# Maskless photolithography for rapid Organ-on-a-Chip prototyping and microvascular engineering

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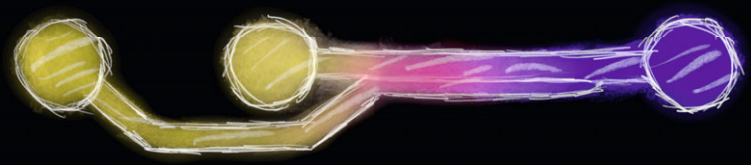
## Abstract

There is an urgent need for accurate human *in vitro* models and considerable societal pressure to reduce the number of animals used in biomedical research and drug development. At the same, investment by the pharmaceutical industry in developing new drugs is enormous yet many candidate drugs based on animal experimentation fail to reach their clinical endpoint in phase 3 trials or are withdrawn after market entry because of unexpected safety issues in humans. There is reasonable concern that current preclinical models often fall short of accurately predicting efficacy or safety in humans. The emergence of human stem cell-based models in combination with microfluidic technology referred to as Organ-on-a-Chip (OoC), is increasingly explored and may present a valuable addition or alternative to animal experimentation. In the research carried out for this thesis, various technical approaches of combining stem cells with innovative OoC and rapid bioengineering technologies are described and developed, aiding OoC development and microvascular engineering, setting the stage for future studies on vascular disease. In the following sections, I describe the context of the research to provide background to the later experimental chapters of this thesis.

# Chapter

## General introduction

## Organ-on-a-Chip technology and microvascular engineering

## Reduction and replacement of animal models

A paradigm shift towards methodologies based on human *in vitro* models is ongoing in biomedical research. Driven by the unmet need for more relevant and accurate models of human physiology, researchers in our lab and others are exploring alternatives to address the limitations of animal models and, where possible, reduce their use[1–6]. Animal models are increasingly considered a double-edged sword: they have been the gold standard in biomedical research and drug development for decades but may fail to predict human responses accurately, ultimately resulting in high drug attrition rates and adverse drug reactions. Regardless, biomedical research remains reliant on animal models for the identification of potential drug targets during preclinical phases[1,2,7–9]. This is in part because of regulatory requirements regarding evidence of effectivity in disease models and data on drug biodistribution, but also because there are few validated alternatives. However, molecular and cellular mechanisms controlling physiology and pathology in animals may differ significantly from those in humans. In addition, animals are often inbred and thus do not reflect the genetic diversity seen in humans. Numerous instances have been reported where drugs, biopharmaceuticals and vaccines that appeared promising in preclinical studies failed in human trials, due to lack of efficacy or severe toxicity[1,7,10]. Moreover, animal-based development of therapeutics such as monoclonal antibodies or gene therapies is rather inefficient because these compounds are highly specific for human target sequences and/or conformations[4].

The mounting pressure from the public to find alternatives for animal experiments is also driving changes in biomedical research. Ethical considerations and a growing awareness of and demand for better animal welfare are major factors that illustrate the importance of the "3Rs": Replacement, Reduction, and Refinement. These principles were devised to minimize and optimize the use of animals in biomedical research while ensuring ethical standards and scientific integrity. While acting as a legal and ethical framework to guide researchers, the 3Rs also describe the need for alternatives and, for some, the ambition of complete replacement of animals[3,5,11,12]. Among the frequently mentioned options to reduce animal experiments is the development and use of Organ-on-a-Chip (OoC) models. This has been an important focus of the research I report in this thesis.

## Human *in vitro* models and Organ-on-a-Chip technology

Human *in vitro* models have the potential to improve biomedical research, or at least complement research on animal models as to minimalize their use in case there is no *in vitro* or *in silico* alternative. A plethora of new technologies and strategies are now emerging as promising alternatives that can better mimic human tissue and organ functions. These new approach methodologies (NAMs) include both human *in vitro* and *in silico* methods. They aim to reduce the reliance on animal models by employing novel and innovative technologies,

thereby aligning with the principles of the 3Rs[3,13–15]. The *in vitro* models include:

- **2D cell culture**: For several decades, human cells have been cultured as monolayers on 2D flat substrates such as Petri dishes or culture flasks. These simple systems are cost-effective and optimized for cell proliferation and batch generation; however, 2D culture often results in the loss of tissue-specific functions and/or dedifferentiation, compromising its physiological relevance.

- **Static 3D models**: Static 3D models offer greater structural complexity than 2D models, which can better sustain tissue functionality over extended periods of culturing. They are designed to mimic organ architecture and function and usually include an extracellular matrix (ECM) that provides structural integrity, physiological mechanical properties, and more realistic cell-ECM and cell-cell interactions.

- **Organoids:** Miniaturized and simplified 3D models of organs, produced from stem cells. They can self-assemble and organize into structures that resemble the architecture and function of organs, making them useful for disease modeling and drug testing with appreciable throughput.

- **Organ-on-a-Chip models (OoCs), also known as microphysiological systems (MPSs):** Microfluidic devices containing living (human) cells in perfused, micrometer-sized chambers to model the physiological functions of tissues and organs in a miniaturized format. The possibility of growing 3D structures, co-culturing multiple cell types, and inclusion of mechanical stimuli and fluid flow enables these devices to possibly recapitulate complex organ-level functions and interactions, potentially providing improved disease modeling and more accurate predictions of human responses to drugs.

- **Multi-Organ-on-a-Chip devices (Body-on-a-Chip)**: Fluidically coupled OoCs that can mimic whole-body physiology and drug distribution. As such, they could provide an integrated platform for studying complex (inter-organ) physiological interactions, systemic effects and drug pharmacokinetics/pharmacodynamics (PK/PD)[16–19].

The OoC field was created through the convergence of microelectromechanical systems (MEMS), lab-on-a-chip (LOC) technology and tissue engineering. MEMS are microdevices that incorporate electronics and moving parts and were made possible in the 1960s to 1980s by adaptation and evolution of engineering processes used in semiconductor fabrication (e.g. photolithography, chemical deposition processes and etching)[20]. In the 1980s and 1990s, the microfabrication techniques for MEMS resulted in the development of LOC devices that miniaturized analytical and laboratory processes and enabled researchers to perform them on a microscale[21]. Concurrently in the 1990s, the development of polydimethylsiloxane (PDMS)-based soft lithography led to facile fabrication of microfluidic devices (via replica molding), microcontact printing and micromolding[22–24]. Finally, in 2010, the first PDMS-

based OoC was created: a biomimetic Lung-on-a-Chip that modeled the functional alveolar-capillary interface of the human lung[25]. This compartmentalized chip consisted of two microfluidic channels separated by a membrane on which human lung endothelium and epithelium were grown on opposite sides. The chip incorporated two side-chambers that could be actuated by cyclically applying a vacuum, thereby simulating human breathing via stretching of the membrane. This OoC was commercialized and resulted in “Emulate”, nowadays a renowned developer and supplier of commercial OoCs[26]. It should be noted that many earlier “on-chip” cell culture efforts had already been published years before the seminal paper on the Lung-on-a-Chip, but that chip nevertheless is considered as the first true OoC[27–41]. Since then, OoCs have been used to model and mimic a variety of different organs, including the intestine, vascular system, brain and heart (Figure 1A, B, C and E, respectively). OoCs can include fluid flow, various types of sensors (Figure 1E), mechanical stimulation (Figure 1F), and may contain individually addressable compartments to model different organ/tissue components in one chip[18,19,42,43].

