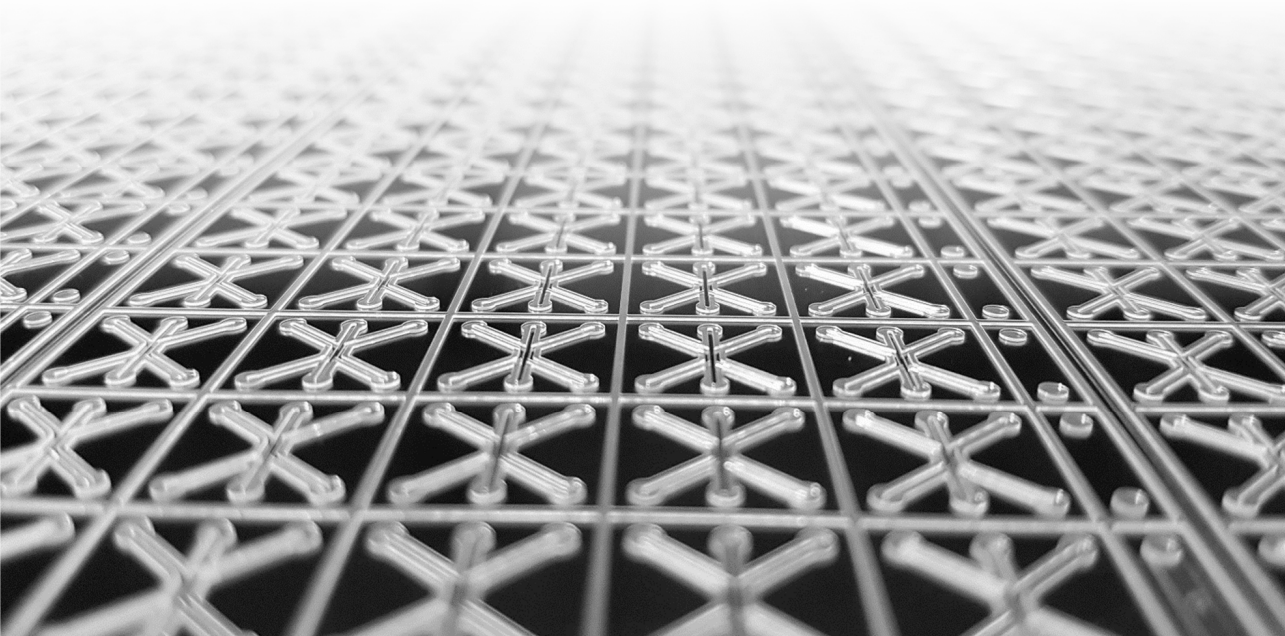
Microfluidic 3D cell culture for high throughput screening

Trietsch, S.J.

Chapter 6

Model development on the OrganoPlate



Introduction

There is an urgent need for more predictive models in preclinical drug development. While 3D cell cultures and complex co-cultures can offer more physiologically relevant tissue models, they have been generally associated with cumbersome techniques, low throughput and poor compatibility with established assays. Microfluidic techniques and especially organ-on-a-chip technology, show great potential to enable the application of complex, perfused 3D cell culture in a high throughput setting.

The field of microfluidics and organ-on-a-chip has offered many impressive feats of engineering and provided ingenious model systems. A wide range of models has been designed to capture particular aspects of the modelled tissues including detailed geometrical characteristics of e.g. vasculature[1], combinations of multiple tissue types[2] including the microbiome[3], mechanical actuation as pioneered by Ingber et al[4] or specific readouts[5]. The various application in the field have been extensively reviewed showing a multitude of devices that offer high flexibility and programmability of the devices and exquisite control of the tissues and liquids in the devices.[6–8] Most platforms use tubing or other off-chip actuators to achieve the needed control, one exception being the surface tension driven platform developed by Beebe et al.[9] In spite of these great achievements, a lack of widespread adoption of these platforms is apparent.

At the same time, there is a need for robust organ-on-chip platforms as the impressive developments in stem cell research and developmental biology will allow breakthroughs in biomedical and pharmaceutical research. For example, induced pluripotent stem (iPS) cells will allow to create different cell types and tissues from individuals.[10] Organoid technology is rapidly setting new standards in cell culture, with a focus of maintaining the stem cell niche, while allowing tissues to proliferate and differentiate at the same time.[11] These techniques are being expanded to model diseases and for usage in the clinic. We are convinced that these biological progress is greatly complemented by the precise control that is made possible with application of microfluidic techniques.

The OrganoPlate is a microfluidic 3D cell culture platform based on a 384 well plate footprint[12]. A single titerplate has 40 to 96 microfluidic networks embedded in the bottom layer of the device. Each of the microfluidic networks connects with various wells of the titerplate that are functioning as in- and outlets for the microfluidics. These inlets are either used to administer an extracellular matrix gel or growth medium that is perfused alongside the gel. The gel is patterned using phaseguide-

based capillary pinning, resulting in stratification into lanes for either gel or perfusion flow.

