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

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Abstract

The research presented in this thesis focused on developing bioengineering methods for in-house prototyping and fabrication of human stem cell-based Organ-on-a-Chip (OoC) and microvascular models. These methods can be performed cleanroom-free in regular biochemistry labs, and are based on a benchtop system for maskless photolithography (Alvéole PRIMO) that employs a digital micromirror device (DMD) and UV light to project arbitrary photomasks. The need for these methods is driven by limitations of animal models, ethical concerns, and regulatory pressure. Rapid OoC prototyping and advanced microvascular engineering to create functional Vessel-on-Chips (VoCs) was achieved using the PRIMO system. In addition, the system was used to perform and develop several high-throughput photopatterning methods that form the foundation for a reference-free traction force microscopy (refTFM)-based contraction assay to study single-cell contractility. Importantly, the bioengineering methods can accelerate OoC and VoC development because of the possibility to rapidly prototype and test them, thereby helping to iteratively align the intended models with their Context-of-Use (CoU). Moreover, combining the methods with patient-derived hiPSCs and gene-editing provides tools for disease modeling and drug development. To this end, steps were taken towards developing a vascular disease model by generating and characterizing hiPSCs and hiPSC-derived endothelial cells (hiPSC-ECs) from a patient with “retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations” (RVCL-S), a rare autosomal dominant small vessel disease. In this chapter, I discuss the results presented in this thesis, its limitations, suggestions for improvement, and future perspectives.

Chapter

General discussion

Advancing Organ-on-a-Chip development through rapid prototyping

There is no universal *in vitro* model or OoC that can answer all biological questions and hypotheses, even for one organ or domain. Methods that simplify prototyping and fabrication of human *in vitro* models and OoCs are potentially useful because they enable iterative design improvements, reduce fabrication time, and allow customization tailored to specific biological applications. Clearly defined Contexts-of-Use (CoUs) are therefore crucial during the early stages of development to develop relevant OoCs that ultimately can be adopted by end users. Rapid prototyping methodologies such as described in **Chapter 2** increase bioengineering accessibility and can help to fine-tune and optimize OoC features based on their CoU. To perform rapid OoC prototyping, easy-to-use equipment, protocols and compatibility with conventional biochemistry labs are essential requirements. We therefore explored the PRIMO system[1] and its maskless photolithography capabilities to develop a cleanroom-free workflow for OoC prototyping, as described in **Chapter 2**. Development of this workflow was possible because the PRIMO system is maskless and has an easy-to-use graphical user interface to expose digital photomasks of arbitrary design using its DMD. Moreover, because the system is connected to an inverted microscope with an automated stage and uses the microscope optics to perform exposure, we were able to incorporate conventional glass coverslips as the supporting substrate to pattern SU-8 microstructures. Because we eliminated the use of traditional silicon wafers and used glass coverslips instead, backside exposure was made possible. This strategy enabled grayscale photolithography of negative photoresists, eliminated edge bead removal and thus, leveled the substrate in the microscope holder. Ultimately, this simplified the microfabrication process by allowing facile fabrication of SU-8 microstructures on glass coverslips, and because of the compatibility with soft lithography, allowed rapid fabrication of microfluidic chips that could be seeded with cells (i.e. OoCs). The workflow could be performed in regular laboratory space and had a turnaround time of 6-8 hours, as opposed to the time-consuming process of cleanroom-based fabrication of patterned silicon wafers and its associated procedures (e.g. mask design, mask fabrication and the communication and logistics between stakeholders). For example, the short turnaround time we achieved using the developed workflow made OoC prototyping significantly more feasible during the course of a typical PhD project. However, while this cleanroom-free approach is suited for OoC prototyping (via mold fabrication and replication), it does not replace cleanroom-based microfabrication; a particle-free environment and specialized high-tech photolithography equipment are critical to fabricate sensors, integrated circuits and other advanced micro- and nanostructures.

To demonstrate the flexibility of the workflow, we prototyped different OoCs, including a Vessel-on-Chip (VoC) and a multi-level chip to culture neurons where compartmentalization of the somas and neurites was possible. Moreover, we fabricated a PDMS substrate containing microgrooves that induced alignment of vascular smooth muscle cells (vSMCs)

when cultured on top, showing that topographical cues to guide cellular behavior can be fabricated.

The flexibility of the workflow combined with the maskless photolithography capabilities of the PRIMO system, enables faster iteration through OoC design and testing/optimization phases because new chips can rapidly be designed and fabricated when required. This faster iteration and increased efficiency also encourage interdisciplinary collaboration between engineers and biologists because their ideas and designs can be tested and optimized quicker. Equally important, the developed workflow lowers barriers to entry for researchers that want to explore advanced *in vitro* modeling because it can be performed in-house. By streamlining and simplifying device fabrication, our approach contributes to the broader adoption of OoC technology by enhancing accessibility and reducing dependency on specialized infrastructure such as cleanrooms. Importantly, once a suitable and working prototype has been developed, as showcased in this thesis, manufacturing could be scaled up by using bulk production methods such as injection molding and hot embossing.

Since the workflow allows fabrication of custom microfluidic chips, designs containing specific features and requirements can be fabricated (e.g. microfluidic channels of specific length, width and height, different compartments, or topographical cues), as demonstrated in **Chapters 2 and 3**. This contrasts with commercially available chips where chip designs are fixed; the flexibility and versatility of the workflow and the PRIMO system can thus be beneficial to provide specific chip requirements and optimization due to the possibility of rapid prototyping. However, as initially used, the manual steps involved in the workflow compromised reproducibility to some extent: While the glass coverslips are a very convenient and low-cost alternative to silicon wafers and support backside exposure, they can break easily if not handled carefully. We mitigated this issue by using petri dishes to mount the glass molds, thereby simplifying the soft lithography process and handling of the fragile glass substrate. Moreover, by replicating the glass molds using an epoxy resin, we increased durability and scaled up the soft lithography process (as briefly described in **Chapter 3**). Overall, this greatly improved the reproducibility, scalability and robustness of the workflow.