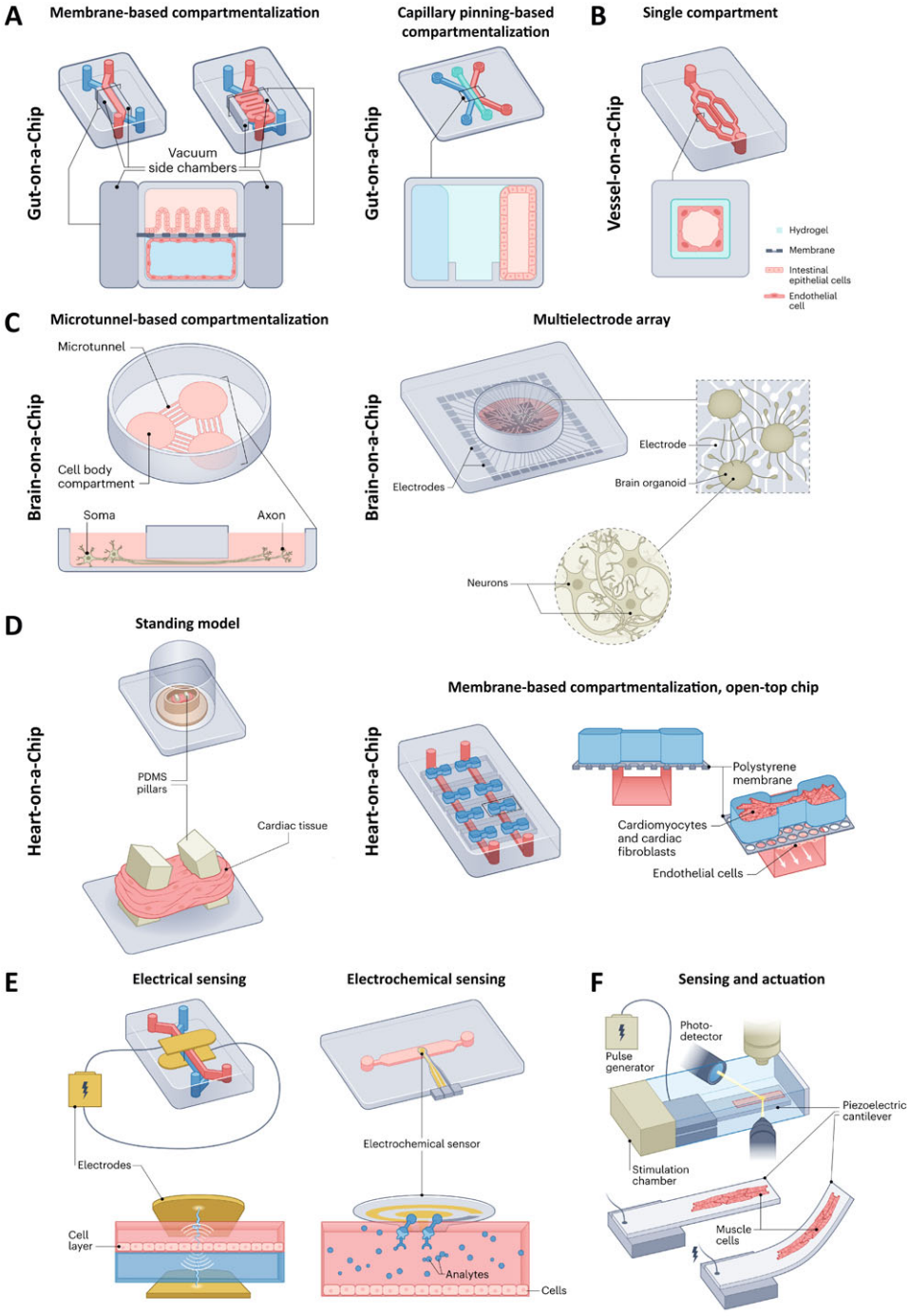

A
Membrane-based compartmentalization
Gut-on-a-Chip
Vacuum
side chambers
Capillary pinning-based
compartmentalization
Gut-on-a-Chip
B
Single compartment
Vessel-on-a-Chip
Hydrogel
Membrane
Intestinal
epithelial cells
Endothelial
cell
C
Microtunnel-based compartmentalization
Brain-on-a-Chip
Microtunnel
Cell body
compartment
Soma
Axon
Multielectrode array
Brain-on-a-Chip
Electrodes
Electrode
Brain organoid
Neurons
D
Standing model
Heart-on-a-Chip
PDMS
pillars
Cardiac tissue
Membrane-based compartmentalization, open-top chip
Heart-on-a-Chip
Polystyrene
membrane
Cardiomyocytes
and cardiac
fibroblasts
Endothelial cells
E
Electrical sensing
Electrodes
Cell
layer
Electrochemical sensing
Electrochemical sensor
Analytes
Cells
F
Sensing and actuation
Pulse
generator
Photo-
detector
Piezoelectric
cantilever
Stimulation
chamber
Muscle
cells

**Figure 1. Representative Organ-on-a-Chip devices of the human heart, vasculature, intestine (gut) and brain. (A)** Gut-on-a-Chip devices. Left, two microfluidic channels separated by a porous PDMS membrane for compartmentalization, typically made of PDMS coated with a thin hydrogel layer, allowing fluid flow and cell culture in both channels. Intestinal epithelium can be cultured in the top and ECs in the bottom. The parallel vacuum chambers enable pressurization and membrane stretching to simulate peristaltic motion. Right, microfluidic channels employing capillary-pinning of hydrogels for compartmentalization. Cells can be cultured in all three compartments. Bidirectional fluid flow can be introduced in the channels by placing the chips on a rocker. **(B)** A single-compartment Vessel-on-a-Chip with square channel geometry, commonly populated with ECs to generate microvessels. Chips can be connected to pumping systems for unidirectional flow, or placed on a rocker for bidirectional fluid flow. **(C)** Brain-on-a-Chip devices. Left, microtunnels compartmentalize the chip and enable separation of neuronal somas and axons. Right, multielectrode array for measuring the electrophysiological activity of brain organoids (or neuronal monolayers). **(D)** Heart-on-a-Chip devices. Left, engineered heart tissue between two standing flexible PDMS micropillars that fit in a standard 96-well plate. Pillar deformation can be tracked and used to derive the contractile force and beating frequency of the tissue. The platform is suitable for high-throughput screening. Right, open-top chip with the top compartment containing a dog bone-shaped cardiac tissue. The chip is compartmentalized using a porous polystyrene membrane and has a perfusable microfluidic bottom channel that can be populated with ECs. **(E)** Organ-on-a-Chip devices containing sensors. Left, chip containing electrodes to sense the electrical properties of tissues, including resistance and capacitance (collectively referred to as electrical impedance). These properties can be continuously monitored and functional properties such as barrier integrity can be derived. Such transepithelial or transendothelial resistance measurements are commonly performed by positioning pairs of electrodes adjacent to the tissue and exposing them to electric fields across a range of frequencies. Right, chip containing an electrochemical sensor that is able to measure analyte concentration by capturing electrical signals that are generated by surface reactions occurring on the sensing electrodes. Electrode surfaces are functionalized with probes for the target analyte, thus enabling specific sensing applications. Multiplexing of multiple functionalized sensors can be performed to simultaneously measure different analytes (e.g. biomarkers). **(F)** Organ-on-a-Chip device that can both sense and actuate. Contractile tissues are cultured on top of a cantilever made of a piezoelectric or electroactive polymer. These materials can be actuated and deformed on electrical input, thereby physically stimulating the tissue. In contrast, these materials can also sense by converting their deformation to an electrical signal. As such, contractile forces exerted by the tissue can be derived. Figure adapted from ref.[43]

## Opportunities for OoCs

NAMs such as OoCs are transformative and may further revolutionize biomedical research due to their capacity to mimic human physiology and their potential for efficient drug development. Importantly, because of the small size that can be achieved with current OoCs, tiny volumes can be used (usually in the microliter range), thereby reducing the required amounts of limited and expensive reagents, and cells[19,43]. Moreover, the development of human induced pluripotent stem cells (hiPSCs) now allows researchers to generate isogenic cell lines carrying patient-derived or genetically engineered mutations with corrected or unmodified variants as controls for disease modeling. Organ-specific cells can be derived from hiPSCs at scale using specialized differentiation protocols and when combined with bioengineering approaches, accurate disease models can be created to study pathophysiological mechanisms or perform drug development in a controlled environment[2,44–48]. It should be noted though that static 3D models do not provide the same level of relevant stimuli compared to OoCs. For example, dynamic fluid flow, vascular

perfusion and interstitial flow are usually not present in these models, and they are thus less suited to model and study transport phenomena, absorption, distribution, metabolism and excretion (ADME) of pharmaceutical compounds, or for PK/PD studies. OoCs are, therefore, more relevant for drug development, and an essential feature of OoCs for pharmaceutical industry is their potential to be employed early in the drug development pipeline. Moreover, they can provide relevant human toxicity and efficacy data during preclinical phases, so potential failures are identified sooner, while successful drug candidates with demonstrated effectiveness in the human model can proceed to clinical trials more quickly[18,19,42,49,50]. This enhanced efficiency potentially leads to improved cost- and time-effectiveness: a necessary requirement for the pharmaceutical industry with the most recent global drug attrition rate hitting an all-time high of 95%, R&D costs of up to 3 billion per drug, and a time-to-market between 10 to 15 years[7,9,49,51].

Personalized medicine, where patient-derived cells are employed together with OoCs, enables detailed analysis of an individual patient's responses to drugs. Therefore, I considered one example of a small-vessel disease called retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations (RVCL-S) in this thesis. Using personalized medicine, guided and informed decisions regarding diagnosis and treatment tailored to the patient can be made[18,19,52]. Interestingly, this approach is also promising when performed at a larger scale where patient-specific models from donors of populations of interest are generated. For example, rare diseases, age-related, or sex-related studies could be performed, while also assessing heterogeneity and inter-individual differences in these groups[18]. Finally, the recent emergence of artificial intelligence (AI) and machine learning (ML) approaches can complement and improve OoC development by increasing the predictive performance of OoCs and assist during prototyping[53–58]. The pharmaceutical industry is already employing AI and ML to optimize and improve aspects such as target identification, lead optimization, and big data analysis of extensive biological and chemical datasets[59]. Therefore, it is very likely that AI and ML methods will increasingly be applied to OoC-derived data to extract maximum information, while it can also be combined with OoCs or other *in vitro* models to validate *in silico* models.

## OoC development: Challenges, bottlenecks and considerations

**Unfulfilled promises despite immense potential**

Many strong claims and promises regarding OoCs have been made by the academic community, implying that OoCs will solve multiple issues currently impacting biomedical research and that the pharmaceutical industry without a doubt will adopt them to perform more efficient, more effective and safer drug development. Much of this is echoed in this thesis as I believe these promises can become reality, provided the challenges in OoC

development, validation, qualification and implementation are properly acknowledged and addressed[7,49,60,61]. In the next section, I briefly introduce current challenges and bottlenecks and provide considerations and recommendations. Moreover, several successes and efforts are highlighted that are pivotal for mainstream adoption of OoCs in biomedical research and drug development.