The platform departs from the trend in the field of microfluidics and organ-on-a chip towards more complex, low throughput devices and aims at providing a robust, easy to use, high throughput system. The passive liquid routing technology of phaseguides is leveraged in combination with gravity driven flow to provide a stand-alone platform that can be used without any specialized equipment or interfaces to the outside world.

The OrganoPlate was first used for (co-)cultures of solid tissues embedded in an extracellular matrix (ECM) including liver and fibroblast cell lines. After proofs of principle were demonstrated for different readouts and dose response curves generation, the scope of tissue types was broadened to include membrane free, perfused barrier type tissues starting with Caco-2 epithelial tubes.[13]

The OrganoPlate was designed as a generic culture platform for complex 3D, perfused (co)-cultures. Moreover, the platform was designed for transfer to and adoption by end-users that are not experts in the field of microfluidics. In this chapter, we will demonstrate the generic aspects of the platform by discussing a number of tissues that were developed as part of or parallel to this thesis. Also, the success of transfer to the end-user is discussed by means of these examples.

It is not the intention to give a comprehensive review of the organ on a chip field or of all platforms being developed by different groups and companies, but rather to give an impression of the potential applications of the OrganoPlate platform and the research efforts that have been undertaken with it.

Liver

One of the earliest tissue types implemented on the OrganoPlate was the liver.[12] This effort was quickly adopted by the Korea Institute of Science and Technology (KIST) in Germany. Mi Jang et al. (Andreas Manz' group) applied the OrganoPlate to develop a liver model and showed the enhanced functionality and metabolism in the OrganoPlate compared to conventional 2D and 3D cell culture.[14] They showed enhanced physiological relevance based on visualization of the morphology and proliferation of cell clusters as well as bile canaliculi formation. Metabolic competence was further demonstrated for albumin and urea production as well as cytochrome P450 1A induction and acetaminophen sensitivity. Increase in sensitivity to toxic compounds, enhance metabolic function and improved morphology was observed when compared to conventional 2D culture, but also

compared to conventional 3D culture, further showing the added value of the organ-on-a-chip model developed. The comparable performance of the platform for the same tissue model in a different lab was one of the first steps towards widespread acceptance and validation of the OrganoPlate platform. Research with a focus on the liver has since been pursued by multiple other groups in Europe and the United Stated including both academic groups and pharma companies.

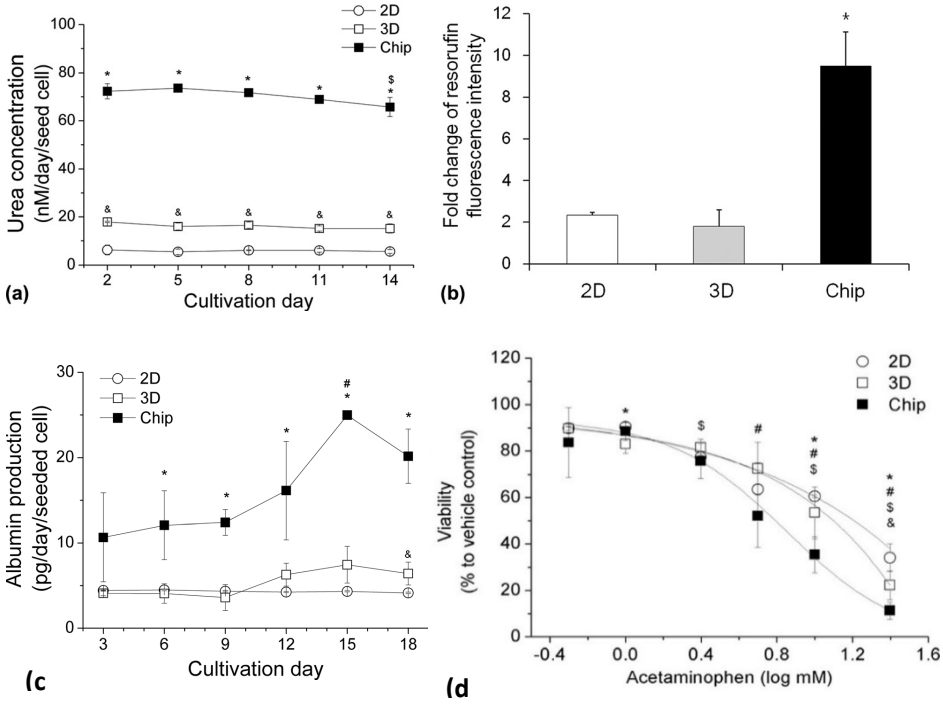


Figure 1 | Characterization of hepatocytes cultured in the OrganoPlate based on urea production (a), cytochrome P450 activity (b), albumin production (c), and acetaminophen sensitivity (d). (* $p<0.05$). Figures adapted from ref 14.

