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"Organoid-in-a-column" coupled on-line with liquid chromatography-mass spectrometry

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Abstract

Organoids are stem-cell derived "mini organs" and are emerging tools for e.g. developmental biology and drug development. We have packed empty liquid chromatography column housings (micro format) with induced pluripotent stem cell (iPSC) derived hepatic organoids, and coupled these "organoid-in-a-column" units directly to liquid chromatography-mass spectrometry. The system has been charged with heroin, followed by on-line monitoring of the drug´s phase 1 metabolism. Using a C4 stationary phase for reverse phase liquid chromatography, small molecule measurements were not significantly affected by liver organoid medium matrix (consisting of e.g. fetal bovine serum). Enzymatic metabolism of heroin was reliably detected using a triple quadrupole instrument over a period of two days, demonstrating increased metabolite production over time and serving as a proof-of-concept for on-line coupling of organoids and mass spectrometry.

Keywords: Organoid; Induced pluripotent stem cells; On-line; Liquid chromatography; Mass Spectrometry; Metabolism

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Introduction

Drug discovery and development is an extremely costly process, and the number of new drugs reaching market per billion dollars spent on drug development is steadily declining [1]. Moreover, many new drugs can be ineffective (or even toxic) to a large portion of target patients [2]. Key bottlenecks of efficient drug development include the limited predictive value of traditional cell cultures and animal models regarding human trials [3]. Furthermore, reliable technologies for predicting personalized drug metabolism, a key issue in toxicology, are lacking.

Organoids are emerging tools for drug discovery and development. These miniature 3D models of actual organs can be grown from human stem cells, and therefore carry potential for being relevant and personalized testing materials [4].

Typically, organoids feature multiple cell types specific of the organ they mimic, and have biological properties and morphologies that (to varying degrees) resembling the model organ [5]. Current liver organoids contain parenchymal cells and endothelial cells in a rather unorganized special arrangement. In iPS derived liver organoids, the parenchymal cells (hepatocytes) elicit an immature fetal phenotype [6]. Nevertheless, within these limitations, liver organoids can be tools for assessing aspects of drug metabolism, in particular phase 1 metabolism, and toxicity.

To analyze the metabolic capability of organoids, LC-MS is the method of choice. Mass spectrometry (MS) analysis of organoids is today commonly performed indirectly, or "off-line", i.e. samples are collected and handled semi-manually prior to MS [7-9]. This process is time consuming and prone to variations based on sample handling. Direct on-line analysis of organoids would offer the advantage of increased automation and precision and hence reduced sample variation [10].

We here describe the first on-line system for detecting organoid-derived metabolites of drugs using MS, also featuring liquid chromatographic separation. The system is related to that described by van Midwoud and co-workers [11], who performed on-line analysis of drug metabolism of liver slices, using custom-built microfluidic chips and UV detection. However, we have simplified the work flow, created an incubation device without the need for

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specialized parts and coupled the system directly to a MS. Consisting of simple parts and standard commercial instrumentation, the here presented system is a scalable on-line MS analysis platform for monitoring drug metabolism in liver organoids upon drug challenges.

Experimental

Consumables and hardware

Stainless steel (SS) unions, reducing unions (1/16" to 1/32"), SS ferrules and nuts (all for 1/16" tubing and for 1/32" tubing), SS tubing (1/32" outer diameter (OD), 0.020" inner diameter (ID) and 0.005" ID) and 1/16" SS screens (1 µm pores) were purchased from VICI Valco (Schenkon, Switzerland).

SST Vipers (130 µm x 650 mm) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Polytetrafluoroethylene (PTFE, Teflon) tubing (1/16" OD, 1/32" ID) was purchased from Darwin microfluidics (Paris, France). A chromatographic column (1 mm x 5 cm) packed with Kromasil C4 (3.5 µm, 100 Å) was purchased from Teknolab (Ski, Norway). Luer lock syringes (10-20 mL) were purchased from B. Braun Melsungen AG (Hessen, Germany).