**Materials and fabrication methods**

Several materials are currently being used to fabricate OoCs, with PDMS being the most common. PDMS is a silicon-based elastomeric material that is suitable for soft lithography, rapid mold replication and prototyping. In addition, PDMS is biocompatible, gas-permeable, optically transparent and can be bonded to different substrates via plasma activation; this has facilitated its widespread use in OoCs[18,22–24,29,62–65]. Whilst very versatile, PDMS is prone to drug absorption, and this is a significant concern and challenge for use in drug development or safety testing since the effective dose may be reduced. However, only hydrophobic compounds are absorbed significantly, whereas other drugs and biologics do not undergo PDMS absorption. Several strategies can be used to reduce or eliminate absorption, including coating of the PDMS surface, quantification of the absorption, or using alternative materials[65–70].

Commercially available chips are usually made from materials other than PDMS because prototyping stages are complete, they are non-absorptive, and more importantly, such materials are compatible with bulk fabrication methods like injection molding and hot embossing. Compared to soft lithography-based fabrication, these methods allow controlled mass production since they offer reproducible and consistent chips using conventional thermoplastics that include polystyrene (PS), polycarbonate (PC), polymethylmethacrylate (PMMA) and cyclic olefin copolymer (COC). Often, these fabrication methods are combined with lamination and bonding methods to create the final multi-material chips[67,71–74]. Nevertheless, PDMS is still used in (commercial) chips but alternative materials such as castable polyurethanes, styrenic block copolymers, off-stoichiometry thiol-ene (OSTE) polymers and poly(octamethylene maleate (anhydride) citrate) (POMaC) are being developed and implemented for OoC development because they solve issues like drug absorption and enable higher throughput production while remaining suitable for prototyping[65,67,75–78].

**OoC development needs a holistic and interdisciplinary approach**

OoC development involves biology, physics and chemistry and thus is inherently interdisciplinary. Extensive collaboration and effective communication between all scientific stakeholders (e.g. biologists, engineers and chemists) is required to create useful chips that accurately mimic human biology. Because of this interdisciplinarity, the development process of OoCs is complicated: every step requires expertise in each discipline and is therefore prone to delivering non-functional prototypes. Knowledge gaps between researchers from

different disciplines and differences in research methodologies can challenge efficient OoC development and reduce the utility of the final OoC[79–84]. Biologists and engineers employ different research methodologies which should be considered during OoC development. Biologists aim to understand the behavior and function of biological systems using empirical and established experimental methods and are usually more conservative, whereas engineers aim to develop novel functional systems by using principles of design, optimization and iteration[84–87]. When developing an OoC, these unique differences need to be exploited for a synergistic approach that transcends disciplinary boundaries and that leads to a functional device with relevant biological features.

Researchers designing and testing OoCs need to understand the global concepts of all the disciplines involved: the biologist should understand basic chip engineering and physics concepts, while an engineer should understand basic cell biology and tissue architecture. This was the postulate in the consortium in which work of this thesis took place: the NWO Gravity project NOCI (Netherlands Organ on Chip Initiative). This interdisciplinary approach allows better assessment of what is realistically possible, and although rather an oversimplification, it illustrates preliminary requirements for starting conceptualization. Moreover, proper biological questions and identification of key research goals during the conceptualization phase are crucial[88], and additionally, a long-term and holistic vision should be devised and maintained throughout the OoC development process. Hence, it is necessary to identify the purpose of the chip, the end-users and their needs, the required chip features, the necessary assays, and readouts. Other considerations such as the choice of chip material, geometry/architecture of the chip (e.g. channel and compartment dimensions), sensors, the type of cells, and biomaterials/scaffolds to use, are equally critical when conceptualizing the intended OoC[42,88]. An overview of the whole OoC development process is illustrated in Figure 2. While it is clear that the aforementioned concepts are crucial for effective OoC development, OoC qualification and adoption are often overlooked and should be considered during the development process and be part of a long-term and holistic vision.

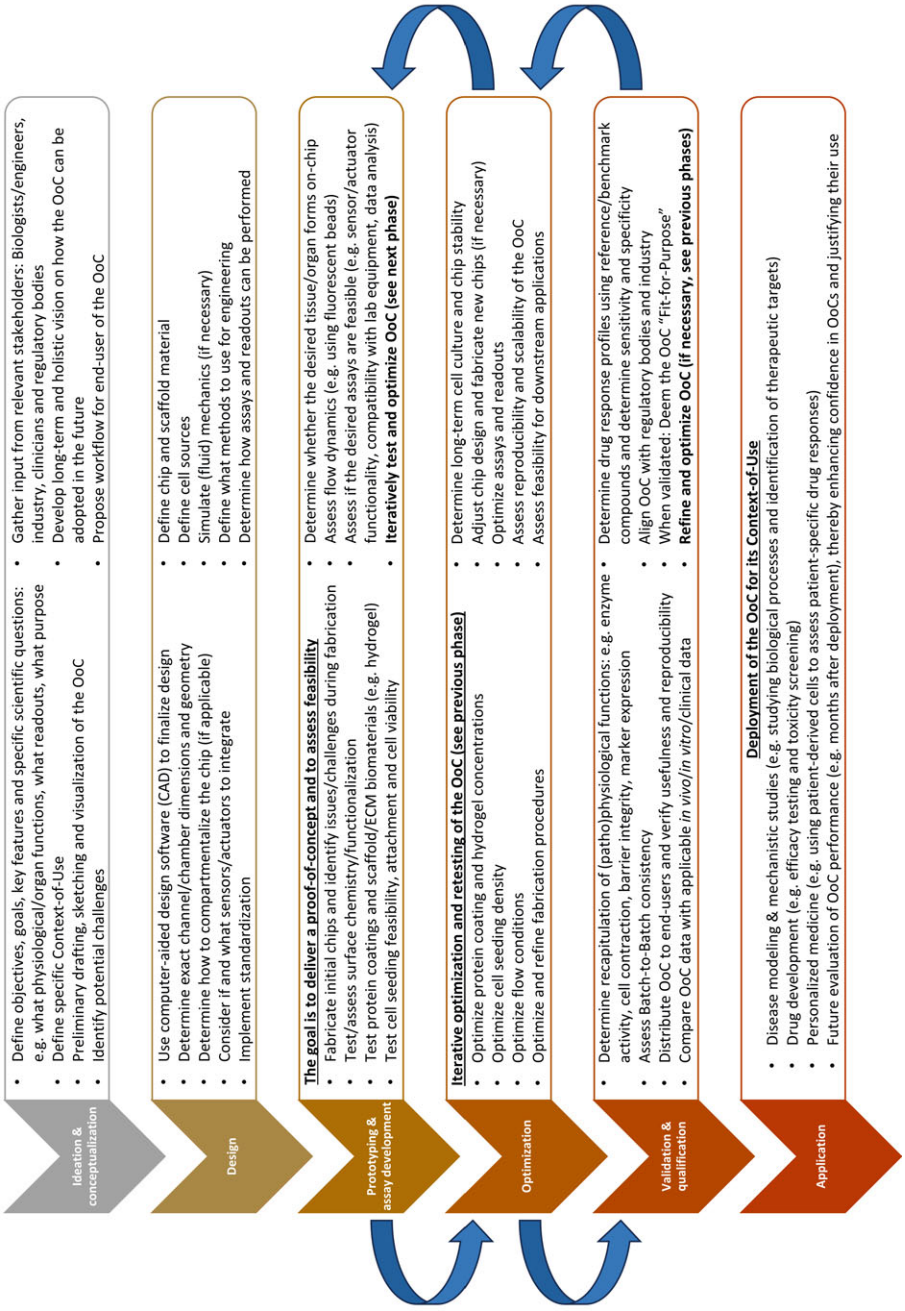



**Figure 2. Schematic of the Organ-on-a-Chip development process.** Stepwise workflow for the development and deployment of Organ-on-a-Chip (OoC) devices. The process progresses through six phases: Ideation & Conceptualization, Design, Prototyping & Assay Development, Optimization, Validation & Qualification, and Application. Iterative feedback loops (curved blue arrows) during the Prototyping, Optimization, and Validation phases are necessary to improve and refine the OoCs. This workflow ensures functional and Fit-for-Purpose OoC devices for diverse contexts such as disease modeling, drug development, and personalized medicine.

## Roadmap towards OoC adoption

### Translational challenges and the need for validation, standardization and regulation

For OoCs to revolutionize biomedical research—by improving drug development and disease modeling, and contributing to the 3Rs—widespread adoption by industry and regulatory bodies is required[18,89–91]. Many different aspects are required for adoption; key elements are summarized in Table 1. Adoption of OoC technology is heavily reliant on translational science as data and insights derived from OoCs need to be valorized, meaning that ultimately, they should benefit patients and society. Biomedical research often meets what is referred to as the "valley of death" between basic scientific discoveries and clinical applications, because promising and innovative research cannot be translated within a reasonable time frame and cost effectively[92,93]. A major hurdle in OoC development is that whereas researchers, industry leaders and regulatory bodies recognize the potential of OoCs, they are reluctant to adopt them in their drug development pipelines. This is mostly because of the inherent uncertainty that comes with the development of novel technology and the conservative reliance on animal models in biomedical research and drug development. Only when OoCs demonstrate utility, performance, reliability and relevance that are equivalent or superior to existing models, can they be qualified and considered as complementary or as replacements (Figure 2)[8,60,61,89,90,94–97].