Neurons

The culturing of neurons has always suffered from a poor availability of primary material and lengthy and sensitive culture methods. The use of iPS derived neurons has the promise to alleviate a part of these issues, but the desire for faster, more robust and more reagent-efficient culture techniques has remained strong. Because of this, multiple initiatives were started to use the OrganoPlate for neuronal models.

At the Luxembourg Center for Systems Biology, Lucumi Moreno et al. (Ronan Fleming group) used the OrganoPlate to differentiate iPS derived neuroepithelial stem cells into functional dopaminergic neurons.[15] After a 30 day differentiation protocol in the OrganoPlate, the cells were characterized based on morphology, immunocytology and electrophysiology. The high biological fidelity of these cells in combination with automation compatibility and low reagent consumption made the use of this platform highly favorable for neuronal culture.

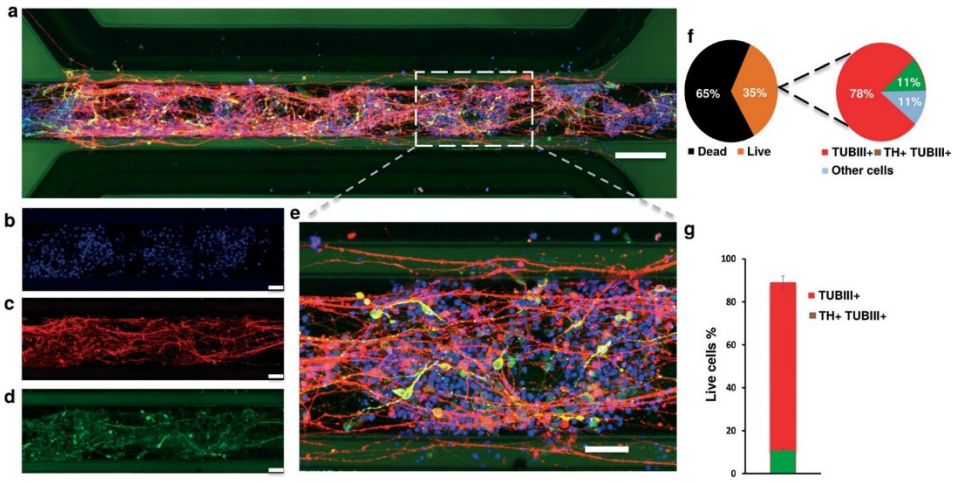


Figure 2 | hiPS derived neurons after 30 days of culture in an OrganoPlate (a,e), stained for nuclei with Hoechst (b), class III β tubulin (TUBβIII) as a neuronal marker (c), and tyrosine hydroxylase (TH) as a marker for dopaminergic neurons (d); (a-d: scale bar 50 µm, e: scale bar 100µm). Viability and differentiation efficiency (f,g). Figure reprinted with permission15.

In the work described by Wevers et al. (MIMETAS labs in collaboration with Westerink Group University of Utrecht, GSK, Sanofi, AbbVie and BASF) this research was followed up by the co-culture of iPS derived neurons with glia cells.[16] The use of various commercially available cells eases adoption of the described protocols by end-users. A protocol was developed where network formation was observed within 24 hrs. Analysis of the network formation over time was demonstrated and the cells were further characterized using immunofluorescence. Calcium imaging was used to further characterize the networks formed by the neurons by visualizing the spontaneous activity of the neurons. The usability of this platform for compound evaluation and toxicity testing. Several model compounds with known mode of action on viability, neurite outgrowth and electrophysiological activity patterns were assayed including methyl mercury, endosulfan, 2,5-hexanedione, GABA and TTX.

The developed neuronal models enable research into various disease areas as well as neurotoxicity using relevant but available cells and scalable readouts to meet a largely unmet need in drug development.