Reagents and solutions

Formic acid (≥ 98%) was purchased from Merck (Darmstadt, Germany). Water (LC-MS grade) and acetonitrile (ACN, LC-MS grade) were purchased from VWR International (Oslo, Norway).

For liquid chromatography (LC) Mobile phase (MP) reservoir A contained 0.1% formic acid in HPLC water (v/v). MP reservoir B contained ACN/HPLC water/FA (90/10/0.1%, v/v/v).

PhD Inger Lise Bogen (Oslo University Hospital) supplied solutions of heroin, 6-MAM, morphine. Fetal bovine serum-free medium and L15 base medium was prepared according to [12], and is hereafter referred to as organoid medium.

Organoids

iPSC line AG27 was differentiated to form liver organoids as described by Harrison et al. (manuscript in preparation). iPSC cell lines HPSI1213i-babk_2 and HPSI0114i vabj_3 were

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differentiated toward liver organoids using a modification of the protocol by Ang et al. [13], by Aizenshtadt et al. (manuscript in preparation).

Instruments

The Dionex UltiMate 3000 UHPLC system and the TSQ Vantage MS with the HESI-II ion source were purchased from Thermo Fisher Scientific. The syringe pump (AL-1000) was bought from World Precision Instruments (Sarasota, FL, USA). A 2-position 10-port valve (for 1/32", C82X-6670ED) was purchased from VICI Valco. A SUB Aqua 5 Plus water bath was purchased from Grant Instruments (Cambridge, UK).

Organoid-in-a-column

For fabrication of the column housing, an approximately 5 cm long piece of Teflon/PFTE tubing (1/16" OD, 1/32" ID) was cut and assembled with nuts and SS ferrules. To one end of the tube, a union with a 1 µm screen (VICI Valco) was connected.

A volume of 1-22 mL of medium containing organoids was then filled into a Luer-lock syringe, and the syringe was subsequently connected to the open end of the column. With the syringe mounted on a syringe pump, the medium was pumped through the column housing at a flow rate of 100 µL/min. Once the entire contents of the syringe was passed through the column, the inlet was fitted with a screen and a union. A schematic of an organ-in-a-column is shown in **Figure 1**.

For idle conditions, a new syringe was filled with fresh organoid medium and connected to the organ-in-a-column, so that the flow was the opposite way as during filling. The pump was set to 0.20 µL/min. After filling, the organoid column was submerged in a water bath at 37 °C.

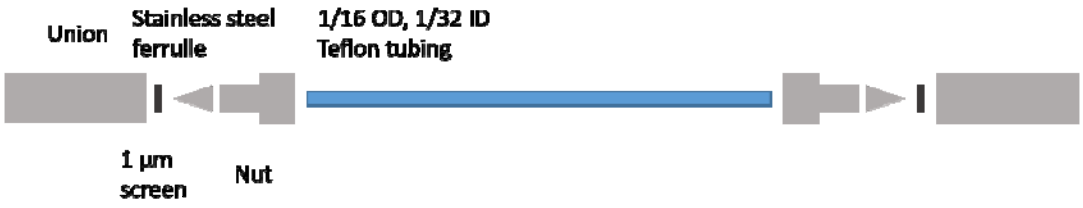

Figure 1. Column housing for "organoid-in-a-column".

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Organoid-in-a-column coupled with liquid chromatography-mass spectrometry

The pump/syringe/organoid-in-a-column system described above was connected to LC-MS instrumentation. Eluate from the column was transported to a valve system for fractionation. The valve system contained two loops, which were filled sequentially. As one loop was being filled, the contents of the other loop were transferred to a 5 cm x 1 mm C4 LC column for separation prior to MS detection. The 20 µL loops were overfilled with an additional 5 µL to ensure that any LC solvent left in the loop would be flushed off. See **Figure 2** for a schematic of the setup. For studying heroin metabolism, 100 µM solutions in medium were pumped at 0.20 µL/min, with fractionation each 120th minute. See **Table 1-3** for LC-MS conditions.