Rigorous and robust qualification and validation efforts will help translate OoC technology so it can be deemed "Fit-for-Purpose", convincing relevant stakeholders to adopt it. For OoC qualification, defining a clear "Context-of-Use" (CoU) helps identifying and maintaining the scope, purpose, intention, and desired goal of the OoC (Table 1, Figure 2). Since OoCs are simplified organ models with inherent limitations—and therefore can only be used to address specific questions—establishing a CoU helps in qualifying them for predefined applications. This ensures that the OoC is tailored to address scientific, industrial, or regulatory needs, such as drug toxicity testing or disease modeling. Building on the defined CoU, validation and benchmarking are essential to demonstrate the ability of OoCs to reliably, consistently, and accurately replicate human (patho)physiology and tissue characteristics[8,90,96–98]. These efforts also support the development of relevant assays and readouts, enhancing confidence in their use for targeted applications.

To streamline the development and adoption of OoCs, standardization plays a critical role. By providing standards, metrics, guidelines, protocols, and quality controls, standardization enables meaningful comparisons between OoCs, clinical data, and other models, while also facilitating compliance with regulatory requirements and facile implementation in existing workflows[8,96,97,99–101]. Economical considerations further influence the accessibility and adoption of OoC technology. Addressing aspects such as patenting, production costs, scalability, and affordability ensures that OoCs can meet the needs and demands of end-users while remaining feasible for commercialization and widespread use. Finally, collaboration

between stakeholders, which includes researchers, regulatory agencies, clinicians and industry leaders, are crucial for advancing OoC development. These partnerships align OoC designs with end-user requirements, ensure regulatory acceptance, and drive innovation through sharing expertise and resources (Table 1)[8,79,81,82,89,96,97,101].

**Table 1. Key elements required for OoC adoption.** Table contains considerations and recommendations as described in refs.[8,49,90,96,97,101,102] and is supplemented with the authors' personal knowledge and expertise.

| Category | Key points | Examples |
|---|---|---|
| **Context-of-Use (CoU)** | • Identification and description of the utility, purpose and relevance of the OoC within a specific, predefined context<br>• Description of conditions for successful use and expected outcomes<br>• Description of its intention as standalone or whether it will be used in conjunction with additional technologies and methods | • Liver-on-a-Chip for evaluation of metabolic conversion and/or hepatotoxicity assessment and/or pharmacological efficacy<br>• BloodBrainBarrier-on-a-Chip for evaluating the permeability of small-molecule drugs and/or therapeutic antibodies<br>• Heart-on-a-Chip for cardiotoxicity screening<br>• Kidney-on-a-Chip to assess drug excretion<br>• Modeling a specific disease on-chip using hiPSC-derived cells and gene editing<br>• Patient-derived OoC for personalized medicine (e.g. oncology or rare diseases) |
| **Validation** | • Determine sensitivity, specificity, precision, robustness, reproducibility, and stability<br>• Define similarity between OoC endpoints and clinical readouts/outcomes, or endpoints of models currently in use<br>• Demonstrate predictive capability when clinically effective interventions are applied to the OoC<br>• Determine OoC performance using well-characterized benchmark datasets derived from clinical data or other models (e.g. by employing reference compounds) | • Comparing inflammatory markers on-chip to those observed in clinical samples<br>• Compare effects of a standard chemotherapy drug in a Tumor-on-Chip to tumor regression observed *in vivo*<br>• Assessing long-term culture stability of a Brain-on-Chip (e.g. several weeks/months)<br>• Evaluating batch-to-batch consistency of OoCs by repeated replication of results |
| **Standardization** | • Standards describing OoC terminology, definitions and symbols<br>• Technical specifications of engineering and biological components<br>• Specifications describing minimum reporting requirements | • OoC (bio)material standards (e.g. biocompatibility, mechanical and chemical properties)<br>• Cell types to be used in OoCs (e.g. hiPSC-derived, primary, or immortalized), and their requirements<br>• Compatibility with commonly used microfluidic connections and pump systems<br>• Compatibility with existing lab infrastructure (e.g. microtiterplate workflows and microscopes)<br>• Modularity (flexibility of OoCs: e.g. chip interconnectivity, compatibility with fluidic circuit boards, possibility of facile sensor integration, and possibility of upgrading OoC properties without redesigning the whole system) |

**Table 1 (continued). Key elements required for OoC adoption.**

| Category | Key points | Examples |
|---|---|---|
| **Standardization (continued)** | • Specifications describing recommended study designs, how to report protocols, and data management practices<br>• Standardized metrics and protocols to evaluate OoC performance<br>• Guidelines that outline the requirements to use OoCs for various applications | • Guidelines on sterilization and leakage<br>• Engineering and fabrication guidelines (e.g. recommended materials and approaches during different phases: prototyping, upscaling and mass production)<br>• Guidelines on how to make protocols reproducible<br>• Frameworks and guidance for regulation and application, including:<br>  • Drug development<br>  • Drug repurposing<br>  • Disease modeling<br>  • Mechanistic studies<br>  • Toxicology<br>  • Cosmetics<br>  • Food<br>  • Personalized medicine |
| **Economical considerations** | • Intellectual property protection<br>• Commercialization<br>• Scalability<br>• Identify and determine target market | • OoC innovations should be patented before being published. This helps to commercialize novel technologies. Especially in academic settings this is critical. Moreover, this will attract investors due to increased chances of future profitability, and increases competition between chip developers, thus driving innovation<br>• After successful prototyping, mass production is only possible if proper scaling is available<br>• The target market/end-users should be identified. OoC technology is specific and the number of end-users are limited, this should be taken into account (anticipated market penetration)<br>• Cost-benefit analyses (e.g. R&D costs, projected cost reduction in drug development due to successful OoC deployment, cost-per-datapoint) |

**Table 1 (continued). Key elements required for OoC adoption.**

| Category | Key points | Examples |
|---|---|---|
| **Stakeholder collaboration and engagement** | • Collaboration between academia, industry and regulatory bodies (e.g. FDA and EMA)<br>• Networking<br>• Training<br>• Standardization efforts (as described above)<br>• Dissemination | • Regulatory bodies, end-users (e.g. industry) and OoC researchers should closely interact during OoC development. This enables guiding and advising of OoC researchers, ensures proper adherence to guidelines and standards, and as such, increases chances of developing a useful OoC that meets the requirements for adoption<br>• FDA Modernization Act 2.0<br>• Giving trainings and workshops to researchers on OoC development using an interdisciplinary approach<br>• Giving trainings and workshops to researchers on OoC adoption<br>• International consortia that focus on OoC development and adoption (e.g. EUROoCS, hDMT, International MPS Society)<br>• Decrease competition between pharmaceutical companies in terms of OoC adoption in drug development pipelines, so that adoption can be accelerated (e.g. the IQ MPS Affiliate)<br>• Create guides for regulatory frameworks and standardization (e.g. the 2024 Focus Group Organ-on-Chip Standardization Roadmap)<br>• Roche Institute of Human Biology<br>• Include long-term and holistic visions in PhD and postdoc trajectories that focus on OoC development: Anticipated and desired scientific impact, valorization and OoC adoption should be described and evaluated at regular intervals during the trajectory<br>• Informing the public of research results and demonstrating the relevance of OoC development (e.g. organize workshops and exhibitions to enthuse young/potential researchers such as high schoolers, writing simplified summaries of published papers and create websites in plain English). Such efforts will help to improve the science literacy of the general public and help understanding why OoCs are needed, while also recruiting motivated researchers |
| **Fit-for-Purpose** | • Indicates sufficient validation and qualification for the defined Context-of-Use | • See Context-of-Use examples |

**The need for standardization**

The number of successful OoC applications for drug development is increasing as demonstrated in several studies where industry and academia have collaborated. For example, a landmark study published in 2022 by Emulate and academic partners showcased the use of 870 Liver-on-a-Chips to predict drug-induced liver injury of 27 known hepatotoxic and non-toxic small-molecule drugs with a sensitivity of 87% and a specificity of 100%. More importantly, the Liver-on-a-Chip met the qualification guidelines published by the IQ MPS Affiliate on liver injury induced by specific small molecules designated as benchmarks. The authors also showed that the level of performance achieved could generate over 3 billion dollars annually for the pharmaceutical industry through increased small-molecule R&D productivity[103]. Another recently published study by researchers from the Roche IHB and academic partners demonstrated integration of organoids and OoC technology. The resulting functional "mini-colons" had mucus-producing goblet cells and displayed colonic cell differentiation, patterning and maturation as seen *in vivo.* Furthermore, the model could be used to mimic drug-induced toxicity and thus provides opportunities for preclinical assessment of drug safety[104].