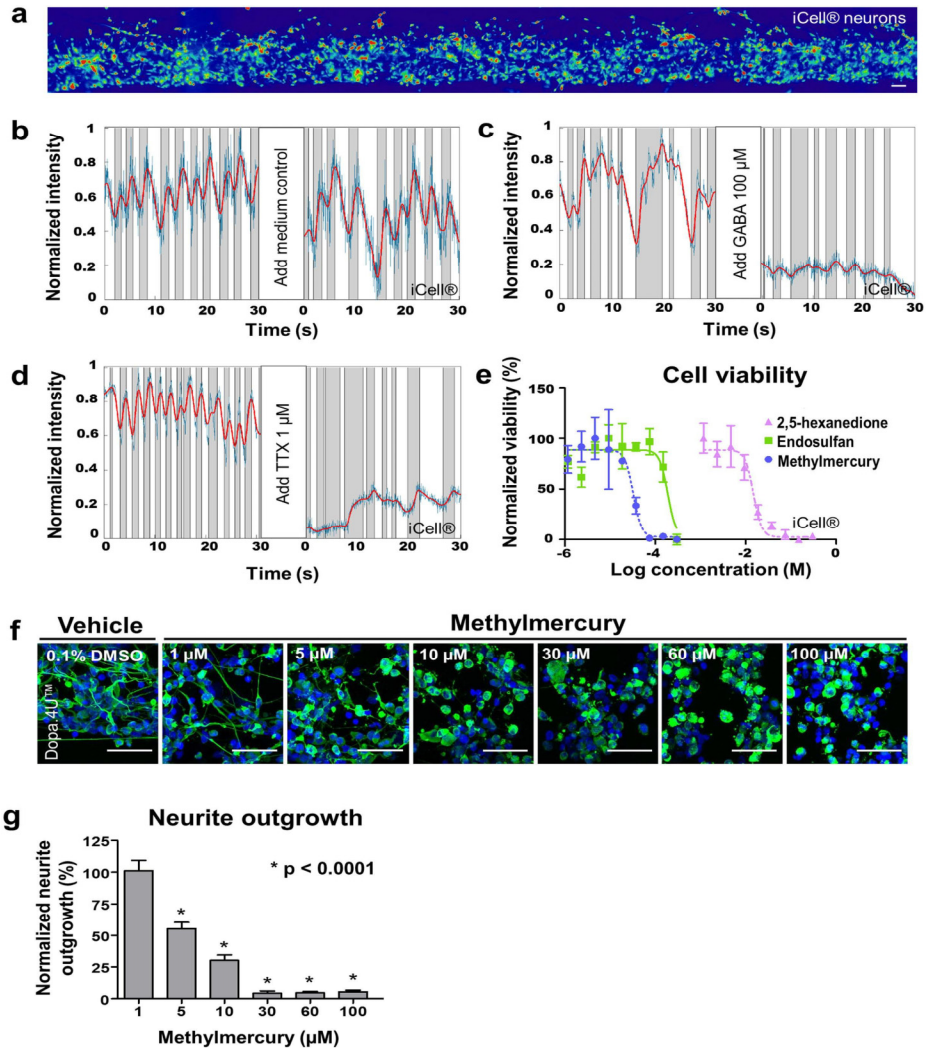


Figure 3 | Overview of assays performed on neuronal models including calcium imaging (a) and spike train analysis of spontaneous and compound modulated activity. Blue and red lines depict unfiltered and filtered traces of an individual neuron, with gray areas indicating inferred spike trains (b-d), and cell viability and neurite outgrowth visualization and analysis after compound exposure. Cells were fixed and stained with β 3-tubulin (neurites, green) and DraQ5 (nuclei, blue) (e-g).[16]

Cancer

Clinical use of the OrganoPlate platform was assessed by culturing tumor tissue and evaluating their response to chemotherapeutic treatments. In work decribed by Lanz et al. [17] (MIMETAS labs and Wang group at Mayo Clinic), protocols were developed to culture breast cancer models in the OrganoPlate. ECM, perfusion and medium conditions were optimized for robust cell culture, and used to grow tumors with known BRCA1 and p53 status. Exposure to various anti-cancer drugs, including paclitaxel, olaparib, and cisplatin, showed an attenuated response. To show the applicability of the platform on primary material, rather than cell lines, cells from patient derived xenografts (PDX) were screened in the platform, showing attenuated response to cisplatin. Based on these protocols, a workflow was proposed for using tumor biopsies in microfluidic devices as an alternative to Xenograft transplantation. . This microfluidic protocol would yield results in 21 days instead of 5-9 months showing its potential for future treatment selection in patients.

A wide range of oncological applications are envisioned for the platform. Culture of tumor tissues in a relevant (and modifiable) microenvironment can assist in gaining mechanistic insights in the pathophysiology. Furthermore, the downscaling and parallelization of 3D culture of these tissues can enable efficient study of combination treatments for different types of tumors, or ultimately for (combination) treatment selection or diagnostic stratification of patient populations.

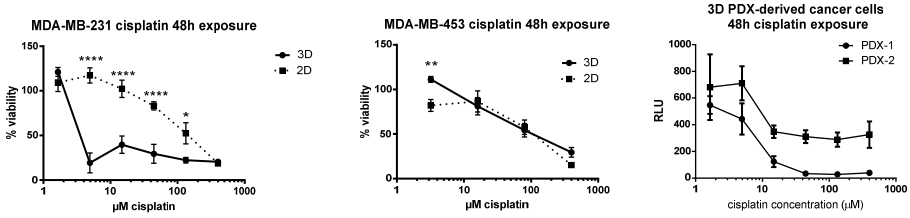


Figure 4 | Response to 48h cisplatin exposure of cell lines in 2D vs 3D and in two PDX derived models show an attenuated effect in some cell lines but not others. The difference in response rate between different PDX derived cells could guide treatment selection. Viability was measured using the luminescent CellTiter-Glo assay (Promega). Adapted from [17]

Endothelium

Blood vessels are a crucial constituent of nearly all tissues and play a major role in most complex diseases. Therefore, incorporation of endothelium is an important step towards physiological relevance. Including vasculature enables an *in-vivo* like supply of oxygen and nutrients even into larger and denser tissues. In addition, the signaling molecules excreted by the endothelial cells provide important cues for the surrounding tissue aiding differentiation and functionality.

