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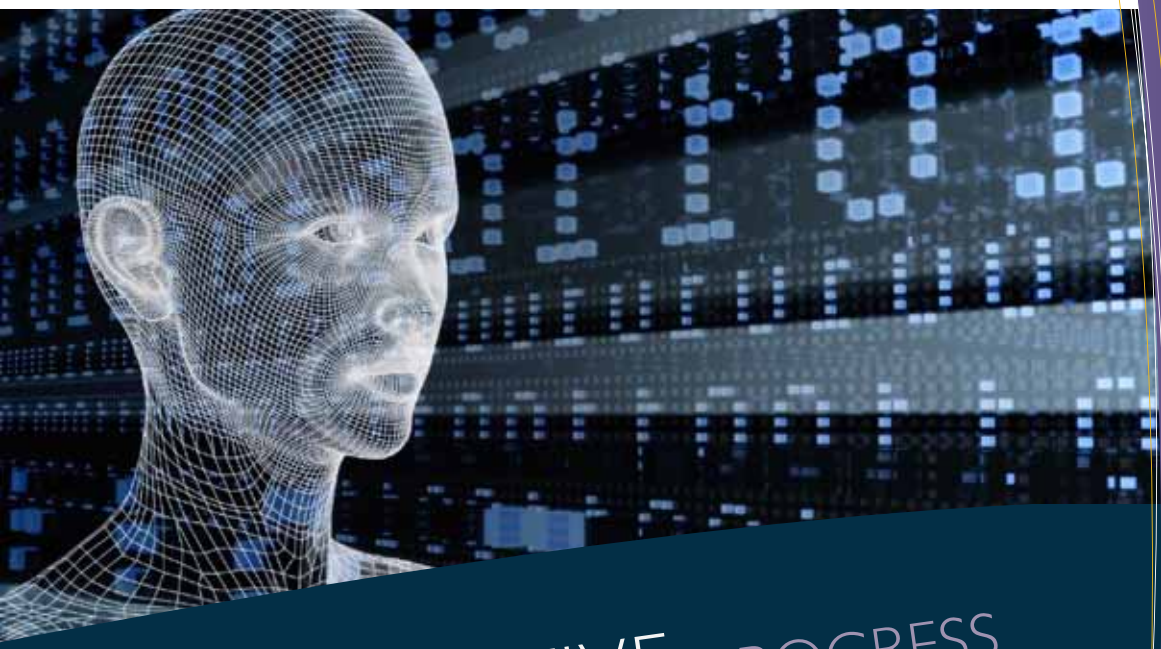
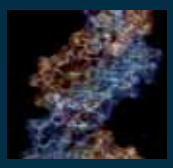


ALTERNATIVE TESTING STRATEGIES PROGRESS REPORT 2012

& AXLR8-3 Workshop Report on a 'Roadmap to Next Generation Safety Testing Under Horizon 2020'



SEVENTH FRAMEWORK PROGRAMME

Advanced Therapies and Systems Medicine

Grant Agreement N° 241958

Multi-Organ-Chips

Developing a Human Chip-Based Platform for Repeated Dose Toxicity Testing

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Background & Objectives

According to the recently published roadmap (Basketter et al., 2012), systemic toxicity testing forms the cornerstone for the safety-evaluation of substances. Pressures to change from traditional animal models to novel technologies arise from the limited prediction of human health effects and animal welfare considerations. This change requires human organ models combined with the use of new technologies in the field of -omics and systems biology, as well as respective evaluation strategies. *In vitro* organ emulation needs an appropriate model for each organ system, i.e., what makes a heart a heart, a liver a liver, etc.? In this context, it is important to consider combining such organs into systems. Miniaturisation of such systems to the smallest possible chip-based scale is envisioned to minimise human tissue demand and to match with the test throughput required in industry (Esch et al., 2011; Huh et al., 2011, 2012). A multi-organ-chip technology has been established based on a self-contained smartphone-size chip format, within a German three-year GO-Bio project. A micro-pump has been successfully implemented into the microcirculation system for long-term operation under dynamic perfusion conditions. Performance of the technology has been proven by 28-day chip-based bioreactor runs of single perfusion circuits combining human 3D liver equivalent and human foreskin tissue cultures. The inclusion of organ equivalents for the intestine, kidney and bone marrow will extend the multi-organ-chip (MOC) use to ADMET testing in an upcoming further three-year programme.