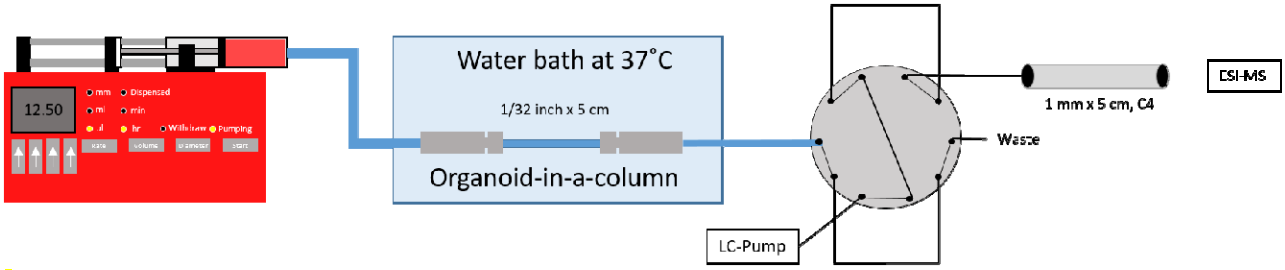

Figure 2. Illustration of "organoid-in-a-column" coupled on-line with liquid chromatography-mass spectrometry. See Experimental section for more details.

Table 1. LC-gradient for on-line studies of "organoid-in-a-column" metabolism of heroin.

Time, min	Flow, µL/min	%B	Purpose
0	50	3	Separation
20	50	20	
20	50	80	Wash
60	50	80	
60	50	3	Re-equilibration
70	50	3	
70	0	3	Save MP
110	0	3	
110	50	3	Re-equilibration
120	50	3	

Table 2. Multiple reaction monitoring (MRM) parameters used for the detection of heroin and its phase 1 metabolites.

Molecule	Parent ion, *m/z*	Product ion, *m/z*	Collision energy, eV
Heroin	370	272	30
6-MAM	328	211	25
Morphine	286	201	20

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Table 3. General MS parameters for detection of heroin, 6-MAM and morphine

Parameter	Value
Capillary temperature	300.0 ℃
Vaporizer temperature	200.0 ℃
Sheath gas pressure	20.0
Ion Sweep gas pressure	0.0
Auxillary air flow	5.0
Spray voltage	Pos: 3000.0 V Neg: 0.0 V
Collision gas pressure	1.0 mTorr

Live/Dead staining

Six days after the final drug metabolism experiments, the organoid-in-a-column was flushed with phosphate-buffered saline (PBS) pH 7.4 to remove the medium. The column was dismantled and the content was collected. Organoids that were stuck to the filters were scraped off and transferred to a glass slide and excess PBS was removed. The organoids were then stained with Live/Dead Cell Double Staining Kit suitable for fluorescence from Sigma Aldrich in the following manner: 100 µL of the staining solution (5 mL PBS, 10 µL solution A and 5 µL solution B) were pipetted onto the organoids. A cover slide was placed on top and the organoids were then incubated at 37 ^{o}C for 15 minutes prior to microscopy.

Results and Discussion

Organoids were packed in liquid chromatography column housings. A micro LC format housing was chosen (1/32" ID and 5 cm length, see **Figure 1**). In separation science, such sizes, belonging to the category "miniaturized LC", are associated with low radial dilution and suited for small sample analysis [14]. Medium containing organoids was syringe-pumped into an open LC column housing at flow rates at lower/mid µL/min ranges (depending on organoid sizes). After the on-line measurements, organoids were flushed from the column using the same instrumentation; Pre-loading and post-flushing inspection of the organoids by microscopy revealed tissue intactness.

The low pressure "organoid-in-a-column" was coupled with high-pressure LC through a fractionation valve set-up. LC-MS is generally operated at high pressures e.g. 50-400 bars, which is incompatible for organoids. A SS-valve was used to collect and send fractions to the

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LC-MS system, not unlike that used for two-dimensional LC separations [15]. The valve system set-up also isolated the organoids from the high-pressure analysis system (see **Figure 2**).