While these major successes are promising, many OoCs are custom-made, not easily implementable in existing workflows and/or require expertise to be used. Moreover, the low throughput of many OoCs makes it difficult to implement them at scale or in existing drug development paradigms. This is in contrast to commercial chips that are ready to use and sometimes standardized to a certain extent. Nevertheless, like custom chips, many commercial chips are not standardized, and until recently, there was no true consensus on standardization in the field. Hence, OoC standardization, improved throughput and automation are required[71,99,100,105]. The need for standardization resulted in the European Committee for Standardization (CEN) and the European Committee for Electrotechnical Standardization (CENELEC) establishing the Focus Group on Organ-on-Chip (FGOoC) to define a roadmap on OoC standardization for the coming years. This roadmap was published in 2024 and is intended as a guide containing overviews and descriptions of existing standards and standardization initiatives, and recommendations and opportunities for drafting new OoC standards[102]. Global standardization efforts will result in increased adoption of OoCs and transform it into "turnkey" technology, thus providing end-users with easy-to-use OoCs (Table 1).

Interestingly, industry first addressed the need for standardization more than a decade ago, as was exemplified by the Organoplate platform produced by OoC developer "Mimetas" which became commercially available in 2013[26]. This platform is based on a standard 384-well glass bottomed plate containing arrays of independent microfluidic chips (up to 96 chips). As such, the chips are fully compatible with standard microscopes, plate readers and (automated) liquid handling equipment, and are therefore easy-to-use and suitable for high-throughput screening[106]. The high-throughput and parallelization capabilities of the

Mimetas platform have been successfully demonstrated in various studies including high-throughput screens for vascular permeability, antibody transport, and liver function[107–109]. The platform has also been employed in high-throughput angiogenesis assays with more than 4000 microvessels and a library containing 1537 kinase inhibitors[110]. Platforms such as these are interesting for the pharmaceutical industry because they support studies at scale and can provide many replicates, rendering them especially suited for drug discovery. While very beneficial, increased throughput generally translates to simpler chips and lower physiological complexity, making this tradeoff important to consider when defining a CoU[49,95,98,105].

In summary, standardization is critical for adoption of OoCs, and to realize their full potential and to implement them in drug development pipelines, throughput, parallelization and automation should be developed further while maintaining sufficient complexity.

## Bioengineering to create human *in vitro* models

### Bioengineering

Microfabrication and tissue engineering are critical not only for developing accurate, small-scale devices and structures that enable researchers to generate and study human cell-based models (e.g. OoCs), but also due to their potential for large-scale tissue engineering and regenerative medicine applications. Microfabrication can be used to create precise patterns and structures at the micro- and nanoscale, and innovative adaptations and implementations of methods such as photolithography, soft lithography, 3D (bio)printing and 2-photon polymerization (2PP) now allow the generation of relevant biological structures with resolution at nanometer scale, thus enabling fabrication of controlled environments that mimic biological systems[58,64,72–74,111–119]. Aspects of this have been core to the research in this thesis. In the field of tissue engineering, these methods provide tools to construct scaffolds that support cell growth, differentiation, and tissue formation. These scaffolds can be made from different materials, such as biocompatible polymers, hydrogels, and biodegradable compounds, which not only provide structural support but also offer biochemical and mechanical cues to guide cellular behavior. For example, by modulating the topography, stiffness, and/or porosity of the scaffolds, researchers can influence (stem) cell differentiation, vascularization, ECM deposition and geometry, which are essential features for developing functional tissues[120–122].

Furthermore, sensors fabricated using micro- and nanofabrication enable detection of specific biomolecules, such as proteins, nucleic acids, and metabolites, with high sensitivity and specificity. These sensors can be embedded in wearable devices, point-of-care diagnostics and implantable systems to continuously monitor physiological parameters and detect disease markers, but can also be miniaturized and integrated into OoCs to provide on-chip sensing and diagnostics[123–127]. The combination of microfabrication and other bioengineering methods has also paved the way for creating smart and responsive biomaterials. These advanced materials can change their properties—for example, stiffness, shape, or chemical activity—in response to biological signals or external stimuli, such as temperature, pH, deformation, or light. For instance, hydrogels with tunable mechanical properties can be designed to degrade or stiffen in response to enzymatic activity in the body, facilitating tissue repair or controlled drug release. Similarly, microfabricated surfaces with dynamic features (e.g. micropatterns or topographical cues) can direct cell migration, proliferation, morphology, and differentiation[122,128–130]. As these fields continue to advance, microfabrication and bioengineering strategies are expected to further revolutionize biomedical research and clinical applications. They provide new avenues for tissue engineering, understanding complex biological phenomena and developing novel therapeutic and drug discovery approaches. This thesis involved the development of bioengineering methods that enable rapid fabrication of OoCs, microvascular models and

2D cell culture substrates that are suitable for controlled cell attachment and in the future, contraction measurements.

**Rapid prototyping accelerates model development**

Conventional methods of chip fabrication, such as photolithography can be time-consuming and expensive, limiting the number of iterations possible during early stages of development. For example, microfabrication of photoresist-based molds requires access to a cleanroom, silicon wafers, custom photomasks, and specialized equipment as well as training to perform photolithography. Moreover, extensive communication between the chip producer and the end-user is frequently required to streamline the prototyping process, thus making chip testing during each iteration time-consuming. OoC development—and bioengineering in general—can benefit from rapid prototyping since it enables accelerated iteration of different designs. Rapid prototyping strategies include maskless photolithography, 3D (bio)printing, 2PP and soft lithography, and allow researchers to quickly design, produce, test and modify microfluidic chips and constructs with significantly reduced costs and faster turnaround times[73,131–134]. These facile methods offer opportunities to explore various configurations and fine-tune features such as flow channel properties, chip compartments, and biomaterials to optimize the chip or construct for specific aims (Figure 2). Since rapid prototyping shortens the timeline from conceptual design to functional testing, experimental results can be obtained faster, and models can directly be refined afterwards. This accelerated development process also enhances the potential for high-throughput testing, as multiple prototypes can be produced and tested in parallel[133–135]. This scalability is a significant advantage for pharmaceutical industry, where rapid testing of new compounds or treatments across various organ models can improve the drug development pipeline. Successful and optimized designs can subsequently be produced at larger scale and employed for further research and development (Figure 2).

**The PRIMO system: A bioengineering toolbox based on maskless photolithography**

Maskless photolithography is a well-known method for rapid prototyping that involves direct projection of desired patterns onto a photoresist. There are several methods for this, including using an electron beam, a focused laser beam or spatial modulation of UV light via a digital micromirror device (DMD)[136–138]. The direct writing capability of DMD-based systems is similar to that of other maskless approaches and was first demonstrated in 1999[139,140]. These systems eliminate the need for physical photomasks and are therefore, inherently faster than traditional mask-based photolithography. Maskless photolithography has been applied to various substrates, resulting in the fabrication of sensors, microfluidic- and cell culture devices, as well as optical microlenses[141–150]. Additionally, DMD-based systems have been employed to create microstructures with complex geometries and submicron features[151–153]. As such, these systems are suited for rapid prototyping of OoCs, especially when combined with soft lithography and cleanroom-free approaches. However, most current DMD setups

are custom-built, making them difficult to replicate or adapt without significant expertise in optics, mechanics, electronics, and computer programming. Moreover, conventional maskless photolithography is predominantly carried out using silicon wafers[136,137,154].

User-friendly bioengineering systems such as commercially available 3D (bio)printers or maskless photolithography setups require no additional specialized equipment or facilities, and can simplify OoC and *in vitro* model development. In fact, such systems can be installed in regular biochemistry labs and hence have lower entry barriers for biomedical researchers who want to start with bioengineering, while providing them with rapid prototyping capabilities and cost-effectiveness. For instance, the DMD-based Alvéole PRIMO system for maskless photolithography enables microfabrication, hydrogel patterning, and photopatterning using a single benchtop setup and digital photomasks that can easily be designed in a vector graphics editor[155–157]. This is the system I used in my thesis research. It is compatible with several substrates such as glass and polystyrene and has been employed in numerous studies where photopatterning of ECM proteins was performed to manipulate cell morphology in 2D. The high precision and level of spatial control that photopatterning provides, allows researchers to interrogate fundamental cellular processes that include cytoskeletal dynamics, mechanoresponses, and cell migration[158–164]. Due to its speed, ease, precision and maskless features, photopatterning is a faster and easier alternative to conventional microcontact printing[156]. For example, uniform single-cell arrays of contractile cells (e.g. cardiomyocytes) having predefined morphology can be created. When combined with elastic substrates, cell contractility can be studied by employing traction force microscopy (TFM) in a high-throughput fashion[165–170]. Finally, since the maskless projection is performed using 375 nm UV light, conventional photoresists can be used for microfabrication, thus providing possibilities to prototype and fabricate OoCs. Moreover, hydrogel patterning is possible when a photoinitiator and crosslinkable hydrogel is used, enabling tissue engineering on-chip or engineering of 3D *in vitro* models[114,154,156,157,171].

Because the PRIMO system provides opportunities to perform advanced bioengineering using a single setup, it was used extensively for the development of the bioengineering methods reported in this thesis, as briefly mentioned earlier.