Deliverables & Milestones

Miniaturised Bioreactor and MOC Prototyping

Fundamental paradigms of the *in vivo* behaviour of human organs can be translated into rational design principles for dynamic multi-micro-organ bioreactors for *in vitro* substance testing within three categories: device (control unit and chip support), architecture (micro-fluidic chip and incorporated tissue culture space) and process (organoid homeostasis and exposure regime). On the basis of these three design principles, we have developed the MOC technology concept and manufactured a dynamic laboratory micro-bioreactor system, shown in Figure 1.

Furthermore, we prototyped and produced a micro-fluidic chip-generation allowing for the simultaneous culture of two different organoids in a common micro-fluidic circuit. The MOC is a self-contained sensor-controlled smartphone-size device. The incorporated micro-fluidic chip is the shape of a standard microscope slide, with a total height of less than 3 mm. It fits into a support for use in CO_2 incubators and is shown in Figure 2.

Figure 1. A bench-top bioreactor at operation in the laboratory.

Figure 2 (above). A 3D-CAD design of the MOC including the support (red colour).

Figure 3 (below). A fully equipped MOC fixed in an autonomous support (red colour).

Additionally, an autonomous support (Figure 3), which maintains and monitors the temperature, has been developed for the incubator-independent incubation of MOCs over long periods.

The MOC is designed to operate two identical micro-fluidic circuits simultaneously. Figure 4 is a schematic drawing showing the positioning of the crucial elements at a glance.

The micro-fluidic channels comprise 10

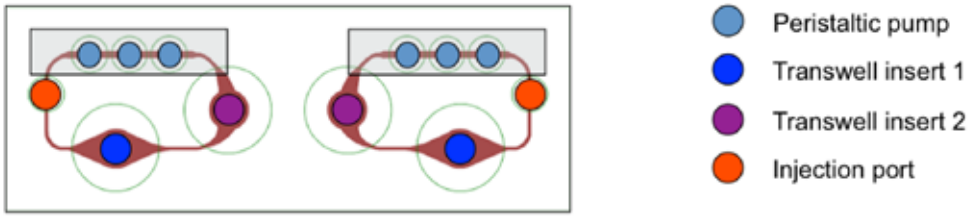

Figure 4. Scheme of the two microcirculation circuits on a single multi-organ chip (top view).

µl of fluid per circuit. A peristaltic on-chip micro-pump reproducibly operates the micro-circulation system interconnecting two tissue culture spaces. Micro-fluidic channels and tissue culture spaces are embedded within a polydimethylsiloxane (PDMS) layer. The layout supports both flexible integration of conventional miniaturised tissue culture formats, such as Transwell inserts, and special organotypic matrices into the tissue culture spaces. The latter have a medium storage capacity of 150 µl each. The volume of 3D organoids incorporated into these spaces with Transwells or tissue-specific matrices can be varied to keep the tissue-to-fluid ratio at an organotypic level. The micro-fluid perfusion rate can be adjusted to physiological ranges by pump settings. Daily media exchange can be carried out via the tissue culture spaces. The tissue culture space diameter is about 6 mm and provides heights of up to 2 mm. Thus, live tissue imaging can be carried out throughout the entire organ culture by means of, for example, two-photon microscopy, as the chip base slide is fabricated from microscopically transparent material.