A C4 stationary phase for liquid chromatography was suited for metabolite and medium analysis. Organoid medium is highly complex and contains considerable amounts of e.g. albumin. Sample complexity and presence of proteins can cause unpredictable chromatographic performance. A C4 stationary phase (considered relatively compatible with proteins) allowed for repeatable chromatography of medium spiked with drugs and metabolites (see **Figure 3**). In addition, we speculate that the rather low pore size of the particles employed (100 Å) aided in separating macromolecules from small drugs via size exclusion. It should however be mentioned that the ion source of the mass spectrometer would become contaminated with the complex mixture, calling for extra attention to maintenance and/or additional source protections. Serum-free medium could be used to avoid the uncontrollable composition of fetal bovine serum (FBS), but will contain high amounts of proteins from supplements and other necessary medium components. Hence, proteins are an inevitable part of medium and need to be dealt with accordingly.

The mobile phase composition was also a key parameter regarding robustness, as e.g. methanol as an organic modifier was associated with column clogging and poor performance when chromatographing the organoid medium.

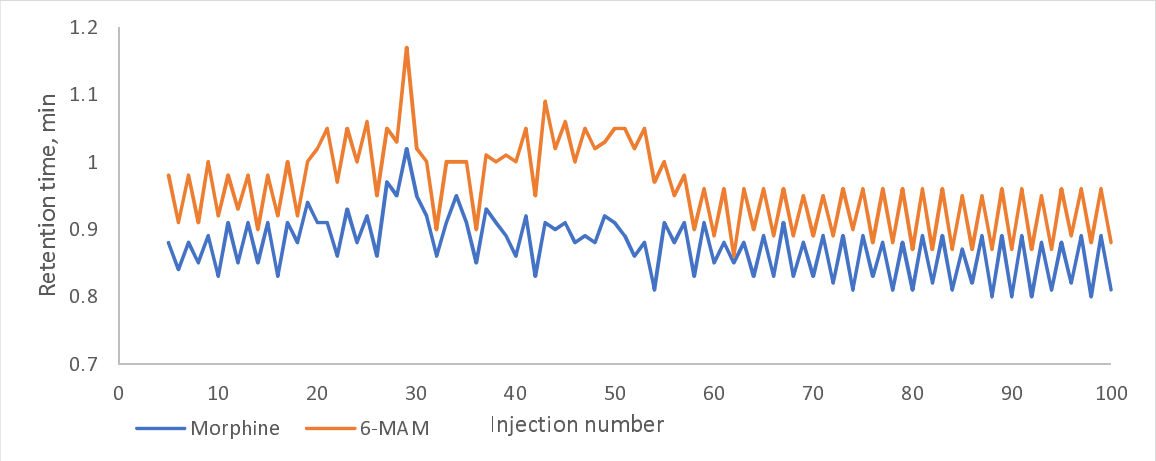

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Figure 3. Retention time variability of heroin metabolites using "organoid-in-a-column" coupled on-line with liquid chromatography-mass spectrometry (100 injections). Zig-zag pattern due to a bridge tubing, creating slightly longer distance from one sample loop to the MS, thereby extending the retention time.

The on-line system was suited for observing enzyme-based drug metabolism of organoids. Heroin was chosen as a model compound as it is a well-studied drug with a simple and well-understood metabolism [16-18]. It was believed that this would simplify the qualitative measurement of its metabolites, as established methods are available. In initial experiments, organoid medium + 1 µM heroin was applied to the organoid-in-a-column at a flow rate of 1 µL/min. Heroin and 6-MAM were detected, but the latter was likely detected due to spontaneous decomposition of heroin to 6-MAM [17]. For evaluating enzymatic drug metabolism of the "organoid-in-a-column", a lower flow rate of 12.5 µL/h was used with a heroin concentration of 100 µM. A total time of 120 minutes per injection was used. Ample signals were obtained for heroin and metabolites 6-MAM and morphine for each fraction analyzed by LC-MS. The final extracted chromatogram for morphine is shown in **Figure 4**. For 6-MAM and morphine, the peak height and area increased substantially over the course of 22 injections (**Figure 5**). The concentration of 6-MAM was expected to increase until all heroin was consumed, as it was added to the medium and stored at room temperature during the experiment. This point was reached after eight injections (corresponding to 16 hours). The concentration of morphine, which only is formed through enzymatic activity, increased beyond this point, pointing towards an upregulation either of the metabolizing enzymes or the transportation of drugs and metabolites in and out of the cells or both.