Other issues common with in-house chip production using PDMS-based soft lithography are the lack of standardization and the required expertise to properly fabricate chips[2–4]. Some steps of the chip fabrication procedure such as PDMS demolding, PDMS trimming using a knife and using biopsy punchers to create inlets and outlets, result in nonuniform chips due to variation introduced by the manual fabrication steps[5,6]. In addition, pipet tips are often used in the inlets/outlets to create larger cell culture medium reservoirs, or used as adapters to connect tubing[7–10]. These manual procedures are nonstandard and complicate handling and laboratory workflows such as microscopy and cell culture medium replacement, and might leak when connected to pump systems[6,7]. Commercially available chips do not have these limitations, are easy-to-use and are thus superior in this regard.

While our workflow mitigates exposure to dust or other particles by covering the substrates during photoresist spin coating, it cannot be fully prevented. Moreover, the spin coating process and subsequent baking steps are labor intensive and require practice. Dry film lamination of photoresists on glass substrates can solve these issues and provides predefined thickness of the photoresist, eliminating minor variations in the coating thickness as occur during spin coating[11–13].

Maskless photolithography offers great versatility but is limited to single-height features with straight and vertical sidewalls because of the projection of a single 2D plane. Multi-level structures with different heights are possible as demonstrated in this thesis, but these are more challenging to produce because sequential spin coating and realignment steps are required. Moreover, while we successfully performed grayscale photolithography using a negative photoresist (SU-8) and backside exposure, and could control the structure height, it has limited applications for OoC development because the process is difficult to control. In contrast to negative photoresists, positive photoresists have well-characterized response curves that are often linear, thereby allowing controlled grayscale photolithography[14]. Nevertheless, we observed a near-linear response in SU-8 when using an optimized laser dose, explaining the fact that we could controllably control microstructure height. Importantly, it should also be noted that SU-8 grayscale patterning was made possible because glass coverslips were used instead of silicon wafers. If topside exposure was used, the structures would detach from the glass substrate during development.

3D printing methods offer exceptional control over structure height and geometry because they print constructs layer-by-layer or use volumetric approaches. Advanced non-planar structures and geometries can be created, including curved channels, overhangs and embedded features[15,16]. While 3D printing is suitable for rapid prototyping of microfluidic chips and/or OoCs and has certain advantages over maskless photolithography, there are drawbacks that need to be considered. 3D printing has a typical feature resolution of 100 µm or higher depending on the printing method used, while maskless photolithography has a resolution of 2 µm or lower. Other methods such as 2-photon polymerization (2PP) offer submicron resolution, but this comes at the cost of very slow processing speeds. Moreover, 3D printing suffers from high surface roughness, whereas maskless photolithography results in smooth surfaces. High surface roughness can cause bubble formation during microfluidic applications due to large numbers of nucleation sites. In addition, cytotoxicity and low optical clarity due to the resins used result in difficulties when using 3D printing for OoC development. Moreover, when using 3D printed constructs for soft lithography, impeded curing of PDMS can occur because of the resins used when proper post-processing of the construct is not performed[15,16].

In summary, both methods are very suitable for rapid OoC prototyping and the choice of which depends on what features are required. Maskless photolithography, however, offers ample versatility and flexibility and offers facile fabrication opportunities to prototype OoCs.

This is mostly because it is based on traditional photolithography, it is compatible with soft lithography, a wide range of features can be fabricated, and it is not cytotoxic. Finally, when considering the on-chip hydrogel patterning developments described in **Chapter 3**, it becomes clear that even more versatility and flexibility is possible when employing maskless photolithography-based *in vitro* modeling.

Facile microvascular engineering for Vessel-on-a-Chip development

After the successful development of the OoC prototyping workflow described in **Chapter 2** we explored on-chip hydrogel patterning to create a VoC containing perfusable microvasculature with predefined characteristics. To this end, we first fabricated microfluidic chips using the OoC prototyping workflow. **Chapter 3** is therefore an immediate application of **Chapter 2** and demonstrates the usefulness of in-house rapid OoC prototyping. We did not need an external supplier for our master mold because we were able to fabricate our glass master mold using the PRIMO system and could subsequently replicate it using an epoxy resin to increase durability, and to scale up chip production. This helped to rapidly fabricate large batches of chips that could be stored indefinitely. Notably and while not described, we initially had a different chip design that turned out to be too complicated for our use case and we could easily prototype a new, simpler chip (i.e. the chip that was used in **Chapter 3**). This new chip had a height of 50 µm, containing a central compartment to house perfusable hydrogel scaffolds connected to the microfluidic main channel.

ECs have an intrinsic capacity to self-organize into microvascular networks and this capacity enables biology-driven engineering of perfusable VoCs. When ECs are mixed with a suitable hydrogel and added to a microfluidic chip, they form a microvascular network inside the chip through vasculo- and angiogenesis[17]. While very useful, the network geometry, topology and vessel diameter cannot be controlled. Technology-driven engineering of microvascular networks provides control over these features and enables the creation of predefined features. We therefore employed hydrogel patterning using the PRIMO system on the in-house fabricated chip and could easily engineer predefined hiPSC-derived perfusable microvasculature with vessels of different caliber (ranging from 10 µm to 100 µm) by subsequently seeding the hydrogel scaffolds with hiPSC-derived endothelial cells (hiPSC-ECs). We were able to pattern 20 chips per hour (3 channels per chip), and these patterned chips could be stored in PBS indefinitely, indicating that this method is fast, scalable and can be used to prepare patterned chips in bulk. This was due to the patterning speed of the system. Nevertheless, the patterning speed comes with a trade-off: the achievable geometry of the hydrogel structures is limited due to the photolithography-based projection of a 2D plane. The hydrogel features can only have straight and vertical sidewalls, which is the same limitation encountered when using the PRIMO system for chip microfabrication.