Modelling Human Liver and Skin Equivalents

Tissue culture spaces are designed to maintain various 3D tissues seeded into Transwell inserts sized for 96-well plates with 10 µm thick micro-porous membranes separating the culture spaces from the fluid flow below (Corning, Lowell, USA; Millipore, Billerica, Ma, USA). This allows and supports a stable dynamic gradient of nutrients and metabolic waste products within any of the possible human organoids cultured in the inserts. Firstly, we qualified the robust maintenance of human full-thickness skin equivalents or foreskin biopsies in micro-fluidic chips operated with skin equivalents only over a minimum of 14 days each. We developed a 3D liver tissue preparation procedure mixing HepaRG cells (Jossé et al. 2008) and primary human stellate cells (Bhatia et al.1999) in a 12:1 ratio, shown in Figure 5.

Aggregates were formed in a 24-hour incubation procedure in AggreWell plates (Stemcell Technologies, Grenoble, France). The aggregates formed were subsequently cultured in low-attachment plate cultures for another four days under static culture conditions. Five 3D human liver tissue

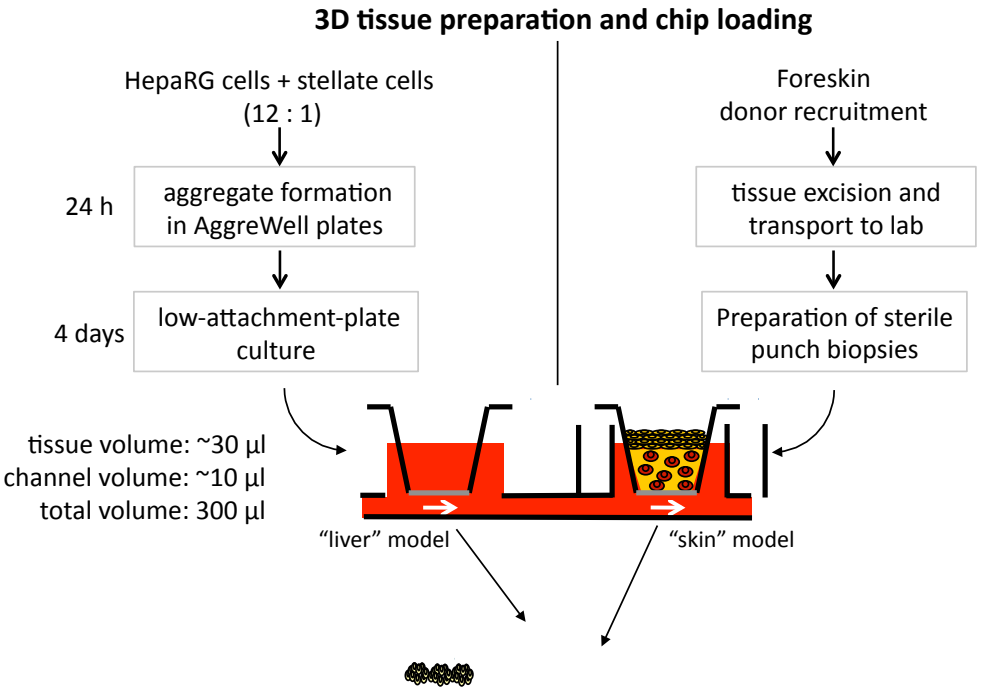

Figure 5. Experimental setup at a glance.