Expectedly, the kinetics were slower than that observed with e.g. liver microsomes and S9 fraction experiments. The retention time of the analytes remained stable throughout the experiment, see **Figure 6**.

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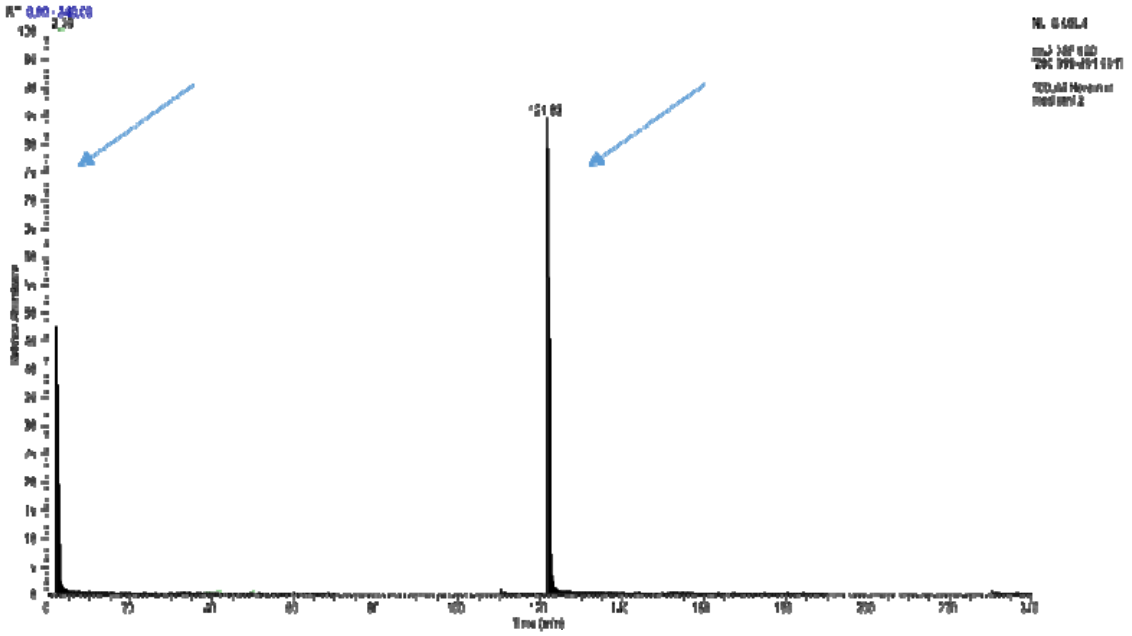

Figure 4. LC-MS chromatogram of the two morphine peaks (*m/z* 286 → *m/z* 201) derived from organoid-in-a-column exposed to heroin. Injections were made at t=0 and t=120 min.