Similar to the benefits it provides for chip prototyping, 3D printing offers fabrication of well-

defined, uniform vascular constructs with great control over structure height and geometry. It is therefore superior to maskless photolithography in this regard. In addition, while grayscale photolithography using the PRIMO system has been performed to control hydrogel height and geometry[18], it does not match the level of control that can be achieved with 3D printing or 2PP[15,16]. However, integrating 3D printing with microfluidics is challenging. The use of "open-top" chips with a relatively large compartment for printer access is usually required, and, depending on the specific model being developed, may also require sealing the chip after printing. Furthermore, the smallest features achievable with 3D printing are typically greater than 100 µm due to limited printing resolution, and the process is time consuming. In contrast, the method we used enables rapid on-chip patterning and takes only 30 seconds per hydrogel compartment, while providing high resolution (<10 µm). Notably, 2PP is another commonly used printing method and offers exceptional submicron resolution, but is notoriously slow.

Like the hydrogel patterning method we used, 2PP and some bioprinting methods (e.g. digital light processing-based printing) rely on synthetic hydrogels and photoinitiators. Therefore, points that need to be considered include the phototoxic nature of photoinitiators and corresponding challenges when cell encapsulation is necessary. We seeded cells after the patterning process was complete, and in that case, phototoxicity was not a concern. However, when mixing cells with the prepolymer solution and subsequently performing the patterning procedure, phototoxicity becomes an issue due to the generation of free radicals and UV exposure. Methods such as photoablation, cavitation molding, template casting and micromolding do not suffer from this limitation as they often use native hydrogels, but these methods are slow and labor intensive[16,19].

Importantly, we demonstrated the flexibility of the on-chip hydrogel patterning method by showing that we were able to pattern arbitrary microvascular scaffolds, pattern functional microscopic valves inside patterned hydrogel channels, and that we could compartmentalize the chip by two simple modifications; adding an additional outlet and only patterning the central part of the hydrogel chamber. The digital photomasks for the microvascular scaffolds, valves and compartmentalization were easily designed, adjusted and tested. Moreover, we could control the mechanical properties of the hydrogel by modulating the UV laser dose. These features enable advanced vascular modeling and offer cell co-culture possibilities. For example, the valves can easily be modified to be stiffer or more elastic, or to have different geometries or sizes, and thereby enable modeling of, for example, thrombosis and associated events.

Hydrogel-based compartmentalization of the chip allows modeling of structures such as the blood-brain barrier. To demonstrate this, we used patterned GelMA as the vascular compartment and seeded this compartment with hiPSC-ECs. We were then able to fill the side compartments with a fibrin gel containing hiPSC-derived astrocytes. This method of compartmentalization differs from capillary pinning-based methods because it is performed

by localized hydrogel crosslinking and does not need a capillary pressure barrier. However, akin to capillary pinning-based methods, it is also membrane-free and allows cells to freely interact. Finally, it is important to note that the on-chip hydrogel patterning we performed, was done in enclosed microfluidic compartments that were flushed afterwards to remove uncrosslinked prepolymer solution. This method is not limited to custom chips and can also be performed on commercially available chips, thus providing great flexibility to perform microvascular engineering on such chips. Moreover, it offers the possibility to modify existing chip designs by adding hydrogel structures and scaffolds to these chips. For example, additional compartments could be added, or functional structures such as vascular valves or hydrogel check valves[20].

Future work could use this on-chip microvascular engineering approach to create VoCs that support functional readouts such as vascular leakage. The model we developed supports a leakage assay since the fluorescent dye is only able to diffuse laterally, simplifying imaging and estimation of the apparent permeability. In addition, we believe that a hybrid approach that is both biology-driven and technology-driven is the proper strategy to engineer vascular models. For example, larger vessels could controllably be engineered using the described approach and smaller vascular beds created through self-assembly could anastomose onto these larger vessels. This approach would resemble the *in vivo* situation more accurately and would be very suitable to create advanced VoCs and vascularized organoids and microtissues. In addition to this, mural cells such as vSMCs and pericytes should be incorporated to increase the relevance of the engineered microvasculature.

We showed that the integral approach of both rapid chip fabrication and hydrogel patterning is beneficial to develop advanced VoCs that can easily be tailored to specific needs and requirements, without the need for specialized equipment and facilities. These features both contribute to a rapid prototyping and holistic philosophy.

Towards a single-cell contraction assay using accelerated photopatterning

Because cell contractility reflects physiological and pathological behavior of the heart and vasculature, we aimed to develop a single-cell assay to study contraction. Measurement of cell contraction is relevant for functional disease phenotyping and to study drug responses. Because the PRIMO system offers high-resolution photopatterning (i.e. down to 1 µm) based on light-induced molecular adsorption of proteins (LIMAP, a two-step photoscission-based mechanism)[21], we hypothesized that a reference-free traction force microscopy (refTFM) substrate could be developed by photopatterning fluorescent microdots that act as fiducial markers, and that photopatterning of single-cells with controllable morphology on top could subsequently be carried out. This simple *in vitro* model contrasts with more complex OoC models but can provide insight in contractile function and drug responses at the single-cell level, and in a high-throughput fashion.