aggregates were loaded into one Transwell insert of each micro-fluidic circuit. The other Transwell insert of each circuit was loaded with a punch biopsy of human foreskin, which exactly fitted the size of the insert. The combination of a human liver and a human skin equivalent resulted in 30 µl of total tissue per circuit. A 28-day culture duration was chosen for the experiments to comply with a timeframe of relevant OECD guidelines for chemical testing, such as OECD TG407, 410, 412, or 419. Preliminary immunohistostaining of Cytochrome P450 isoforms 3A4 and 7A1 for liver equivalents, and Tenascin and Collagen IV for skin equivalents was carried out at the start and the completion of each experiment. Other metabolic and genetic analyses are ongoing. The experimental set-up was performed in four replicates (n = 4). Preliminary data suggest a survival of the liver equivalents within the chosen 3D architecture (Figure 6) with hepatocyte activity both for drug metabolism (CYP 3A4) and bile acid synthesis from cholesterol (CYP7A1). In contrast to well-known fast *ex vivo* degradation of skin biopsies in static tissue cultures, immunohistostaining of the 28-day foreskin culture suggests a functional survival of the epidermis, the maintenance of an intact epidermis-dermis interface with a basal lamina and the preservation of different intradermal structures, such as vessel walls, in the dynamic MOC cultures (Figure 6).

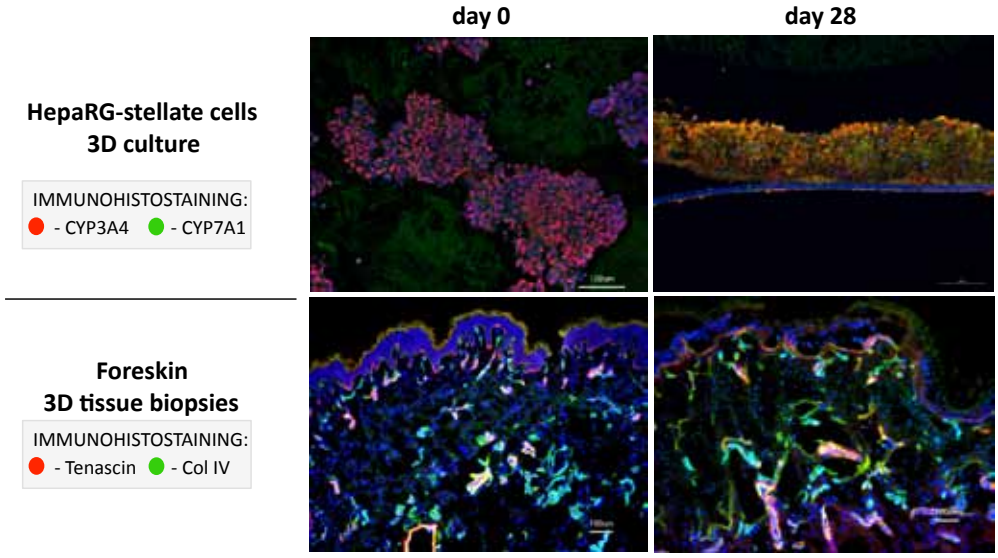

Figure 6. Results of a preliminary immunohistochemistry screen of liver equivalents and foreskin biopsies before and after the 28-day MOC culture.

Developing an MOC-Based Biological Vasculature

In order to further advance the MOC technology toward human multi-organ models which fully emulate human biology, the integration of a biological vasculature seems to be the most important step reflecting Mother Nature. Over the last ten years a great deal of experience with a human vascularisation of biological scaffolds has been established by Heike Walles et al. at the Fraunhofer Institute in Stuttgart, Germany. The researchers developed the so-called BioVaSc®, a decellularised biological vascularised scaffold (Mertching et al., 2005; Schultheiss et al., 2005; Linke et al., 2007). By integrating such scaffolds into a specially optimised bioreactor (Mertsching & Hansmann, 2009) with dynamic media circulation, it was possible to fully repopulate the previous vessel system with human endothelial cells establishing fully contained/closed micro-vasculature. It has been shown that such a bioreactor-based BioVaSc® is a perfect background for the self-assembly and long-term maintenance of different human organ equivalents, such as liver, intestine, trachea, and skin (Schanz et al., 2010).

