemented with laser ablation and used to produce porous tubes. The endothelialized tubes were then embedded in ECM and ECs would sprout into the surroundings through the pores. [102]

4. CONCLUSIONS

Organ- and body-on-a-chip systems are a leap towards better modelling of human physiology. Their main advantage comes from the possibility to precisely control the environmental parameters under which the cells are cultured. The structural arrangement of different cell types in co-culture can be fine-tuned through design geometry or fabrication methods selected, which aids in creating organ models consisting of several tissue types. Connecting multiple organs-on-a-chip enables modelling of cellular signalling and metabolism.

The main issues to overcome are related to the unique nature of each organ-on-a-chip model: while a diverse range of different, feasible designs adds to the potential of the chip technology, some degree of standardization would be beneficial in model validation. Recapitulating organ structures and functions in microscale is accompanied by the question of scaling: how to scale cell types in a single organ-on-a-chip, or single organ compartments in a multi-organ-on-a-chip, in relation to each other? Another smaller but still a significant issue is the widespread use of PDMS as the chip material, as it absorbs small hydrophobic molecules such as signalling factors.

The diffusion range of gases and molecules in tissue is 100–200 µm, and tissues larger than that cannot survive in culture. Therefore, vasculature is needed to cover mass transport in longer distances. The perfusion-controlled on-chip platform is suitable for vascularization of tissue constructs, as flow has important effects in vascular network formation and remodelling. Engineered vasculature is created using approaches based on self-organization of endothelial cells or on seeding pre-patterned vessels with cells. The best option might be the combination of both: Larger blood vessels could first be built with pre-patterning methods to ensure there is a rudimentary perfusion throughout the tissue. New, smaller vessels could then sprout and expand the network through angiogenic processes. This is a necessary step, as fabrication of capillary-sized vessels using pre-patterning methods is difficult, if not impossible at the moment.

A vascular-parenchymal interface is present in almost all organs. Vascularization should therefore be an integral part of almost any organ-on-a-chip, even if adequate perfusion could be achieved without it. *In vivo*, angiogenesis and organ development proceed simultaneously, guiding each other. The endothelial barrier provided by vascu-

lature helps separate different medium types in a multi-organ device; a universal blood surrogate suitable for culturing all the organ types might not be needed. Using endothelialized, vessel-like structures would provide a more biomimetic way of organ-organ interconnection than plastic tubing. Instead of fixed structures, modular multi-organ-on-a-chip designs often rely on tubing that can be moved on demand in order to connect organ modules into the system. Endothelialized PDMS tubes can perform this function. However, publications on vascularized body-on-a-chip devices are still scarce.

Vascularization in organ-on-a-chip context can serve purposes in creating better organ models, studying vascular function and network formation, and connecting multiple organ compartments to each other in body-on-a-chip platforms. Application-wise, vascularization is important in pharmacokinetic studies, as a drug molecule might experience changes when it passes through the blood vessel endothelium into its target tissue. Advances in on-chip vascularization will also be valuable in clinical context, for example in understanding cancer metastasis and creating large tissue engineered constructs to be used as implants.

The main merit of organ-on-a-chip platforms is their versatility as the concept can be applied in numerous ways for different purposes. It can also be seen as an obstacle, prolonging the time the technology needs to become established. As with all *in vitro* models, organ-on-a-chip development requires balancing between throughput or feasibility and physiological accuracy, and the purpose of the model will determine which functions to prioritize. In the future, it is likely that there will be widely used standard chips for some applications and plenty of custom-made chips for more specific needs. When it comes to engineering vasculature, controlled flow is indispensable, and organ-on-a-chip devices definitely have the cutting edge.

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