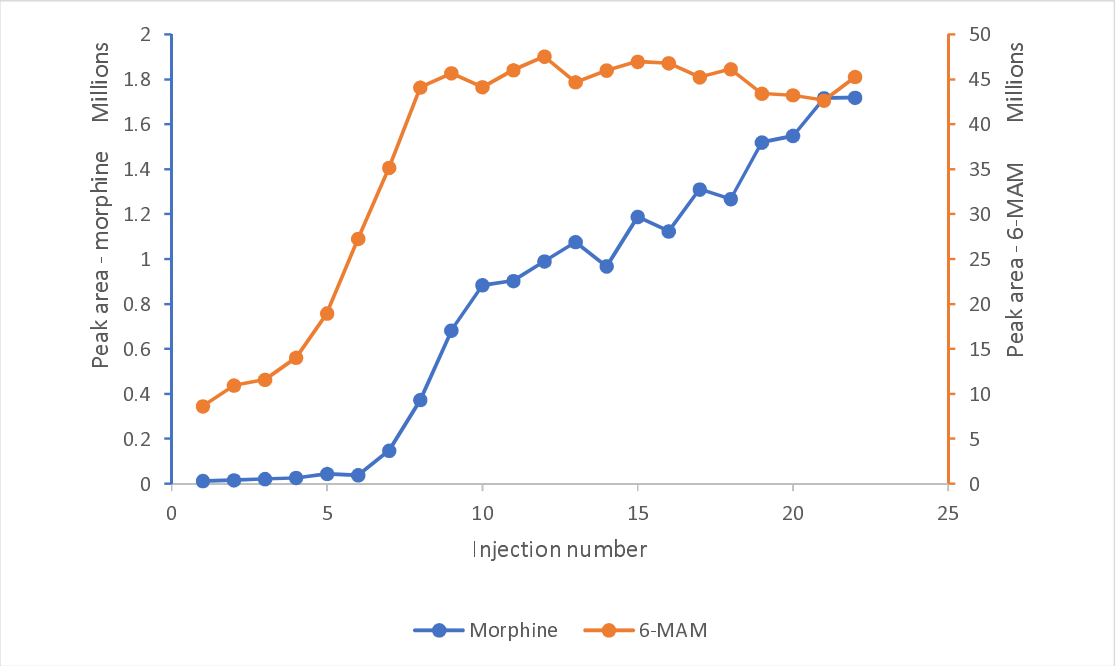

Figure 5. Peak area as a plot of injection number. Peak area of morphine and 6-MAM was increasing substantially throughout 22 injections. Morphine concentration increased even after 6-MAM reached a plateau.

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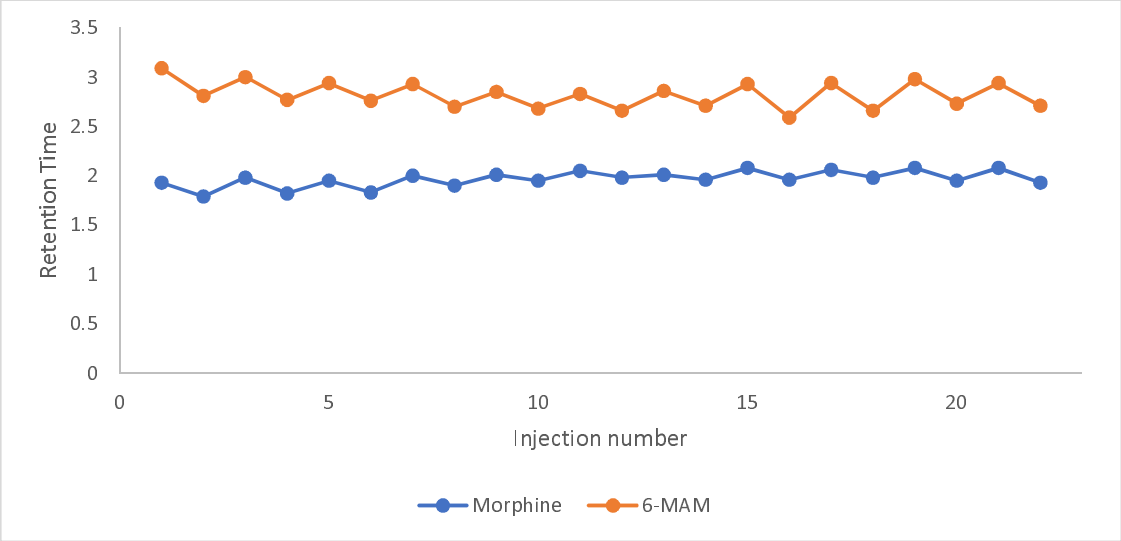

Figure 6. Plot of retention times for morphine and 6-MAM from the 22 injections made. Retention time remained stable- Zig-zag pattern due to slightly longer distance to MS from one sample loop.

Organoids were able to survive the conditions of the experiment. After the on-line measurements of heroin metabolism, the organoid-in-a-column was left under perfusion of medium for six days. Then, the organoid column was taken apart and its content collected for staining. A live/dead staining kit was used to stain live and dead cells (calcein acetoxymethyl/propidium iodide). **Figure 7** demonstrates that organoids could survive the fairly harsh settings applied, indicating that the developed system may work for long-term cultivation under appropriate conditions.