Furthermore, the researchers at the FhIGB could demonstrate in a recent study that such a biological background ensures the indefinite homeostasis and complete metabolic capacity of cultured human liver tissues. A constant, high level of albumin secretion could be detected in the repeatedly exchanged media after an outgrowth and tissue assembly period of about seven days (Figure 7a). Liver specific metabolic competence was demonstrated by the repeated challenge of the human

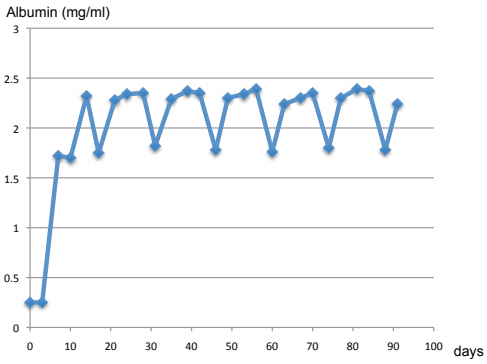

Figure 7a. Albumin synthesis in the BioVaSc-based human liver cultures.

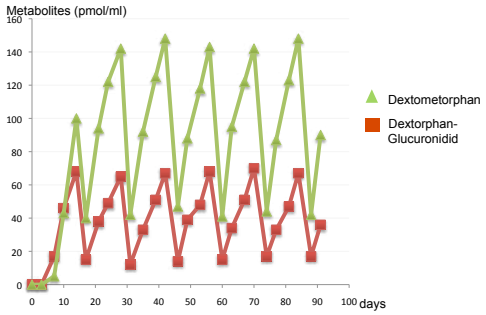

Figure 7b. Stable metabolism of small molecule drugs.

liver equivalents with dextometorphan, a cough suppressant, which is metabolized *in vivo* by the human liver into the phase II metabolite dextorphan-glucuronidid. Again, this metabolic activity is stable over the entire experiment (Figure 7 b).

Over the last three years, the teams of the FhIGB and the TU Berlin have joined forces to adopt the approaches to the significantly miniaturised MOC technology described above. Full and complete coverage of all micro-fluidic channels of the MOCs with human primary endothelial could be achieved by special surface modifications. Figure 8 demonstrates a representative section of such a tightly closed human endothelial vessel wall layer of the micro-capillary network on the chips continuously and functionally maintained over 40 days.

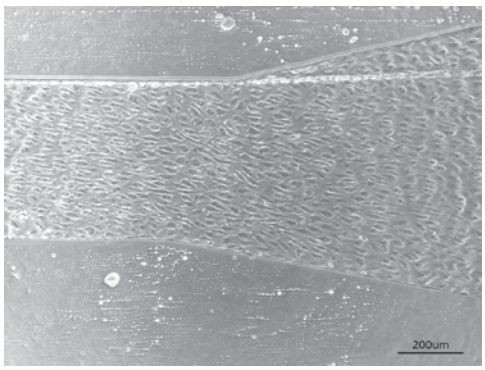

Figure 8: Light-microscopy image of a representative segment of the micro-fluidic channel of a MOC after 40 days of dynamic culture.

Challenges & Next Steps

The combination of the fully vascularised micro-fluidic channel system of the MOC with a capacity of neo-vascularisation of the tissue culture spaces is one of the remaining challenges to translate human long-term organ homeostasis from larger size bioreactors into the MOC format. Furthermore, the experimental throughput (number of simultaneously operated chips) and degree of process automation are still technical hurdles for transfer into industrial scale MOC operation. Therefore, organ-specific matrices and an automated robotic platform for large-scale chip-operation are under investigation.

In parallel, the teams are working on the integration of intestine, kidney and hematopoietic organ equivalents into the chips. A major programme compound is the technical realisation of any relevant exposure regimen with a highly flexible software background for easy data mining and processing.

The recently announced coordinated NIH/NCATS initiative to develop translational 'human-on-a-chip' platforms backed by the FDA (*science20.com/news_articles/biomimetics_human_body_chip-92403*) has identified attractive research groups targeting chip-based disease model development within the US. This is an attractive opportunity to create research alliances with partners within that portfolio based on our advanced MOC technology.