## Vascular engineering and opportunities for hiPSC-based models to study the brain vasculature

### Human *in vitro* models of the brain

The human brain is the most complex organ in the human body. Although only constituting 2% of total body mass, it receives 20% of cardiac output and consumes 20% of the total oxygen and glucose supply. Nearly 100 billion neurons communicate through electrical and chemical signals. Different types of neurons exist, including excitatory, inhibitory, sensory and motor neurons. Glial cells are also abundantly present, that is astrocytes, oligodendrocytes and microglia[172,173].

There are many different types of brain disease and for many, animal models have been developed. These animal models have provided valuable insights into brain disease mechanisms but are limited because animal brains, even of non-human primates, differ significantly from human brains. Many efforts have been made to model the brain and the blood-brain-barrier (BBB) *in vitro* using OoCs and other technologies and/or organoids. These models are simple when compared to the *in vivo* situation as they do not incorporate all relevant cell types and lack intricate architectural features of the brain[43,174,175]. Nevertheless, especially when combined with hiPSCs, they can be used for building human models to study complex neurodegenerative diseases such as Parkinson's, Alzheimer's and Huntington's disease.

Interestingly, compartmentalized *in vitro* models provide opportunities to study axonal degenerative mechanisms and develop treatments for regeneration[176]. For example, neuronal somas can be separated from axonal projections via compartmentalization (Figure 1C, left schematic), and axonal injury can be simulated by cutting, compression, aspiration or laser-based methods[176]. In addition, electrophysiological studies are very relevant to investigate neuronal function and can be performed by culturing neurons and/or brain organoids on top of multielectrode arrays (MEAs, Figure 1C, right schematic). For example, neuronal network dynamics and their activity in response to drugs can be studied[43]. Notably, MEAs also provide opportunities to study highly complex and multifactorial brain diseases such as epilepsy or migraine.

### The vascular system

OoCs are particularly well-suited for studying the vasculature, since microfluidic chips support fluid flow which is essential for proper vessel function and integral to controlling the behavior of endothelial cells (ECs) that line blood vessels. The vascular system is crucial for maintaining tissue homeostasis, facilitating nutrient and gas exchange, transport of signaling molecules and proteins, waste removal, and immune cell trafficking. Moreover, it is also a primary route for drug delivery and is thus an important target for therapeutic interventions[177–179]. This closed circulatory system comprises interconnected vessels of

varying diameters, forming a large hierarchically organized network that includes arteries, arterioles, capillaries, venules, and veins, through which the blood flows (Figure 3A, center part of schematic). In contrast, the lymphatic system is a unidirectional and blind-ended network that is related and connected to the vasculature. It consists of lymphatic vessels, lymph nodes and lymphoid organs (e.g. thymus and bone marrow). Its main function is returning lymphatic fluid to the vascular system: an interstitial fluid mainly composed of lymphocytes, cellular waste and debris, plasma proteins and other cell types[179–181].

The interior of blood and lymphatic vessels is lined with ECs that are specific to the vessel and organ in which they reside. Large arteries and veins have a continuous lining of ECs (*tunica intima*), a basement membrane, elastic fibers and smooth muscle cell layers (*tunica media*), and an outermost layer that is composed of connective tissue (*tunica adventitia*, Figure 3A, bottom part of schematic). Capillaries consist of wrapped ECs and can be continuous, discontinuous or fenestrated, having a varying coverage of basement membrane and pericytes (Figure 3A, top part of schematic). For example, as part of the BBB, capillary ECs are highly selective and continuous, thereby tightly controlling the transport of compounds and protecting the brain from toxic compounds, pathogens and circulating immune cells. In tissues involved in filtration and secretion such as the kidney glomeruli, ECs are fenestrated, while in the liver they are discontinuous[180,182].

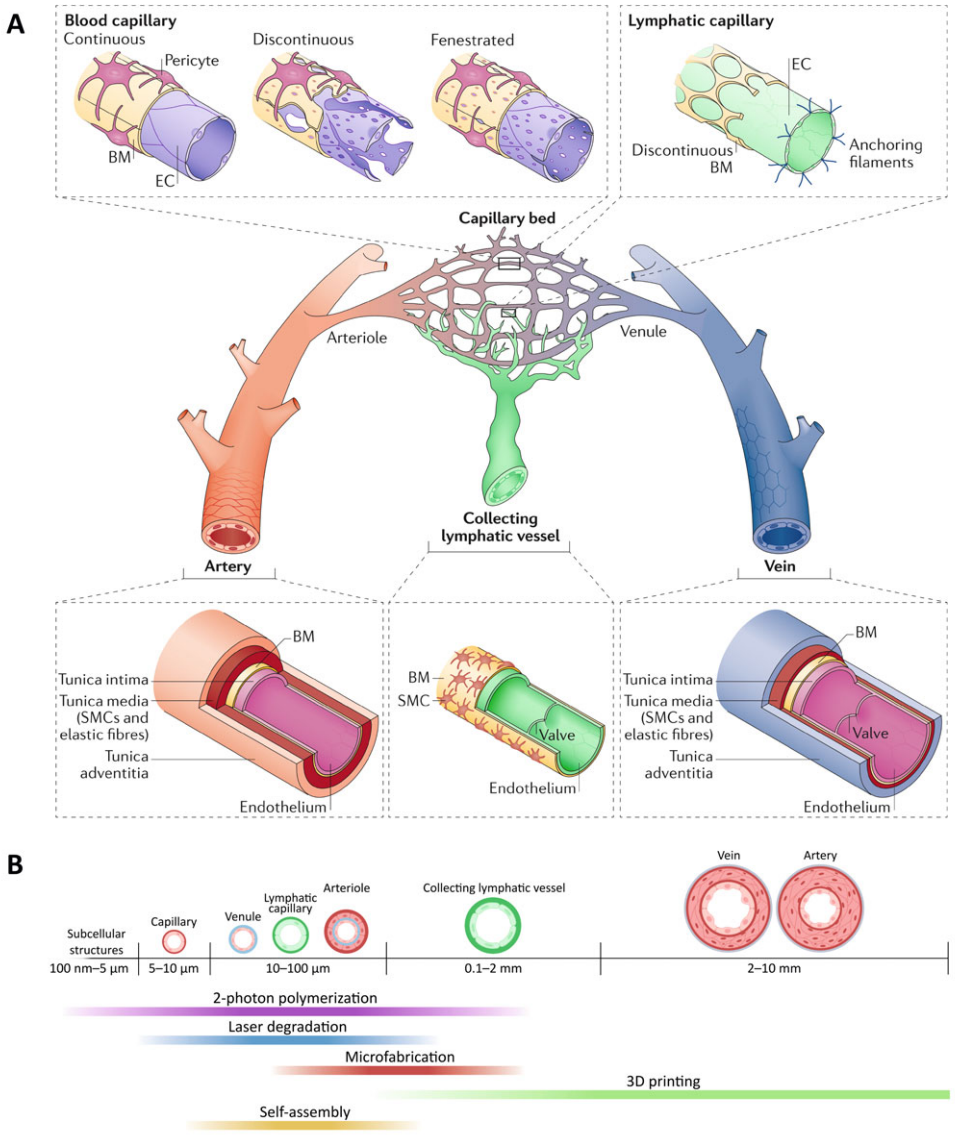
Part of the research described in this thesis provides methods that can be used for microvascular engineering to controllably fabricate and mimic perfusable microvasculature. This allows creating realistic Vessel-on-Chip models (VoCs) applicable to disease modeling, including small vessel diseases.

**(Micro)vascular engineering strategies**

Cardiovascular diseases such as stroke and congestive heart failure are a leading cause of death and disabilities worldwide[183]. Vascular engineering provides opportunities to treat patients because it enables the fabrication of vessel constructs that can be transplanted to replace those damaged by disease or injury (i.e. regenerative medicine). These engineered vessels may also be suitable to study disease and to perform drug development. Vascular engineering can use controlled fabrication methods for a technology-driven approach or use a biology-driven approach which relies on the self-assembly of vascular cells within the device, or a combination of both[179,184,185]. Vascular cells such as ECs have an inherent capacity to self-assemble into vascular networks and exploiting this property allows biology-driven engineering of vascular tissues and models. Moreover, the same property is also used to vascularize other engineered organ models and organoids to support proper perfusion. It should be noted that organoids are another example of biology-driven engineering, where the pluripotency or multipotency of stem cells is used together with specific growth factors to guide cell differentiation and organization to create organ-like structures[186–189].

In contrast to biology-driven approaches, technology-driven approaches provide more control over the features, properties and geometry of the desired construct[43,131,132,190,191]. Commonly used methods for technology-driven vascular engineering are photolithography, soft lithography, 3D bioprinting, 2PP, electrospinning, photoablation and (micro)molding. Interestingly, photolithography-based microfabrication can also be used for patterning hydrogels and cells to engineer vascular models in addition to its chip fabrication capabilities. While versatile, fast, and suitable for rapid prototyping, the geometrical complexity that can be obtained with photolithography is limited (structures are restricted to having straight sidewalls) due to the projection of a single 2D plane. To overcome this, layer-by-layer lithography, 3D bioprinting or laser patterning approaches can be used. 3D bioprinting methods provide exceptional control over geometry because it prints the required constructs layer-by-layer, but they generally have limited resolution (>100 µm feature size) and are time-consuming[111,112,114,192,193]. However, the field of biofabrication is rapidly evolving as is evident by novel and innovative methods that provide increased resolution, cell compatibility and speed of production. For example, volumetric 3D printing offers fast and high resolution layerless printing (~5 µm feature size). Other methods such as photoablation and 2PP are also evolving which enable researchers to construct structures and models with increasing precision and speed[114,115,194–196].

