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Microfluidic 3D cell culture for high throughput screening

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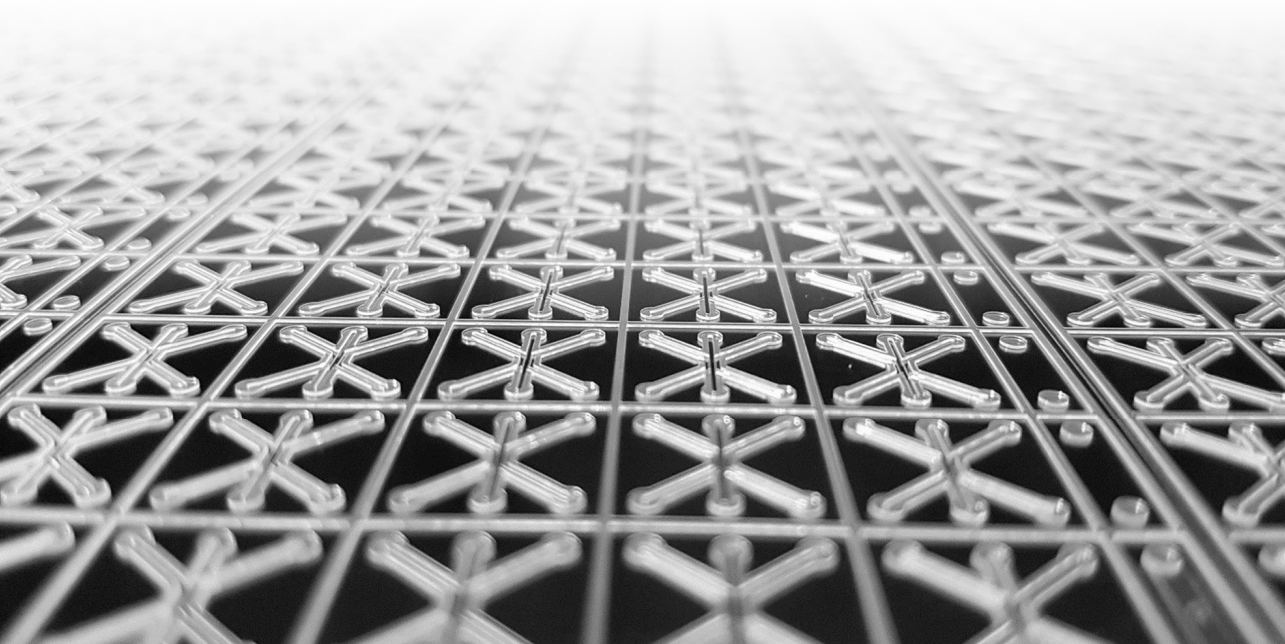
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Chapter 1

General Introduction



Introduction

Development of a single new drug can take 12-15 years and costs over \$1.3bn.¹ Early in the development process large compound libraries are screened for potential new drug candidates based on fast and cost-effective tests. After extensive *in silico* evaluation of many potential molecules and ultra-high throughput molecule to molecule screening, cell based models are used for selection and prioritization of compounds for further testing. Based on these results, candidates progress towards more predictive, but also lengthier and more costly animal models and eventually human clinical trials.

Even after the extensive selection process in preclinical phases, up to 90% of drugs fail during clinical trials. As approximately 70% of the drug development costs are accrued during these clinical phases it is of vital importance to improve the selection of drug candidates that enter clinical trials. Any enhanced predictivity of preclinical models that can prevent late attrition will have a major impact on costs of drug research and development by improving research output and reducing waste of resources.

As approximately 11% of US, and 7% of global, research and development expenses are devoted to drug research and development, the importance of improving efficiency in this field cannot be overstated.²

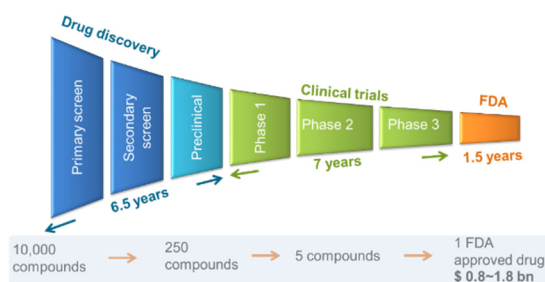


Figure 1 | The drug discovery pipeline. Over 10.000 potential new compounds enter the drug discovery pipeline to get 1 drug to market. Increasingly complex model systems are used to select which drug candidate to move forward through the pipeline towards clinical tests and the market.