Since it is reference-free, it will be easy-to-use and implementable in regular biomedical research facilities equipped with confocal microscopes. In **Chapter 4**, we laid the foundations for this contraction assay by employing and developing a variety of photopatterning methods that in the future can be used to further develop the assay. First, we performed high-throughput LIMAP-based photopatterning of arrays of uniform single vascular smooth muscle cells (vSMCs) displaying *in vivo*-like morphology and anisotropic alignment. This reproducible photopatterning of vSMCs can be applied to many other contractile cell types (e.g. cardiomyocytes). Moreover, since cell morphology can be controlled, different shapes can be tested to study the effects on contractility and anisotropy. We then performed sequential photopatterning where superimposition of arbitrary micropatterns on fluorescent microdots (fluorescent bovine serum albumin, BSA) was made possible. This way, substrate deformation can be tracked via the fiducial markers, while the possibility of arbitrary cell morphology is maintained, an essential criterium for our assay. To address the issue of slow patterning speeds and evaporation issues with soluble 4-benzoylbenzyl-trimethylammonium chloride (photoinitiator, PLPP, Alvéole), and to solve the incompatibility issue of the proprietary photoinitiator gel (PLPP-gel, Alvéole) with soft substrates, we tested and optimized a photografting method. This additive one-step photografting mechanism allowed grafting of fluorescent BSA on top of the anti-fouling layer and was easier and faster to perform than the subtractive, two-step photoscission-based LIMAP method because protein incubation after patterning was not required. Moreover, photografting has the advantage that it is not oxygen-driven (as is the case with LIMAP) and eliminates the need for oxygen diffusion, thereby improving photopatterning speed. More specifically, in contrast to LIMAP, it requires the absence of oxygen and therefore allows the use of gas-impermeable “ceilings” (e.g. glass or polystyrene) to prevent evaporation and as such, permits photopatterning of large areas. Prepatterned substrates could be prepared in batch and adapted to a multiwell plate format so that they are ready-to-use for end users.

An important consideration is that the sequential photopatterning method performed was based on the two-step LIMAP method, but that it should be based on the one-step photografting method to facilitate faster photopatterning. We hypothesize that this is possible because the anti-fouling strategy is the same and that sequential grafting should thus be possible. However, this needs to be tested in future studies. It is important to note that we delivered proof-of-concept for different photopatterning methods and demonstrated that they can be used for the development of a refTFM-based contraction assay. Nevertheless, several steps need to be undertaken to develop the actual assay. For proof-of-concept we used glass substrates, but soft PDMS-based substrates are critical to perform refTFM. Briefly, soft PDMS can be spin coated on a glass supporting substrate and the photopatterning methods we described can subsequently be applied to it. An existing refTFM analysis paradigm such as “Cellogram” can then be employed to derive traction fields[22,23]. However, it should be noted that upscaling to a multiwell plate format is necessary to increase usability and throughput. Finally, contraction assays with cells of interest can be

performed by seeding them on the patterned substrate and following the refTFM paradigm.

Patient-derived hiPSCs could be used to generate these cells of interest and photopatterned in single-cell arrays on top of a prepared refTFM substrate. For example, dysfunctional contractility of cardiomyocytes, a feature observed in many cardiomyopathies, can then be studied at the single-cell level. In addition to absolute force quantification, we envision that the contraction assay can be used to quantify other relevant features such as the contraction frequency, duration and velocity by extracting these parameters using rapid image acquisition. Furthermore, when fully developed, the photografting method is not limited to refTFM substrate fabrication, but suitable for fast photopatterning in studies were micropatterned cells are required.

Opportunities to study migraine and migraine-related diseases using vSMCs

While the anticipated refTFM-based contraction assay is applicable to study cardiomyocyte contractility and other diseases where contractile dysfunction is an important driver, studying vSMC contractility is very relevant to understand many vascular diseases. Blood vessels contain layers of vSMCs that control their diameter and vascular tone by responding to vasoactive compounds (e.g. endothelin-1, nitric oxide, angiotensin II). Deregulated vSMCs can lead to vascular disease, including hypertension and atherosclerosis, and can switch from a contractile phenotype to a synthetic phenotype, leading to stiffening and reduced contractile capacity of vessels. Interestingly, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is a small vessel disease that could be of interest to study using the contraction assay. CADASIL is caused by mutations in the *NOTCH3* gene and patients suffer from stroke, migraine with aura and vascular dementia. The disease mainly affects cerebral arterial vSMCs by abnormal accumulation of Notch3 protein and causes cerebral vessel dysfunction[24–26].

Notably, since migraine with aura is a major clinical hallmark of CADASIL and since it is a monogenic disease of vascular origin, it also provides an interesting opportunity to study migraine itself. Migraine is a neurovascular disorder, and its cause is much debated; it is currently believed to be neuronal, with vascular effects being secondary. However, there are many vascular hypotheses stating that migraine is triggered by cerebral blood vessels[26–30], and CADASIL is therefore an important disease to consider. Patient-derived CADASIL hiPSC-vSMCs could be used in the contraction assay and their basal cell tone and contractile properties could be studied and compared to isogenic corrected cells. Moreover, their response to vasoactive compounds and potential therapeutic drugs could be tested. In migraineurs, calcitonin gene-related peptide (CGRP) antagonism is an important therapeutic intervention because CGRP is a migraine trigger and potent vasodilator released by the trigeminovascular system during migraine attacks. The contraction assay could therefore be used to study responses to migraine-relevant compounds such as CGRP, endothelin-1

and pituitary adenylate cyclase–activating polypeptide (PACAP). In addition, the assay could be used to perform pharmacological studies to characterize vSMC responses to existing anti-migraine medication such as gepants and monoclonal antibodies targeting CGRP and/or its receptor[26,31–35]. In similar pharmacological studies, it could be employed to develop novel anti-migraine drugs by characterizing vSMC responses. Importantly, since the assay is refTFM-based, post-assay analysis of the cells is possible (e.g. immunofluorescence imaging). Therefore, both their functional contractile response and marker expression can be studied after pharmacological treatment.