The fabrication method dictates the achievable features of the final vascular construct and therefore, its properties, tradeoffs and limitations are crucial to consider. More specifically, the dimensional, hierarchical and topological features of engineered vasculature are dependent on the fabrication method (Figure 3B). For instance, extrusion-based bioprinting is not suitable to engineer capillaries, but can be used to fabricate larger caliber vessels, while 2PP can be used to engineer microvascular structures in the 5-10 µm capillary range. It should be noted that 2PP fabrication times are very long but that 2PP can also be used to engineer submicron structures because of its high precision[114,115,132,179,184]. To estimate the efficiency of a fabrication method, the resolution/time for manufacturing ratio (RTM) can be used and is defined as the spatial resolution divided by the time for manufacturing; such measures help in assessing the possibilities and limitations of fabrication methods. In addition to the fabrication efficiency, it is also important to consider the available and compatible “bioinks” for both the fabrication method and the intended vascular constructs. Bioinks form the scaffold of the final construct, provide structural support, largely determine the construct’s mechanical properties, and can be natural/native hydrogels (e.g. collagen, fibrin, gelatin), (semi)synthetic hydrogels or other synthetic liquid/solid biomaterials. Moreover, bioinks may contain cells during the fabrication process and are often photocurable as this is an essential requirement for light-based fabrication methods[114,120–122,128,129,131].


A
Blood capillary
Continuous
Pericyte
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Discontinuous
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EC
Discontinuous BM
Anchoring filaments
Capillary bed
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Venule
Collecting lymphatic vessel
Artery
Vein
BM
Tunica intima
Tunica media (SMCs and elastic fibres)
Tunica adventitia
Endothelium
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SMC
Valve
Endothelium
BM
Tunica intima
Tunica media (SMCs and elastic fibres)
Valve
Tunica adventitia
Endothelium
B
Vein
Artery
Subcellular structures
Capillary
Venule
Lymphatic capillary
Arteriole
Collecting lymphatic vessel
100 nm–5 µm
5–10 µm
10–100 µm
0.1–2 mm
2–10 mm
2-photon polymerization
Laser degradation
Microfabrication
3D printing
Self-assembly

**Figure 3. Vascular anatomy and engineering. (A)** The human vasculature is composed of complex and hierarchical vessel networks. Large vessels are the arteries (red) and the veins (blue) and have a continuous lining of ECs, a basement membrane, and layers of smooth muscle cells (SMCs). The smaller vessels collectively form the microvasculature and include arterioles, venules and capillaries. Blood capillaries feature ECs that can be continuous, fenestrated, or discontinuous, having varying coverage of basement membrane and pericytes. The lymphatic system—also part of the vasculature—contains blind-ended lymphatic capillaries and collecting vessels (green) that remove lymphatic fluid from tissues. Lymphatic capillaries are composed of specialized ECs that facilitate fluid entry under high interstitial pressure. Collecting lymphatic vessels have a sparse SMC coverage and contain valves that support lymphatic pumping and prevent backflow. **(B)** Vascular engineering requires the appropriate fabrication method(s) to achieve the desired features and properties; tradeoffs between resolution, speed and dimensional control need to be made. In addition, the biomaterials/bioinks and cell types used need to be considered in order to engineer vasculature that displays relevant human vascular characteristics. Figure adapted from refs.[132,180]

### Human induced pluripotent stem cell-derived ECs and Vessel-on-Chips

There is a vast number of options to engineer vascular constructs and models, as outlined above, but when engineering a VoC, a basic microfluidic chip needs to be designed and fabricated first. Importantly, when subsequent on-chip fabrication of microstructures is required, this chip should be compatible with relevant fabrication methods (e.g. 3D bioprinting, 2PP or photolithography). Moreover, ECs are an essential component of VoCs and can be hiPSC-derived, immortalized or primary (e.g. human umbilical vein- or brain microvascular ECs). There are several protocols to generate hiPSC-ECs which are mostly based on 2D monolayer cultures to which growth factors and small molecule inhibitors are added to induce mesoderm formation, followed by endothelial specification, analogous to the development of embryos. Commonly used growth factors for mesoderm induction are bone morphogenetic protein 4 (BMP4) and Activin A, but the small-molecule CHIR99021 (a glycogen synthase kinase-3 inhibitor) that modulates Wnt-signaling can also be used, either alone or combined with BMP4[197,198]. For endothelial specification, vascular endothelial growth factor (VEGF) and/or fibroblast growth factor-2 are usually combined with a small-molecule Activin receptor-like kinase-5 inhibitor (SB431542), or by adding Y-27632, a rho-associated kinase inhibitor. Other methods include using forskolin for cyclic AMP-mediated protein kinase-A activation, and modulation of the ETS variant 2 (ETV2) transcription factor[197–201]. In contrast, non-monolayer cultures such as cell aggregates called embryoid bodies, and more recently, vascular organoids, can also be used to generate ECs[199,202,203]. Interestingly, further specification to tissue-specific ECs is possible during differentiation and enables vascular modeling of tissues and organs of interest[204].

EC differentiation protocols can be used with patient-derived hiPSCs harboring disease-specific mutations and can be genetically corrected to generate matched isogenic controls. Alternatively, disease-causing mutations can be introduced by gene editing in healthy hiPSCs, again to generate isogenic lines. Vascular engineering in combination with hiPSC-ECs opens many opportunities for *in vitro* modeling of vascular diseases[2,6,205]. When

compared to other *in vitro* models, VoC technology is most suited for compound screening, drug development and basic research because elements like flow, compartmentalization and vascular barriers can be incorporated in chips (Figure 4). These functionalities enable modeling of physiological phenomena especially when combined with appropriate vascular cells, are promising for personalized medicine and vascular disease modeling. VoCs can be based on engineering approaches (technology-driven) such as photo- and soft lithography, photoablation, 3D bioprinting, 2PP, cavitation molding, viscous finger patterning, template casting and micromolding, or can be based on self-assembly (biology-driven)[114,131,132,184,190,206], as mentioned earlier. In VoCs, compartmentalization is often incorporated to introduce vascular and parenchymal sides (Figure 4A) and/or to enable barrier integrity assays where a fluorescent dye diffuses from the donor compartment (vascular side), through the endothelial barrier to the acceptor compartment (parenchymal/hydrogel side). This compartmentalization can be achieved by using physical membrane-based separation (e.g. a PDMS or polystyrene membrane)[43,103], or via capillary pinning where a hydrogel is pinned by using capillary pressure barriers to control the liquid-air meniscus (Figure 4A)[106,207–210]. It is worth mentioning that chips or constructs where a vessel is created that is directly surrounded by a hydrogel (e.g. bioprinted, micromolded or template-based) can also be considered compartmentalized, depending on the context (Figure 4B). For assessing cell characteristics and functionality, different VoC readouts are available, depending on the design and its capabilities. Commonly used readouts are barrier integrity (vascular permeability), transendothelial electrical resistance (TEER, Figure 1E, left schematic), endothelial marker expression, morphology, angiogenesis, vasculogenesis, tubulogenesis, EC viability, leukocyte adhesion and/or transmigration, gene and/or protein expression, and cytokine expression[43,184,205].