Improving preclinical screening

Cell culture models have been the foundation of preclinical efficacy and toxicity screening of drug candidates. Conventional cell culture models consist of monolayers of adherent cells on plastic and are easy to culture and use in high throughput screens. However, these models fail to recapitulate many important aspects of the *in vivo* situation. A lack of physiological cell-cell interaction, 3D morphology, extracellular matrix, interaction with other cell types and complex tissue architecture leads to a poor predictivity for efficacy and safety *in vivo*.

The preclinical drug discovery pipeline shows a gradual increase in complexity and predictivity from *in-silico* modeling, to chemical molecule-to-molecule screens, to conventional cell based models, to animal models. The lack of predictivity of conventional cell based models, however, causes a gap in this selection process, leading to many inappropriate drug candidates to be passed onto animal and, much worse, clinical trials.

Many scientific efforts have been directed at overcoming this gap, and ensuring a more effective selection of drug candidates before progressing into later drug discovery stages. These efforts have consisted of biological advances in cell culture methods as well as engineering advances in devices and tools to enable implementation of new types of in-vitro models.

Advanced cell based assays

The search for better predictive models for use in pre-clinical drug development has pushed the development of more complex cell based models that better recapitulate the complexity of tissues and organs in human physiology. Primary screens make use of new insights gained from advances in genetics and molecular biology to guide compound class selections and the advent of induced pluripotent stem cell technology has provided access to previously unavailable cell types which are patient specific and have defined genotypes. However, when used in conventional 2D assays, these models still lack a large degree of the physiology found *in vivo*.

Ever since the landmark paper by Weaver et al in 1997, which showed vastly different drug response of breast cancer cells cultured in 3D rather than 2D, 3D cell culture has gained momentum.³ 3D cell culture can be performed by embedding

cells in an extracellular matrix. Synthetic hydrogels, or hydrogels extracted from tissues can be used to support cells. Cells mixed with these gels can bind in a more physiological manner. More importantly, the cells can migrate through the gel to form clusters with other cells and form larger tissue constructs. Tuning the physical and chemical properties of the cell's microenvironment, especially in combination with co-culture, can yield complex tissues that better capture the tissue complexity found *in vivo*. 3D cell culture has not only been shown to yield a different morphology, but also modified gene expression, metabolic activity and sensitivity to compounds.

Currently, the superior physiological relevance of 3D cell culture is widely recognized, but its implementation in preclinical testing and screening is still limited. Reasons for this are largely practical in nature: current technologies lack compatibility with imaging and automation and results are difficult to interpret. In addition, although platforms exist to grow tissues in hanging drops or low adhesion plates, these offer only limited advance over current status quo, as perfusion of tissues and complex co-culture options are still lacking. In other words, a platform is needed that addresses aspects of physiological relevance and end-usability in an equally satisfying manner in order to enable the end-user to make the jump towards a next generation of phenotypic models.

Microfluidics

Lab-on-a-chip (LOC) is a field of science and engineering that focusses on miniaturized tools for (analytical) chemistry and life sciences. Since its emergence in the 1990s⁴, the field of lab-on-a-Chip technology has held the promise of translating the immense advantages gained by miniaturization in electronics to the fields of chemistry and the life sciences.

By miniaturizing entire laboratory workflows on a single chip, a great reduction in labor, cost and reagent consumption is envisioned, as well as allowing large-scale parallelization of experiments in a small space. In addition, downscaling can result in unconventional physical and chemical parameters such as extreme surface to volume ratios causing dominance of surface related properties over bulk properties and laminar flow causing dominance of diffusion over advection. Leveraging these properties enables processes that would be physically impossible at conventional scale.