Van Duinen et al. (MIMETAS labs, Hankemeier and van Zonneveld groups Leiden University) developed a high throughput methodology for culturing and assaying membrane free, perfusable endothelial tubes in a way akin to the methods described in this thesis for intestinal tubules.[18] Van Duinen et al. cultured 96 tubules in parallel from endothelial cells that were fully leaktight for 150kDa TRITC dextran and showed limited permeability for 20 kDa FITC-dextran. The barrier integrity of the endothele tubes was quantified in the cross-platform standard of the apparent permeability (P_{app}) in cm/s. This eases comparison of the data acquired in the microfluidic platform with conventional assays. Moreover, authors show response of the endothele barrier to several cytokines including Vascular Endothelial Growth Factor (VEGF), Interleukin 1 beta (IL1β), Interleukin 8 (IL8), Interferron gamma (INFγ), Tumor Necrosis Factor alpha (TNFα) and retinoic acid (RA)

In recent work by van Duinen et al. (MIMETAS labs, Hankemeier and van Zonneveld groups, Leiden University, *in preparation*) tubes are exposed to cocktails of growth factors to induce angiogenesis. In the 3-lane design, gradients of signaling molecules are formed and maintained in a stable manner to induce a directional network of perfusable sprouts into the gel. The effect of different combinations of medium components and the characteristics of the gradient is quantified based on the level of sprouting and the morphology of the sprouts. Recently, this work has been applied to co-culture endothelial cells with other cell types: the vascularization from a larger vessel into ECMs is combined with the culture of other tissues to provide perfused vascularization of complex 3D tissue constructs.

Another possibility of this platform is the combination of endothelium with other cell types. Endothelium can be co-cultured with pericytes to construct a comprehensive model of microvasculature. Endothelium is also being combined with neuronal tissues to mimic the neurovascular unit and kidney epithelium towards mimicking the entire kidney clearance pathway.

Finally, co-culture with monocytes is being explored to study monocyte recruitment and transmigration. The range of models comprising a vasculature component thus shows that endothelial models are not only important for vascular research purposes, but for nearly any tissue model, diseased or healthy.