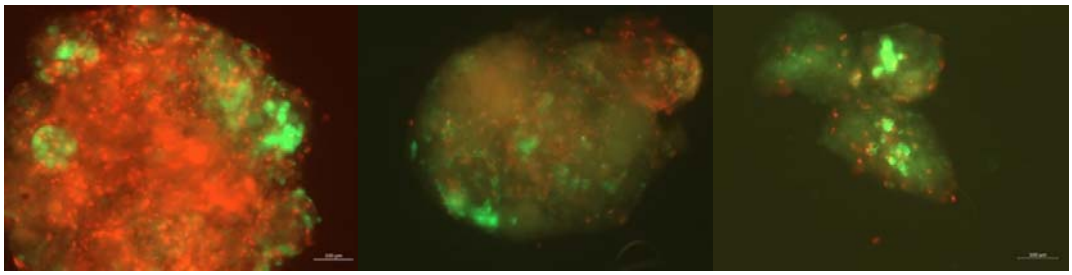

Figure 7. Live/dead staining of some organoids after organoid-on-a-columns experiments. Organoids stained could survive (middle, right) even after exposure to high concentrations of heroin and left under perfusion of medium without oxygenation for more than 7 days. However, not all organoids survived (left). Scale bar is 100 µm.

A set-up for measuring organoid metabolism shows promise through a quite simple concept, namely "packing" organoids in a LC column housing, which is readily adaptable for on-line

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analysis. Initially, we probed the use of commercial organ-on-a-chip housings (e.g. ones with PDMS-based materials), but with little success. During the time of this work, most commercial housings have been designed for e.g. confocal microscopy experiments, with little attention to coupling options, fragility, and potential leakages that can occur in microfluidic coupling. We also experimented with in-house developed chip designs, which however, had issues related to poor retention of the organoids. LC column housings, on the other hand, are specifically designed for avoiding leakages and simple "packing", and come in a variety of diameters and lengths, with readily available fittings.

The "organoid-in-a-column" variant allowed for automated LC-MS action, with a proof-of-principle shown with heroin metabolism. We will expand the technology to systematically test phase 1 metabolic pathways, eg. of CYP-activity tell-tale molecules, e.g. diclofenac and imidazole.

We are exploring the addition of various on-line sample clean up steps, e.g. coupling with electro-membrane extraction (EME) [19, 20] or solid phase extraction (SPE) [10]. This may allow for cleaner extracts to enter the LC-MS system. We believe this will be a necessity for an upscaled version or when performing thousands of injections on a system. We therefore view the organoid-in-a-column-LC-MS system as an early stage concept and invite our colleagues in the field to investigate the coupling of mass spectrometry and organoids.

To assess the crosstalk between organs, we are currently investigating organoid-in-a-column coupling of two different tissue types in series, i.e. as a two-column system of for example islets of langehans and liver.

As the size and with it the number of cells can vary greatly between organoids, proper standardization of loading is difficult to achieve. While larger organoids should give a theoretical higher metabolizing ability, cells in smaller organoids, to a larger degree, will be directly exposed to the drug. Which approach is favored, remains to be tested.

As it was difficult to standardize the numbers of organoids in each column, some were loaded with too many organoids. This caused the loaded organoids to form a plug that eventually clogged the system. Therefore, future systems will contain support material, which will aid in the distribution of single organoids throughout the column. Such support

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material could be beads or porous "sponges" of glass, silica, matrigel, alginate, PDMS or other materials and may be engineered with additives to work as sensors for e.g. pO_2, pCO_2, temperature, pH, or even selective sensing of compounds.

Organoid-in-a-column, is a simple approach for testing drug metabolism without the need for highly specialized parts. The technology should therefore be readily available to anyone in need of an *in vitro* system for the metabolism studies of tissue. The addition of an on-line sample clean-up step should make the technology scalable for high throughput applications such as large-scale drug testing.

Acknowledgements

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