Downscaling of laboratory workflows into LOC devices commonly involves engineering of networks of sub-millimeter sized channels. Fluids are routed through these networks so that they interact with actuators, sensors and other fluids to achieve the desired reaction or readout. The devices can be fabricated using various techniques developed by the semiconductor industry including photolithography, thin film deposition, and wet and dry etching. In addition to utilizing semiconductor materials, other materials such as silicone rubbers have been widely utilized for their biocompatibility, flexibility and ease of processing.

Analytical chemistry is a common application of LOC devices. In addition to the obvious benefits of miniaturization for reduction in reagent use, reduced diffusion distances and proximity of analytes to sensor surfaces can vastly improve reaction speed and detection sensitivity.

Chemical synthesis can benefit from miniaturization through increased reaction speeds, access to high electrical fields, and sharp temperature gradients due to reduced thermal capacity.

Finally, biological applications can strongly benefit from the close control of the microenvironment of cells. Monitoring and manipulation of processes at the cellular level are enabled by the tight control over the microenvironment of cells in microfluidic chips, while the small volumes used avoid the vast dilution of cytokines and other signaling molecules produced by cells.

Organ-on-a-chip

The search for ever more comprehensive cell culture models has triggered efforts towards using lab-on-a-chip technologies to recapitulate organ function and physiology. This field is nicknamed 'organ-on-a-chip'. Organ-on-a-Chip adds perfusion flow, control over signaling gradients, mechanical cues as well as allows integration of actuators and sensors. Early work in combination of cell culture with microfluidics was done by the group of Linda Griffith, who added perfusion flow to a transwell type system⁵, Luke Lee, who aimed to mimic a liver lobule⁶, Mike Shuler who connected various tissues^{7,8} and Roger Kamm, who focused on vasculature aspects⁹.

A widely-lauded organ-on-a-chip publication by the Ingber group in 2010 in Science represents a milestone in Organ-on-a-Chip technology that was now regarded as a scientific discipline in its own right, alongside lab-on-a-chip. The paper by Huh et al.¹⁰ presented a lung-on-a-chip. In this device, conventional multilayer soft lithography is used to manufacture a polydimethylsiloxane (PDMS) chip that allows the culture of endothelium and alveolar epithelium on opposing sides of a porous membrane. The endothelial side is perfused with medium and the epithelial side is exposed to air. By applying a vacuum to flanking channels, the membrane can be stretched to include mechanical forces in this lung model. The exquisite control over liquid flows inside the device, as well as the inclusion of the hitherto unavailable factor of mechanical forces, are a striking demonstration of the progress that is being made in the development of such models. A downside of such devices is that they commonly contain just a single tissue and need significant of chip equipment to run.

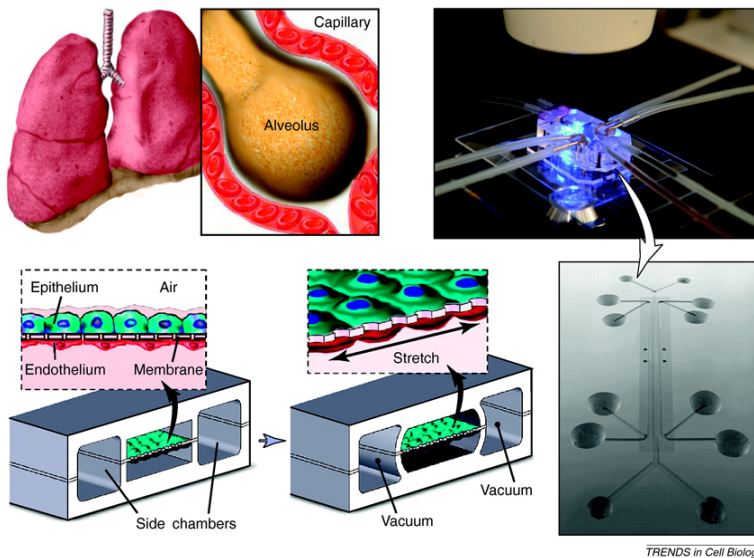


Figure 2 | Lung-on-a-chip concept showing epithelial and endothelial co-culture. Controlled fluidic flow on either side of the membrane is combined with mechanical actuation of the cells by stretching the membrane. This exquisite control over mechanical cues exerted to the cell monolayers is provided by connecting the single device to an off chip pneumatic controlled. (top right).¹¹