Summary

It could be demonstrated that a 28-day 3D multi-tissue culture is feasible. We were able to cultivate an artificial liver equivalent together with a human foreskin biopsy in one perfused MOC over 28 days. The experiments underlined that both cell lines and primary tissues can be used in the MOC platform. In addition, long-term circulation of human blood components, such as plasma, erythrocytes and leukocytes, has been successfully tested for later implementation into tissue cultures. Thus, the MOC format supports the investigation into different non-standard human repeated dose toxicity test designs.

References

Bhatia SN, Balis UJ, Yarmush ML, et al. Effect of cell-cell interactions in preservation of cellular phenotype: cocultivation of hepatocytes and nonparenchymal cells. FASEB 13, 1183-1900 (1999).

Basketter DA, Clewell H, Kimber I, et al. A roadmap for the development of alternative (non-animal) methods for systemic toxicity testing – t4 report. ALTEX 29, 3-91(2012).

Huh D, Hamilton GA & Ingber DE. From 3D cell culture to organs-on-chips. Trends in Cell Biology 21, 745-754 (2011).

Huh D, Torisawa Y, Hamilton GA, et al. Microengineered physiological biomimicry: Organs-on-Chips. The Royal Society of Chemistry. London, UK (2012).

Esch MB, King TL, Shuler ML. The Role of Body-on-a-Chip Devices in Drug and Toxicity Studies. Ann. Rev. Biomed. Eng. 13, 55-72 (2011).

Jossé R, Aninat C, Glaise D, et al. Long-term functional stability of human HepaRG hepatocytes and use for chronic toxicity and genotoxicity studies. Drug Metabol & Dispos. 36, 1111-1118 (2008).

Mertsching H & Hansmann J. Bioreactor technology in cardiovascular tissue engineering. Adv. Biochem. Eng. Biotechnol. 112, 9-37 (2009).

Mertsching H, Walles T, Hofmann M, et al. Engineering of a vascularized scaffold for artificial tissue and organ generation. Biomaterials 26, 6610-6617 (2005).

Linke K, Schanz J, Hansmann J, et al. Engineered liver-like tissue on a capillarized matrix for applied research. Tissue Eng. 3, 2699-707(2007).

Schanz J, Pusch J, Hansmann J, et al. Vascularised human tissue models: A new approach for the refinement of biomedical research. J. Biotechnol. 148, 56-63 (2010).

Schultheiss D, Gabouev AI, Cebotari S, et al. Biological vascularized matrix for bladder tissue engineering: matrix preparation, reseeding technique and short-term implantation in a porcine model. J. Urol. 173, 276-80 (2005).

Publications

Gernaey KV, Baganz F, Franco-Lara E, et al. Monitoring and control of microbioreactors for applications in biotechnology: A review. Biotechnology Journal *(submitted)* (2012).

Marx U. Trends in Cell Culture Technology. *In* Balls M, Combes RD, Bhogal N (Eds.) New Technologies for Toxicity Testing. Landes Bioscience and Springer Science+Business Media, pp. 26-46 (2012).

Pilarek M, Neubauer P & Marx U. Biological cardio-micro-pumps for microbioreactors and analytical micro-systems. Sensors and Actuators B. 156, 517-526 (2011).

Sonntag F, Gruchow M, Wagner I, et al. Miniaturisierte humane organtypische Zell- und Gewebekulturen. BioSpektrum 17, 418-421 (2011).

Sonntag F, Schilling N, Mader K, et al. Design and prototyping of a chip-based multi-micro-organoid culture system for substance testing, predictive to human (substance) exposure. *J. Biotech.* 148, 70-75 (2010).

Marx U. How drug development of the 21st Century could benefit from human micro-organoid in-vitro technologies. *In:* Marx U, Sandig V (Eds.), Drug Testing in vitro: Breakthroughs and Trends in Cell Culture Technology, Wiley VCH, pp 271-282 (2007).

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