Establishing hiPSC lines and hiPSC-derived ECs for vascular disease modeling

While most of this thesis is centered around technological bioengineering developments, **Chapter 5** is biologically oriented around stem cells. Appropriate technologies are crucial to engineer *in vitro* disease models, but well-characterized cells harboring the disease gene mutation of interest are equally important to recapitulate disease features in the disease model. Therefore, we pursued the generation of hiPSC lines and their isogenic controls from a female patient suffering from retinal vasculopathy with cerebral leukoencephalopathy and systemic manifestations (RVCL-S), a monogenic small vessel disease[24,36]. We derived ECs from these hiPSCs and performed basic characterization of both the hiPSCs and hiPSC-ECs, and in addition aimed to determine whether these hiPSC-ECs display intrinsic defects using a simple vascular 2D model as a prelude to more complex VoC models. hiPSCs have been utilized in various settings for disease modeling and drug screening, including 2D cultures, OoCs, tissue engineered models and organoids[17,37–39]. The question arises, however, on how much complexity is needed to answer defined biological questions. 2D cell-based assays—such as the proposed contraction assay in **Chapter 4** and the proliferation and barrier function assays performed on 2D EC monolayers in **Chapter 5**—often provide useful data and can be performed at higher throughput than more complex models, thus being more suitable for compound screening, while also being cost-effective. However, no clear phenotype was observed in the 2D assays we performed in **Chapter 5**. This underscores the importance of several key considerations in hiPSC-based disease modeling. For example, future work needs to incorporate 3D assays as these might reveal a phenotype. A similar approach was used for another small vessel disease; hereditary hemorrhagic telangiectasia (HHT). In this study, no phenotype was evident in 2D vascular cultures but was clear in 3D VoCs where vessels were leaky and pericyte-EC interaction was poor[40].

Another important consideration for disease modeling and when working with hiPSCs is the number of lines and clones required to unambiguously show a mutation is causing a phenotype in cell culture. hiPSC-based disease modeling often requires many biological replicates due to genetic variability between cell lines from different patients when compared to inbred animal models or standard (immortal) cell lines[41]. Recent studies have focused on understanding the statistical power and optimal study design for hiPSC-derived

disease models. These studies have highlighted the increased power of using isogenic paired hiPSCs over non-isogenic ones, particularly in modeling monogenic disorders, by minimizing the variation resulting from individual genetic differences. This aligns with the experimental design employed in **Chapter 5**. However, many published studies seem to be underpowered[42,43]. To ensure findings can be extended to broader patient groups, inter-individual variation needs to be incorporated in the study. The most effective way to achieve this is by including additional donors rather than to increase the number of hiPSC clones in the study design[44]. An innovative and potentially effective approach to do this is by combining different lines in a single dish, referred to as "village in a dish" model systems. These model systems have demonstrated the ability to significantly increase throughput while maintaining consistency in genetic, epigenetic or line-dependent effects underlying gene expression variation, comparable to cells cultured separately[43].

X-chromosome inactivation status (XCI) is another crucial consideration when working with female hiPSC lines, as we did in **Chapter 5**. Aberrant XCI status in female hiPSCs could lead to changes in cellular protein content and differentiation potential[45–47]. While erosion of XCI is primarily seen in human embryonic stem cells (hESCs) and only to a lesser extent in hiPSCs[48], significant variation remains between hiPSC lines. Interestingly, differences in XCI status between clones did not appear to directly correlate with hiPSC-EC differentiation potential, as evidenced by variation in both gene and protein expression of EC-specific markers. This is consistent with other reports demonstrating XCI status-independent differentiation efficiencies into germ cells[49]. Future investigations should aim to elucidate the underlying mechanisms, potentially by exploring strategies to prevent aberrant XCI. This has already been demonstrated in hESCs through changes in media composition using lithium chloride or inhibitors of GSK-3 proteins[50].

Another challenge is the immaturity of hiPSC-derived cells, which can limit the *in vitro* recapitulation of disease phenotypes observed *in vivo*[51,52]. Immaturity may therefore explain the absence of EC defects seen in RVCL-S patient-derived hiPSC-ECs. Several strategies have been tried to enhance the maturity of hiPSC-derived cells, ranging from manipulation and improvement of the extracellular matrix (ECM), metabolism (e.g. altering the energy substrate), 3D culture methods, or chemical stimulation of the cell culture using different reagents[53]. OoCs, and specifically VoCs, offer unique abilities to control these aspects, including microfluidic flow for induction of shear stress, thereby stimulating the ECs.

Other examples that future studies could explore are the use of the RVCL-S hiPSC-ECs to create a VoC model and explore their vasculogenic and angiogenic potential, as for other small vessel diseases[40]. In addition, using VoCs, barrier integrity assays and on-chip TEER measurements could be performed to assess vascular leakage and junctional integrity. Such functional assays would provide more insight in the effects of dysfunctional TREX1 exonuclease on EC function. Interestingly, more complex immunocompetent OoC models would be very suited to study RVCL-S because TREX1 expression has been shown in microglia.