In the paper from Van Duinen et al.¹², we reviewed recent efforts in this field with a particular focus on 3D cell culture. We observed a predominance of vasculature and cancer related models. In addition to these, models have been described for most tissues types present in the body including brain^{13,14}, liver¹⁵, kidney^{16,17}, bone¹⁸, gut¹⁹, muscle²⁰, skin^{21,22} and many more.

In a further step, the ambition has extended to model multi-organ interaction and even develop a Human-on-a-Chip in an ambition to capture systemic effects as an alternative to animal models^{11,15,23}.

In parallel to these efforts, tremendous interest was raised amongst end-users, pharmaceutical industry in particular. The question posed itself now: how are these platforms going to be implemented in an end-user environment?

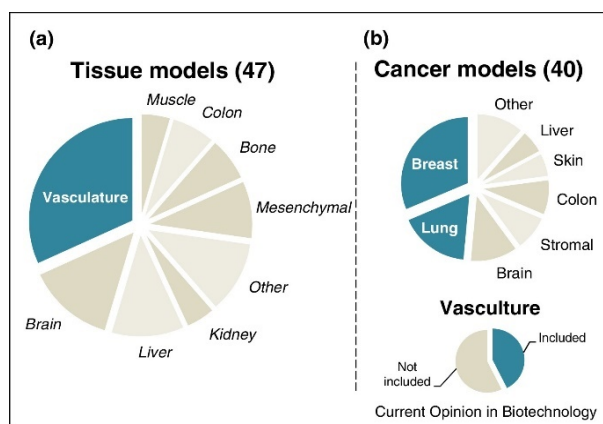


Figure 3 | Overview of the organs modelled in 87 articles published between 2012 and 2015 showing predominance of cancer related and vascular focusses models.¹²

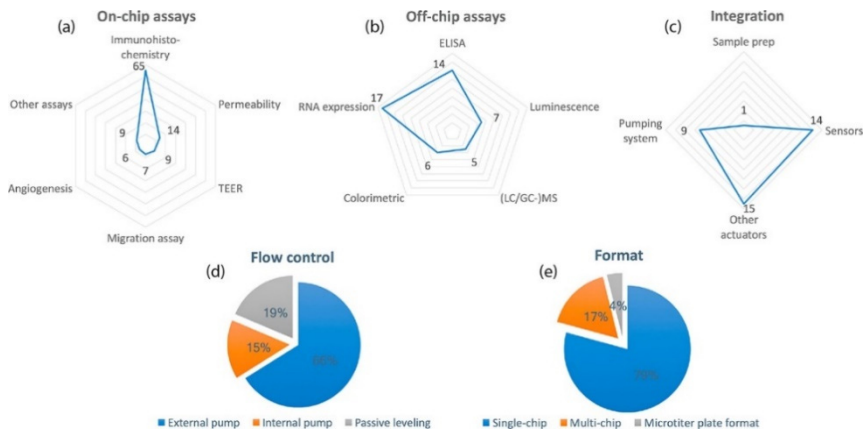


Figure 4 | Overview of assay types, flow control and formats used in 77 Organ-on-a-Chip publications published in 2014-2016. Single chip devices with external flow control make up the majority of published work resulting platforms with a high adoption barrier.²⁴

In a review by Junaid et al²⁴ it was stressed that almost none of the published models fully satisfy end-user usability requirements including assay compatibility, ease of handling and throughput. The majority of available systems are single chip devices designed to carry out experiments with a single copy of an organ model that need to be hooked up to external actuators, severely limiting throughput and scalability.