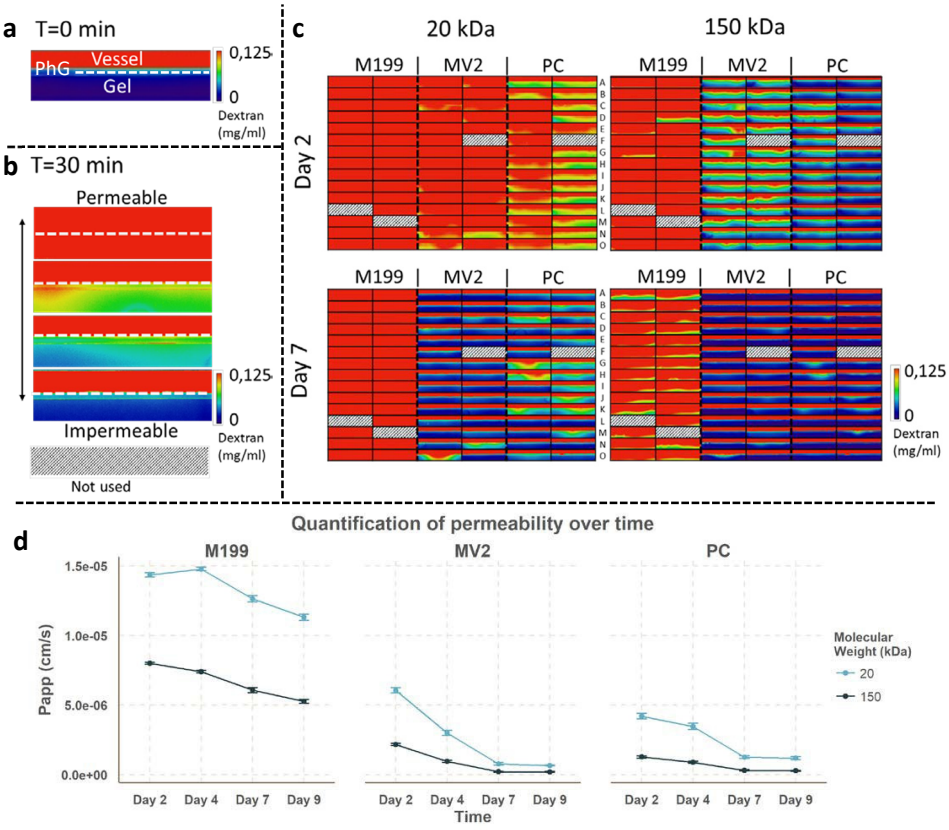


Figure 5 | Representation of the optimization of endothelial culture as described by van Duinen et al including an illustration of the barrier integrity assay with a fluorescent probe diffusion from the vessel into adjacent gel in more permeable vessels. The PhaseGuide (PhG) position is indicated by the white dashed line **(a,b).** Permeability of 86 tubes cultured in three media types is illustrated by the diffusion of fluorescent probes of 20 and 150 kDa at day 2 and 7 in culture. Each rectangle enclosed by a solid black line is and individual chip, with the perfused vessel in the top and gel in the bottom position. PhaseGuide™ location is not indicated in these images. **(c)** The progression of the permeability over time is depicted for each condition by plotting the extracted P_{app} **(d)**.

Kidney

The kidney is amongst the critical organs for attrition of new drugs as a result of nephrotoxic events. Notwithstanding this, no models are available to predict such adverse events in humans other than animals. For this purpose, the MIMETAS lab in collaboration with Wilmer Group (Nijmegen University), Masereeuw group (Utrecht University), Suter Group (FHNW, Switserland) and pharmaceutical companies Roche, GSK and Pfizer set off to develop a kidney model in the OrganoPlate. In a parallel effort to this thesis and similar to the approach described for culture of Caco-2 tubules in Chapter 5, models have been developed to mimic the proximal tubule. Vormann et al. (MIMETAS labs, in prep) developed and validated a nephrotoxicity screening model based on proximal tubules grown in 3D. In addition to developing culture protocols for several different proximal tubule cell sources and assays for measuring barrier integrity and apparent permeability, active transport has been a significant focus within these projects.

Assays were developed to monitor active influx and efflux transport. Fluorescent substrates, and agonists or antagonists for the transporters of interest are used in combination with automated image analysis protocols, to monitor transporter activity. An example of an automated image analysis pipeline is depicted in figure 6. Measuring transporter activity upon exposure to different compounds can give insight into transporter mediated nephrotoxicity and especially drug-drug interaction

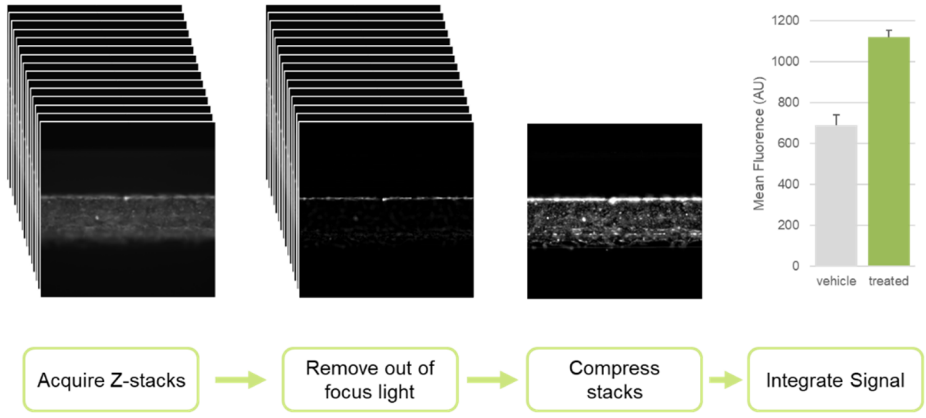


Figure 6 | Automated analysis pipeline to monitor compound uptake into cells. A stack of images is acquired along the z-axis of each tubule. Out of focus light is filtered from each slice and the resulting images are projected into a single image. The signal in the final image is analyzed and represents the uptake of the fluorescent compound into cells throughout the tube.

Organoids

In work described by Schutgens et al. (Clevers group, Hubrecht Institute), their groundbreaking work on organoids is combined with tubular culture in the OrganoPlate[19]. The work elaborated on previous achievements in culturing and expansion of adult intestinal *Lgr5+* stem cells that was also translated to other organoid systems.[11,20–22] In the work by Schutgens, an organoid culture system is described for mouse and healthy and diseased human kidney tissue. By combining this method with the OrganoPlate, the normally spheroidal organoids have been cultured in a tubular fashion as depicted in figure 7. The kidney tubules were leak-tight and polarized, with maintained transporter activity. The change from spheroidal to tubular culture yield access to the basal and apical side of the epithelium, enabling transporter and drug disposition studies.