Incorporating these cells into RVCL-S VoCs would allow investigation of microglial reactivity and endothelial behavior. Importantly, the technological developments described in this thesis could be used together with RVCL-S hiPSC-ECs to generate RVCL-S models tailored to specific needs (e.g. compartmentalized chips with a vascular and immune component, a model containing both neurons and ECs, or a model that supports leakage assays).

Finally, although hiPSCs offer many opportunities for disease modeling, their limitations include(epi)genetic variability and varying differentiation efficiencies aside from immaturity, mentioned earlier[38,51,52]. This can also complicate development and/or validation of readouts due to difficulties in interpreting their results, as we observed in **Chapter 5**. While one of the goals of *in vitro* models is to contribute to replacing animal models, it will take many years to achieve this. During this transitional phase, researchers will need to continue to use animal models, but these will be complemented by human *in vitro* models of increasing complexity and accuracy. It would therefore be extremely useful for RVCL-S research and *in vitro* model development in general, to employ animal cells from established and well-characterized animal models (when available, as is the case for RVCL-S[54,55]) to develop and employ *in vitro* assays, in addition and complementary to hiPSCs[56]. For example, this would make it possible to go from *in vivo* to *in vitro* mouse models, and finally, to human *in vitro* models. Incorporating cells from animal models into *in vitro* models can help ensure that the experimental system accurately recapitulates key aspects of the disease, thus offering a robust proof-of-concept that the disease features are faithfully reproduced. These studies would complement hiPSCs moving towards drug development and personalized medicine and serve as validation of the *in vitro* model by allowing comparison of assay data to *in vivo* data from the animal model the cells originate from. This intermediate and transitional step could help to validate findings from hiPSC-based assays, thereby bridging the gap between animal studies and human cell-based models. Importantly, it would also help convince regulators and industry to adopt the technology, and ultimately, improve translation to patients.

Conclusion

OoC technology is becoming a powerful tool for creating advanced human *in vitro* models, especially together with hiPSCs, and in the near future, artificial intelligence may take this even further. These models have applications in disease modeling, drug development and personalized medicine. In this thesis, we described facile and interdisciplinary bioengineering methods—based on the PRIMO system for maskless photolithography—that support OoC and *in vitro* model development. More specifically, the versatile workflows and methods we developed are suitable for rapid and in-house prototyping of OoC devices, VoC development and for microvascular engineering.

While these methods are deployable in regular biochemistry labs and we made considerable effort to transition them to being as easy and reproducible as possible, interdisciplinarity in

approach and training will be critical to their wide adoption. In turn, this could result in a holistic approach to OoC development and implementation by improved design, testing efficiency and validation of *in vitro* models, tailoring the model to and aligning it with the defined CoU throughout its development process. A relevant example of an interdisciplinary training network is the Netherlands Organ-on-Chip Initiative (NOCI), of which this thesis is part, and where PhD candidates and postdocs are trained to understand the interdisciplinary aspects of OoC development, and are applying their knowledge to conduct *in vitro* research and develop novel OoCs[57].

The generation and characterization of RVCL-S patient-derived hiPSCs and hiPSC-ECs in this thesis, highlights the importance and foundations for disease modeling, and its corresponding challenges. The RVCL-S disease hiPSC-derivatives can be combined with the technological developments in this thesis in future studies, enabling accurate *in vitro* models that recapitulate RVCL-S features. To improve hiPSC-based disease modeling (e.g. for RVCL-S), animal cells could be complementary used to validate *in vitro* models and corresponding assays. This strategy could help to provide compelling data to convince regulators and industry to adopt OoCs and advanced *in vitro* models.

Only when OoCs are qualified and fit-for-purpose, will they be adopted for mainstream use. I believe that the technological developments described here can help to achieve this, but that such technological developments alone are not sufficient. Even when OoCs and corresponding technologies demonstrate significant potential and provide compelling data, trust and confidence is needed to convince industry and regulators. As such, major efforts are required by all stakeholders for OoCs to be truly adopted. Ongoing efforts of OoC developers, industry and regulators such as the FDA modernization act 2.0, standardization roadmaps, the Innovation & Quality Microphysiological Systems Affiliate and the Roche Institute of Human Biology, are encouraging and signify the interest in OoC technology[2,58–61]. Moreover, collaborative networks such as the European Organ-on-Chip Society (EUROoCS) and the human organ and disease model technologies (hDMT) consortium aim to provide infrastructure to increase OoC adoption by connecting OoC developers, industry and regulators, a very welcome development[62,63].