3D cell culture and organ-on-a-chip technology have the potential to alleviate the major problems facing preclinical drug development. For the strengths of both of these concepts to reach pharmaceutical industry, however, significant limitation still need to be overcome. 3D cell culture offers improved physiological relevance, but is currently associated with cumbersome techniques that are poorly compatible with automation in liquid handling and screening. Microfluidic approaches offer the exquisite control of liquids that could overcome these compatibility issues, but most developed platforms have been focusing on increased complexity instead of increased compatibility and throughput. While the level of control such devices offer is impressive, their suitability for screening, or even usability by non-expert biologists, is limited. For organ-on-a-chip technology to be widely adopted, the focus needs to shift from complex single tissue devices, towards easy to use, high throughput designs.

Scope of this thesis

The aim of the research described in this thesis was to develop a microfluidic platform that enables enhanced physiology in cell culture models by combining 3D cell culture, co-culture and perfusion flow in a single approach. Moreover, a primary guiding principle for the development has been the demand for scalable, compatible technology that is easy to implement by a non-expert end-user. To meet these demands the envisioned 3D cell culture system should meet two sets of design criteria: one for physiological relevance and one for compatibility and ease of use.

Table 1 | Design criteria defined for a 3D cell culture platform that can deliver both high physiological relevance and be easily adopted by end-users. By meeting all these criteria, a platform will be capable providing more predictive cell culture models for use in drug discovery research and screening.

Physiological relevance	End-user adoption
3D tissue morphology	Parallel data point generation
Extracellular matrix embedment	Standard lab equipment compatibility
Co-culture of multiple cell types	Stand-alone operation
Integration of tubules and vessels	Scalable throughput
Absence of artificial membranes	Inert and biocompatible materials
Continuous flow	Usable by non-experts

An overview of available microfluidic technologies for lab-on-chip platforms is described in chapter 2. While many platforms are described that could be employed for 3D cell culture, it was found that their reliance on valves, tubes and complex interfaces for a high level of control and programmability, often rendered them difficult to use and poorly suitable for high throughput screening To overcome these difficulties, we have chosen to adopt a powerful passive liquid routing technology called phaseguiding²⁵ to control fluids, gels and cells without increasing complexity for the user.

In chapter 3, phaseguide technology was developed to enable complex liquid routing through extended microfluidic networks using only a standard pipet. This unprecedented level of passive control was achieved by choosing to hardwire the fluidic unit operations into the plate, instead of focusing on user programmability of the device. A decision that is completely in line with the needs of robustness and repeatability in a screening environment rather than flexibility of a research laboratory.

In Chapter 4, phaseguide technology was implemented in a dedicated 3D cell culture device, which we called the OrganoPlate. Up to 96 microfluidic networks were embedded in a standard 384 well titerplate. Each network connected 4 wells with specific function and enabled stratified culture of cells embedded in an extracellular matrix with continuous perfusion. The use of a standard plate format, gravity driven perfusion, inert materials and high quality optical readout ensured the platform offered all the envisioned characteristics of a high throughput screening platform.

In chapter 5, the culture of perfused, membrane free, tubular barrier tissues was added to the applications of the microfluidic 3D cell culture platform. A special focus was put on intestinal tubules with proper tissue polarization and transporter expression. The applicability and throughput of the platform was illustrated by providing the largest organ-on-a-chip dataset published to date according to our knowledge. Combining throughput and ease of use with perfused 3D (co-)culture of extracellular matrix (ECM) embedded and barrier type tissues has set the stage for wide adoption of this organ-on-chip platform in laboratories and screening facilities throughout the world.

In chapter 6, we provide an overview of the models and assays developed on the microfluidic 3D cell culture systems by our and other groups. They include models of solid tissues such as the liver, neurons and cancer, as well as barrier type tissues including endothelium and epithelium. The wide range of complex (co-)culture models as well as the described range of assays demonstrates the flexibility of the system and shows that passive microfluidics can be employed to generate culture models of highest biological complexity.

The ongoing adoption of the technology developed in this thesis by expert and non-expert users is setting the stage for the organ-on-chip platform, which we named OrganoPlate, to become a standard for perfused 3D cell culture that is now even implemented in a screening environment. At the time of writing this thesis, the OrganoPlate platform has been applied in a plethora of tissue models at over 50 different sites and is the basis of the company Mimetas, which is currently market leader in the field of organ-on-a-chip.

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