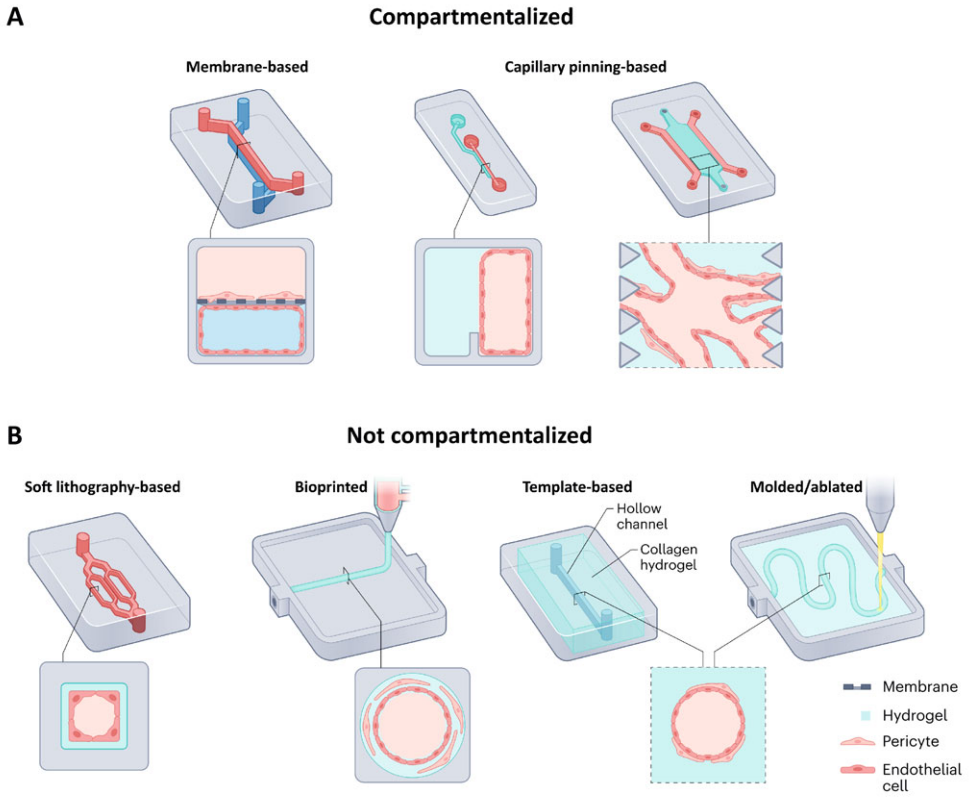


**Figure 4. Different Vessel-on-a-Chip devices. (A)** Compartmentalized chips. Left, two microfluidic channels separated by a porous PDMS membrane for compartmentalization. The chip is typically made of PDMS coated with a thin hydrogel layer and allows fluid flow and cell culture in both channels. The bottom channel may contain ECs, while the top channel may contain tissue-specific cells (e.g. neurons, astrocytes, lung epithelium or intestinal cells). Middle, two microfluidic channels separated using a "phaseguide" for capillary pinning of hydrogels. Tissue-specific cells can be mixed with the hydrogel precursor solution prior to being injected into the chip, after which the hydrogel polymerizes. The endothelialized channel is perfusable and the chip can be placed on a rocker for bidirectional flow. Right, three microfluidic channels separated by trapezoidal microposts for capillary pinning of hydrogels. Usually, the middle channel is filled with a hydrogel containing ECs and mural cells, after which self-assembly of a 3D microvascular network occurs. Perfusion can be performed via the flanking side channels. **(B)** Chips that are not compartmentalized. Left, chip with square channel geometry that can be made of PDMS or an agarose-gelatin hydrogel. Channels can be populated with ECs to create microvessels. Chips can be connected to pumping systems for unidirectional flow, or placed on a rocker for bidirectional fluid flow. Second chip from left, culture chamber with a coaxially bioprinted hollow microfiber that can be endothelialized to create a perfusable microvessel. Second chip from right, tubular channel that can be made by template-based approaches (e.g. using microneedles), or viscous finger patterning. The lumen can be endothelialized to create a perfusable microvessel. Right, micromolding or photoablation can be used to pattern tubular structures within hydrogels that can be endothelialized. Such approaches can be used to controllably engineer perfusable microvascular networks on-chip. Figure adapted from ref.[43]

**Brain-specific vasculature and the blood-brain barrier**

Due to the large metabolic demand of the brain, the brain vasculature plays a critical role in maintaining proper brain function. This vascular network is established during embryonic and postnatal development and displays characteristic features that distinguish brain vasculature from other vasculature in the body. The most distinguishing feature is the BBB, a highly selective barrier that isolates the vascular lumens of the brain vasculature from the brain parenchyma. This vasculature is composed of specialized ECs (human brain microvascular ECs, hBMECs) that are connected by tight junctions (TJs) and adherens junctions (AJs)[211]. The barrier restricts the passage of hydrophilic and potential neurotoxic compounds from the blood into the brain and actively transports lipophilic compounds and metabolic waste out of the brain via efflux transporters, while influx transporters regulate the stringent entry of relevant nutrients and compounds[211–213]. For example, the glucose transporter-1 (GLUT1) is an important influx transporter that actively transports glucose into the brain; since glucose demand in the brain is high, this transporter is highly expressed at BBB ECs[212]. The barrier thus provides a combination of physical, transport, and metabolic barriers that protect and nourish the brain. Brain capillaries are surrounded by a basement membrane and pericytes that support BBB function by supporting AJs and TJs and expressing relevant transporters such as the P-glycoprotein (Pgp) transporter. Moreover, astrocyte endfeet also connect to the capillaries and support endothelial function and communication with neurons. The vascular smooth muscle cells and endothelial cells present in the brain's vasculature, together with the astrocytes, pericytes and neurons form the neurovascular unit (NVU)[211–213].

There is significant interest in *in vitro* models of the BBB because it is a major delivery route for therapeutic drugs that need to act in the brain. The fact that the BBB is very stringent and restricts passage of many compounds makes it very challenging to develop drugs that can cross it. Suitable *in vitro* models to study these transport phenomena are therefore crucial to develop such drugs[107,212]. As part of the research described in this thesis, technological solutions are provided to engineer advanced vascular structures including the BBB because they describe rapid chip prototyping and microvascular engineering strategies. In addition, a compartmentalization method was developed to co-culture hiPSC-ECs with hiPSC-astrocytes, thereby providing additional potential for modeling the BBB. While these advancements are very promising, relevant cells are critical to faithfully model the BBB. Since hBMECs are highly specialized, accurate derivation methods to generate such cells are necessary. As mentioned earlier, tissue-specific ECs can be derived from hiPSCs via addition of relevant growth factors and small molecules[204]. As such, various differentiation strategies to derive hBMEC have been performed[214].

A well-known and initially widely used hBMEC differentiation protocol and subsequent improvements thereof[215–217], resulted in high TEER values and expression of relevant transporters; desired properties that indicate continuous and functional ECs with tight cell-cell junctions. However later research demonstrated that these hBMECs displayed markers

for neuroectodermal epithelial lineage while lacking expression of relevant vascular lineage genes. This resulted in controversies surrounding these studies and highlights the significant challenges of deriving brain-specific ECs[218,219]. Alternative approaches for deriving hBMECs involve co-culturing of hiPSC-ECs with tissue-specific cells to drive the ECs towards a specific fate. This approach has previously been shown to induce an EC tissue-specific identity for several organ systems, including the brain[220–222]. This co-culture strategy underscores the plasticity of ECs and the significance of the environment and cellular interactions in creating tissue-specific ECs.

Importantly, engineered models containing brain-specific vasculature are very relevant for studying small-vessel diseases such as retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations (RVCL-S). This rare disease is caused by truncating mutations in the *TREX1* gene, which encodes a major 3'-5' DNA exonuclease, and leads to disruption of both TREX1 localization and its interaction with the endoplasmic reticulum[223–225]. This in turn leads to dysfunctional microvasculature due to putative endothelial dysfunction and possibly the presence of immunogenic glycans; however, the exact disease mechanism remains to be elucidated. Clinical manifestations of RVCL-S include stroke, migraine, cognitive decline, vascular dementia and progressive visual impairment due to destruction of the retina[226–228]. While many manifestations of RVCL-S are in the brain, vascular dysfunction is also present in other organs[226]. Due to specific mutations and a clear and detrimental effect on ECs *in vivo*, hiPSC-derived RVCL-S ECs can be used for disease modeling by generating these from patient-derived hiPSCs and their isogenic CRISPR/Cas9-corrected counterparts. This process and characterization of the cells is described in this thesis, although the major focus is on technological aspects of OoC development. Genetic correction of mutant hiPSCs and derivation of specific cells such as ECs, are very relevant for disease modeling since it provides the essential healthy control for reference on the same genetic background. When these cells are assayed using *in vitro* models (e.g. in 2D assays or VoCs), differences between corrected cells and the mutant cells are most likely caused by the mutation.

## Aim and scope of this thesis

Microfabrication and bioengineering, as described above, are fundamental to OoC development, and their potential to transform biomedical research is enormous. However, several challenges and limitations hamper the mainstream adoption of OoCs. Among these, technical and logistical challenges complicate rapid prototyping, a critical feature required to develop OoCs and engineer microvasculature. This thesis describes facile microfabrication and bioengineering strategies that accelerate OoC development and microvascular engineering, thereby solving some of the challenges present in the OoC field and providing novel opportunities for vascular modeling that is relevant to human disease.

In **Chapter 2**, I describe a method to rapidly prototype OoCs using the DMD-based PRIMO system for maskless photolithography. This method simplifies OoC microfabrication, reduces prototyping time and can be performed cleanroom-free, thereby increasing OoC prototyping accessibility for non-specialized labs.

In **Chapter 3**, I describe an immediate follow-up and application of the technology developed in **Chapter 2**; perfusable microvasculature was engineered on-chip using hydrogel patterning and hiPSC-derived ECs. Both chip fabrication and hydrogel patterning were performed using the PRIMO system, and in addition to the possibility of co-cultures, advanced functional structures such as microvascular valves could be fabricated.

**Chapter 4** demonstrates high-throughput photopatterning methods that are being used for the development of a TFM-based cell contraction assay that employs patterned microdots. 2-step sequential photoscission-based photopatterning was performed using the PRIMO system, and a successful proof-of-concept of a 1-step photografting procedure is described, enabling faster and large-area photopatterning when compared to the regular 2-step photopatterning procedure.

In contrast to these technological developments, **Chapter 5** describes a critical step in the disease modeling process: the genetic correction of an hiPSC line derived from a patient with a disease of interest, and subsequent derivation and analysis of cells of interest. In this case, hiPSCs were first generated from fibroblasts derived from a patient with small vessel disease RVCL-S after which ECs were differentiated. The resulting isogenic disease and corrected ECs were used to study endothelial defects.

Finally, **Chapter 6** discusses the results and conclusions presented in the preceding chapters and provides future perspectives for OoC development and vascular modeling.

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