References

1. Alvéole Lab. https://www.alveolelab.com/.
2. CEN/CENELEC Focus Group Organ-on-Chip. *Focus Group Organ-on-Chip Standardization Roadmap*. https://www.cencenelec.eu/news-and-events/news/2024/brief-news/2024-07-10-organ-on-chip/ (2024).
3. Piergiovanni, M., Leite, S. B., Corvi, R. & Whelan, M. Standardisation needs for organ on chip devices. *Lab Chip* **21**, 2857–2868 (2021).
4. Reyes, D. R. *et al.* From animal testing to in vitro systems: advancing standardization in microphysiological systems. *Lab on a Chip* vol. 24 1076–1087 Preprint at https://doi.org/10.1039/d3lc00994g (2024).
5. Elvesys group - The PDMS lithography replication process: tips and tricks. https://www.elveflow.com/microfluidic-reviews/soft-lithography-microfabrication/pdms-softlithography-replication/.
6. Cameron, T. C. *et al.* PDMS Organ-On-Chip Design and Fabrication: Strategies for Improving Fluidic Integration and Chip Robustness of Rapidly Prototyped Microfluidic In Vitro Models. *Micromachines (Basel)* **13**, (2022).
7. Rho, H. S., Yang, Y., Veltkamp, H.-W. & Gardeniers, H. Direct Delivery of Reagents from a Pipette Tip to a PDMS Microfluidic Device. https://blogs.rsc.org/chipsandtips/2015/10/09/direct-delivery-of-reagents-from-a-pipette-tip-to-a-pdms-microfluidic-device/?doing_wp_cron=1739961754.7595820426940917968750.
8. Kim, K. M. *et al.* Shear stress induced by an interstitial level of slow flow increases the osteogenic differentiation of mesenchymal stem cells through TAZ activation. *PLoS One* **9**, (2014).
9. Park, J. Y. *et al.* Responses of endothelial cells to extremely slow flows. *Biomicrofluidics* **5**, (2011).
10. Coelho, B. J. *et al.* Hybrid Digital-Droplet Microfluidic Chip for Applications in Droplet Digital Nucleic Acid Amplification: Design, Fabrication and Characterization. *Sensors* **23**, (2023).
11. Joseph, X., Akhil, V., Arathi, A. & Mohanan, P. V. Comprehensive Development in Organ-On-A-Chip Technology. *Journal of Pharmaceutical Sciences* vol. 111 18–31 Preprint at https://doi.org/10.1016/j.xphs.2021.07.014 (2022).
12. BlackHole Lab - Dry film lamination station. https://www.blackholelab-soft-lithography.com/su-8-photolithography-and-pdms-soft-lithography-products/lamination-station-dry-film-microfluidic-microfabrication.
13. Mukherjee, P., Nebuloni, F., Gao, H., Zhou, J. & Papautsky, I. Rapid prototyping of soft lithography masters for microfluidic devices using dry film photoresist in a non-cleanroom setting. *Micromachines (Basel)* **10**, (2019).
14. Khonina, S. N., Kazanskiy, N. L. & Butt, M. A. Grayscale Lithography and a Brief Introduction to Other Widely Used Lithographic Methods: A State-of-the-Art Review. *Micromachines* vol. 15 Preprint at https://doi.org/10.3390/mi15111321 (2024).
15. Lim, K. S. *et al.* Fundamentals and Applications of Photo-Cross-Linking in Bioprinting. *Chem Rev* **120**, 10662–10694 (2020).
16. Moroni, L. *et al.* Biofabrication: A Guide to Technology and Terminology. *Trends in Biotechnology* vol. 36 384–402 Preprint at https://doi.org/10.1016/j.tibtech.2017.10.015 (2018).
17. Nahon, D. M. *et al.* Standardizing designed and emergent quantitative features in

microphysiological systems. *Nat Biomed Eng* (2024) doi:10.1038/s41551-024-01236-0.

18. Pasturel, A., Strale, P. & Studer, V. Tailoring Common Hydrogels into 3D Cell Culture Templates. *Adv Healthc Mater* **9**, 1–8 (2020).
19. O'Connor, C., Brady, E., Zheng, Y., Moore, E. & Stevens, K. R. Engineering the multiscale complexity of vascular networks. *Nature Reviews Materials* vol. 7 702–716 Preprint at https://doi.org/10.1038/s41578-022-00447-8 (2022).
20. Wang, Y., Toyoda, K., Uesugi, K. & Morishima, K. A simple micro check valve using a photo-patterned hydrogel valve core. *Sens Actuators A Phys* **304**, (2020).
21. Strale, P. O. *et al.* Multiprotein Printing by Light-Induced Molecular Adsorption. *Advanced Materials* **28**, 2024–2029 (2016).
22. Lendenmann, T. *et al.* Cellogram: On-the-Fly Traction Force Microscopy. *Nano Lett* **19**, 6742–6750 (2019).
23. Bergert, M. *et al.* Confocal reference free traction force microscopy. *Nat Commun* **7**, 12814 (2016).
24. de Boer, I. *et al.* RVCL-S and CADASIL display distinct impaired vascular function. *Neurology* **91**, e956–e963 (2018).
25. Tan, R. Y. Y. & Markus, H. S. CADASIL: Migraine, encephalopathy, stroke and their inter-relationships. *PLoS One* **11**, 1–14 (2016).
26. Ferrari, M. D., Klever, R. R., Terwindt, G. M., Ayata, C. & van den Maagdenberg, A. M. J. M. Migraine pathophysiology: Lessons from mouse models and human genetics. *Lancet Neurol* **14**, 65–80 (2015).
27. Sutherland, H. G. & Griffiths, L. R. Genetics of Migraine: Insights into the Molecular Basis of Migraine Disorders. *Headache* **57**, 537–569 (2017).
28. Murinova, N., Krashin, D. L. & Lucas, S. Vascular risk in migraineurs: Interaction of endothelial and cortical excitability factors. *Headache* **54**, 583–590 (2014).
29. Mason, B. N. & Russo, A. F. Vascular Contributions to Migraine: Time to Revisit? *Front Cell Neurosci* **12**, 1–10 (2018).
30. Brennan, K. C. & Charles, A. An update on the blood vessel in migraine. *Curr Opin Neurol* **23**, 266–274 (2010).
31. MaassenVanDenBrink, A., Meijer, J., Villalón, C. M. & Ferrari, M. D. Wiping Out CGRP: Potential Cardiovascular Risks. *Trends Pharmacol Sci* **37**, 779–788 (2016).
32. Close, L. N., Eftekhari, S., Wang, M., Charles, A. C. & Russo, A. F. Cortical spreading depression as a site of origin for migraine: Role of CGRP. *Cephalalgia* **39**, 428–434 (2019).
33. Rubio-Beltrán, E. *et al.* Characterisation of vasodilatory responses in the presence of the CGRP receptor antibody erenumab in human isolated arteries. *Cephalalgia* **39**, 1735–1744 (2019).
34. Messlinger, K. The big CGRP flood - sources, sinks and signalling sites in the trigeminovascular system. *Journal of Headache and Pain* **19**, (2018).
35. Dreier, J. P. *et al.* Endothelin-1 potently induces Leão's cortical spreading depression in vivo in the rat: A model for an endothelial trigger of migrainous aura? *Brain* **125**, 102–112 (2002).
36. Richards, A. *et al.* C-terminal truncations in human 3'-5' DNA exonuclease TREX1 cause autosomal dominant retinal vasculopathy with cerebral leukodystrophy. *Nat Genet* **39**, 1068–1070 (2007).
37. Kuehn, M. H. & Zhu, W. *Human IPSC-Derived Disease Models for Drug Discovery*. vol. 281 (Springer International Publishing, Cham, 2023).