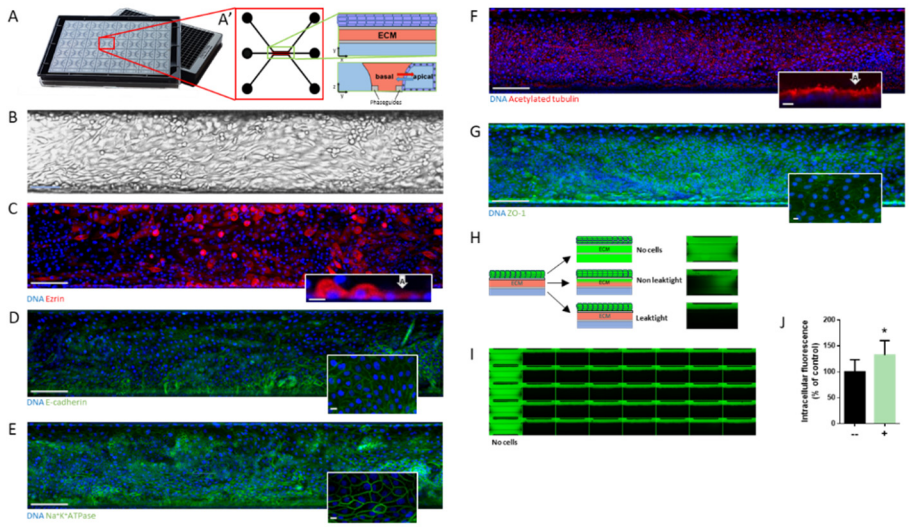


Figure 7 | Organoid derived kidney tubules. Cells are seeded in a manner similar to the process previously described for cell line based epithelial tubes[13] **(A)**, yielding leaktight, polarized tubes with maintained transporter activity **(B-G)**. The tubes show robust performance in barrier integrity assays and respond appropriately in transporter studies **(H-J)**.

Co-culture

One of the strengths of microfluidic 3D cell culture is the ability to perform complex co-cultures. Figure 8 depicts three examples of possible co-culture configurations. In various collaborations, different co-culture methods are being explored. Vasculature has been added to various models including liver, cancer, kidney, and brain. Other combinations include the addition of pericytes and fibroblasts to endothelium, of various non-parenchymal cells including Kupffer cells to hepatic models and glia to neuronal models. Lastly, co-culture of tissue with circulatory cells such as monocytes or non-mammalian cells such as bacteria is being explored as a method to mimic the interaction of tissues with the immune system and microbiome.

It is expected that co-culture of different cell types will become a standard in micro physiological systems. The membrane-free combination of various cell types enables mimicking of the intimate interaction between cells that is crucial in *in vivo* tissues and can yield vast improvement in physiological relevance of models.

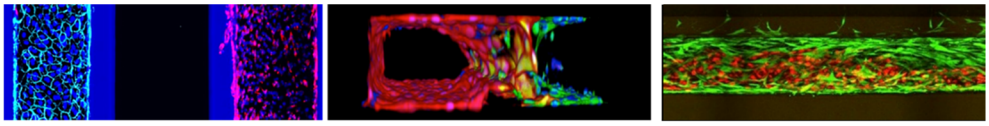


Figure 8 | Examples of co-cultures being explored including adjacent culture of two tubules consisting of kidney epithelium and endothelium (Vascular Endothelial-cadherin labeled Human Umbilical Vein Endothelial cells (HUVEC), green; Acetylated Tubulin labeled Renal Proximal Tubule Endothelial cells (RPTEC), Red, DraQ5 labeled nuclei, blue) (**l**). Endothelium was cocultured with astrocytes towards mimicking the blood-brain-barrier (RFP labeled HUVECs, red; Vimentin labeled astrocytes, green; Draq5 labeled nuclei, blue) (**m**). Another method of coculture was demonstrated by culturing a tubule with an outer layer of pericytes lining an inner tubule of endothelium. (RFP labeled HUVECs, GFP labeled pericytes derived from kidney mesenchymal stem cells, green) (**r**).

Assays

The application of the OrganoPlate in a screening setting relies heavily on the availability of relevant and compatible readouts for assays performed on the models. The work in this thesis describes the use of widefield and confocal imaging of cells, reporting their morphology and expression of proteins. In addition, a dedicated readout for barrier integrity is described for perfused intestinal epithelium tubes. In addition to these readouts, a plethora of on-plate assays has

been developed including calcium imaging, transport assays, absorption spectroscopy, luminescence spectroscopy, 3D morphological analysis, and invasion assays. All of these assays use an optical readout to optimally leverage the high quality optical access to the plate without overly suffering from downscaling.

In addition to on-plate optical readouts, efforts are ongoing to enable electrical readouts of the models in the OrganopPlate. For example, direct electrophysiological evaluation of neurons in the OrganoPlate is made possible by integrating sensing electrodes inside the microfluidic channel. To maintain ease of implementation, the envisioned plate is designed to be compatible with commercially available multi-electrode array instruments. A second electrical readout that is being developed is the use of impedance spectroscopy to assay barrier function. By making electrical contact with the apical and basal side of a barrier type tissue, impedance measurements can be performed across this barrier. The extracted transepithelial electrical resistance (TEER) is a well-accepted characteristic to describe the barrier function of a cell layer. Enabling such readout will ease the comparison between conventional culture models and data acquired using the OrganoPlate as well as allowing for a non-invasive, high throughput method to evaluate barrier function.

In another research effort, aliquots of the medium are taken for mass spectrometric profiling of metabolites, drugs or drug metabolites.

The range of assays performed on models developed in the microfluidic 3D cell culture platform is expected to continue to grow as more tissues are being constructed. The compatibility with standard equipment and high quality optical access, ensure an easy translation of standard assays to on-plate assays. In addition, the ongoing development of peripheral equipment can further increase the range of assays that can be performed on the platform without sacrificing throughput.

Automation

The OrganoPlate was designed with a focus on throughput and compatibility with current lab instrumentation and robotic systems. The standard 384 well plate layout, glass bottom, and gravity driven perfusion not only make it easy to use manually, but also allow their implementation in a fully automated workflow.

One example of the integration of the titerplate for microfluidic 3D cell culture has been recently described by Kane et al.[23] In this work, a fully automated cell culture platform is presented that includes all steps required for cell culture maintenance, compound exposure and optical readout of cellular parameters over time. The use

of the platform is demonstrated by the successful automation of the generation of personalized *in vitro* models from neuroepithelial stem cells. This level of automation enables the characterization of large numbers of microtissues with increased reproducibility and throughput.

It is envisioned that fully automated workflows including plate handling, liquid handling for cell maintenance, exposures and assays, automated incubator hotels providing rocking and high throughput readouts such as high content imaging systems, will provide all the means necessary to perform large scale screens in the preclinical drug discovery pipeline.

Discussion

The research described above shows a wide range of applications of the OrganoPlate. The standalone and high throughput nature of the platform allows easy adoption by end-users, but of course also has forced some compromises and prerequisites.

One prerequisite of the system is the need for ECM. The presence of ECM is often advantageous for mimicking the in vivo microenvironment, but some research efforts have also been focused on culturing cells in 3D without any ECM, for example using hanging drop methods.[24] The fundamentals of phaseguide-based, membrane free culture require however the use of an ECM to maintain the architectures as patterned during seeding.

The dimensions and design of the cell culture compartment have been chosen to be a compromise between ease of imaging and 3D cell culture. Where macroscopic ECM embedded cultures can grow a lot larger[11] and allow for easy reclamation of cells from this culture, the dimensions of the microfluidics channels limit the maximum size of the tissues to hundreds of micrometers and the glass prevents easy physical access to the tissues. The benefit from this setup is, however, that higher throughput is more easily obtained using optical readouts.

The use of phaseguides for patterning cells in the OrganoPlate allows for side by side layering of different cell types, as well as the creation of perfuseable tubes in channels. This method, however, is limited to side by side patterns that are largely predetermined by the plate geometry. While being easy to adopt in a standard laboratory, and not requiring exogenous compounds or some bioinks, this method allows less freedom than 3D printing approaches that allow for the free patterning

of different cell types in three dimensions.[25] On the other hand, however, the achievable resolution is much higher than with current 3D printing methods.

The choice to manufacture OrganoPlates from glass enables high quality optical access, but the result is a rigid device. This is beneficial for handling and robustness, but less compatible with approaches for inducing mechanical ques to the system. Systems using external pressure sources to move flexible membranes incorporated in the microfluidics, such as the ones described by Ingber et al[26,27], are much more adept at providing these ques that can be valuable for certain models.

A final compromise that has been made in the OrganoPlate is the choice for gravity driven, bidirectional flow. Systems using integrated[2] or external pumps[28] to achieve active perfusion are commonly able to achieve much higher flow rates and shear combined with truly unidirectional flow. The gravity driven approach of the OrganoPlate yields a laminar flow that reverses direction at a preset interval. This has been a deliberate choice as it is to our knowledge the only manner to perfuse 40 to 96 tissues in parallel in a manner that is still workable for a non-expert end-user and is compatible with high throughput screening set ups. However, the range of flow profiles that can be achieved is more limited than what is possible with active perfusion.

Conclusion

The microfluidic 3D cell culture platform, the OrganoPlate, has been adopted for the development of a wide range of tissue models including mono and co-cultures of both solid and barrier type tissues. ECM embedded 3D cell culture, continuous perfusion and membrane free patterning of tissues is leveraged to yield models of the liver, brain, tumors, kidney, gut, vasculature and many more. Assays have been developed to make use of the parallel setup of the microfluidic devices and provide data in high throughput.

The ease of use and compatibility with standard equipment, combined with a high flexibility for developing complex tissue models has prompted a shift from engineering of complex devices, to the development of complex biology. This transition marks the onset of real adoption of the platform for biological research, rather than microfluidic engineering.

The most important validation of the usability of the OrganoPlate platform is the fact that is has been adopted by a multitude of end-users in laboratories around the world. Currently the OrganoPlate is being used by over 50 companies, institutes or research groups including most of the top 20 pharmaceutical companies. It is thus expected that the OrganoPlate platform will become an industry standard and will help to improve drug development efficiency and to bring better therapies to patients by using patient derived human cell models.

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