38. Passier, R., Orlova, V. & Mummery, C. Complex Tissue and Disease Modeling using hiPSCs. *Cell Stem Cell* **18**, 309–321 (2016).

39. Shi, Y., Inoue, H., Wu, J. C. & Yamanaka, S. Induced pluripotent stem cell technology: A decade of progress. *Nat Rev Drug Discov* **16**, 115–130 (2017).

40. Orlova, V. V. *et al.* Vascular defects associated with hereditary hemorrhagic telangiectasia revealed in patient-derived isogenic iPSCs in 3D vessels on chip. *Stem Cell Reports* **17**, 1536–1545 (2022).

41. Volpato, V. & Webber, C. Addressing variability in iPSC-derived models of human disease: Guidelines to promote reproducibility. *DMM Disease Models and Mechanisms* vol. 13 Preprint at https://doi.org/10.1242/dmm.042317 (2020).

42. Brunner, J. W. *et al.* Power and optimal study design in iPSC-based brain disease modelling. *Mol Psychiatry* **28**, 1545–1556 (2023).

43. Neavin, D. R. *et al.* A village in a dish model system for population-scale hiPSC studies. *Nat Commun* **14**, 3240 (2023).

44. Germain, P. L. & Testa, G. Taming Human Genetic Variability: Transcriptomic Meta-Analysis Guides the Experimental Design and Interpretation of iPSC-Based Disease Modeling. *Stem Cell Reports* **8**, 1784–1796 (2017).

45. Anguera, M. C. *et al.* Molecular signatures of human induced pluripotent stem cells highlight sex differences and cancer genes. *Cell Stem Cell* **11**, 75–90 (2012).

46. Brenes, A. J. *et al.* Erosion of human X chromosome inactivation causes major remodeling of the iPSC proteome. *Cell Rep* **35**, 109032 (2021).

47. Geens, M. & Chuva De Sousa Lopes, S. M. X chromosome inactivation in human pluripotent stem cells as a model for human development: back to the drawing board? *Hum Reprod Update* **23**, 520–532 (2017).

48. Bar, S., Seaton, L. R., Weissbein, U., Eldar-Geva, T. & Benvenisty, N. Global Characterization of X Chromosome Inactivation in Human Pluripotent Stem Cells. *Cell Rep* **27**, 20-29.e3 (2019).

49. Chang, Y. W. *et al.* Tissue of Origin, but Not XCI State, Influences Germ Cell Differentiation from Human Pluripotent Stem Cells. *Cells* **10**, 2400 (2021).

50. Cloutier, M. *et al.* Preventing erosion of X-chromosome inactivation in human embryonic stem cells. *Nat Commun* **13**, 2516 (2022).

51. Sharma, A., Sances, S., Workman, M. J. & Svendsen, C. N. Multi-lineage Human iPSC-Derived Platforms for Disease Modeling and Drug Discovery. *Cell Stem Cell* vol. 26 309–329 Preprint at https://doi.org/10.1016/j.stem.2020.02.011 (2020).

52. Campostrini, G. *et al.* Maturation of hiPSC-derived cardiomyocytes promotes adult alternative splicing of SCN5A and reveals changes in sodium current associated with cardiac arrhythmia. *Cardiovasc Res* **119**, 167–182 (2023).

53. Ottaviani, D., ter Huurne, M., Elliott, D. A., Bellin, M. & Mummery, C. L. Maturing differentiated human pluripotent stem cells *in vitro* : methods and challenges. *Development* **150**, (2023).

54. Mulder, I. A. *et al.* Increased Mortality and Vascular Phenotype in a Knock-In Mouse Model of Retinal Vasculopathy With Cerebral Leukoencephalopathy and Systemic Manifestations. *Stroke* **51**, 300–307 (2020).

55. Sakai, T. *et al.* DNase-active TREX1 frame-shift mutants induce serologic autoimmunity in mice. *J Autoimmun* **81**, 13–23 (2017).

56. Ingber, D. E. Human organs-on-chips for disease modelling, drug development and personalized medicine. *Nat Rev Genet* **0123456789**, (2022).

57. Netherlands Organ-on-Chip Initiative (NOCI). https://noci-organ-on-chip.nl/.
58. Han, J. J. FDA Modernization Act 2.0 allows for alternatives to animal testing. *Artificial organs* vol. 47 449–450 Preprint at https://doi.org/10.1111/aor.14503 (2023).
59. Baran, S. Perspectives on the evaluation and adoption of complex in vitro models in drug development: Workshop with the FDA and the pharmaceutical industry (IQ MPS Affiliate). *ALTEX* **39**, 271–272 (2022).
60. IQ MPS Affiliate. https://www.iqmps.org/.
61. Roche Institute of Human Biology. https://institutehumanbiology.com/.
62. hDMT consortium. https://www.hdmt.technology/.
63. EUROoCS. https://euroocs.eu/.