Biofabrication of Perfusable Liver Constructs

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Utrecht University
Faculty of Veterinary Medicine

Monique Schuddeboom
Honours Programme Student
Faculty of Veterinary Medicine
Student nr.: 3551393

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Content

Summary

In this study, we aimed for the development of a bioprinted *in vitro* model of the human liver for the prediction of drug induced liver injury. The liver is the main site of drug metabolism, which makes it vulnerable to cytotoxic effects of pharmaceuticals and their metabolites. Liver injury caused by drugs gives rise to around 50% of all acute liver failure cases. DILI still occurs in clinical phases of drug development or even post-marketing, which makes it a serious health concern.

Several *in vitro* systems exist for the prediction of DILI, but none of them allow for an accurate prediction. Even animal models fail, because of the great variability in drug metabolism even amongst closely related species such as mammals.

Our approach is to develop a biofabricated 3D liver construct that can be cultured in a custom designed flow perfusion chamber. Human cells are employed, to increase the resemblance with the *in vivo* liver. Instead of culturing only cells in a 2D fashion, we bioprinted a 3D construct using a collagen-derived hydrogel, which allows for a better resemblance of the complex 3D structure *in vivo*. Bioprinting is already being explored as a technique for mimicking several other tissues, like bone and cartilage. However, a functional bioprinted liver module has not been established thus far.

We were able to develop a method for bioprinting a cell-laden 3D liver construct. As a source of hepatocytes, small organ buds consisting of Hepatic Progenitor Cells, termed liver organoids, or a hepatic cell line (Huh7) was used. To create flow, perfusion chambers were custom designed and produced by stereolithography technology. Liver cells were printed in 5% w/v gelMA hydrogel and subsequently cross-linked by UV-A irradiation. The simultaneous deposition of pluronic F127 as a sacrificial support material, allowed the formation of a porous structure, enabling media to flow through. After printing, liver constructs were followed over time with cell viability assays (Alamar Blue and cell count). As a proof of principle for using our construct as a DILI prediction model, constructs were treated with a toxic compound, after which cell damage was measured with an ATP assay.

Cells in printed gelMA constructs remained viable for at least four days after 3D printing. Acute toxicity of the liver constructs was measurable as a significant increase in ATP levels after Triton X-100 treatment, at 2-3 days post printing. Placement of the porous liver construct in the custom designed bioreactor allowed the perfusion of the construct.

These results indicate that perusable liver bioreactor systems have the potential to better predict DILI compared with the current models.

Introduction

Anatomy of the liver

The liver is the largest gland in the body. Its anatomic location is in the upper right quadrant of the abdomen, directly beneath the diaphragm [1–3] (Figure 1).

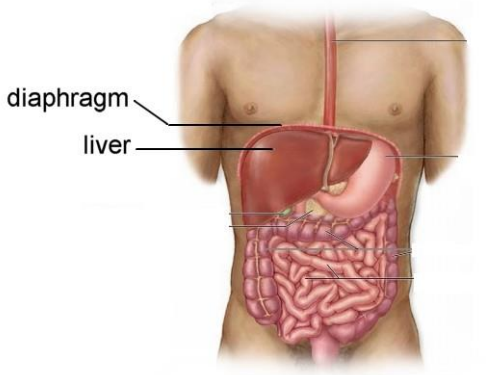


Figure 1: Location of the liver[3].

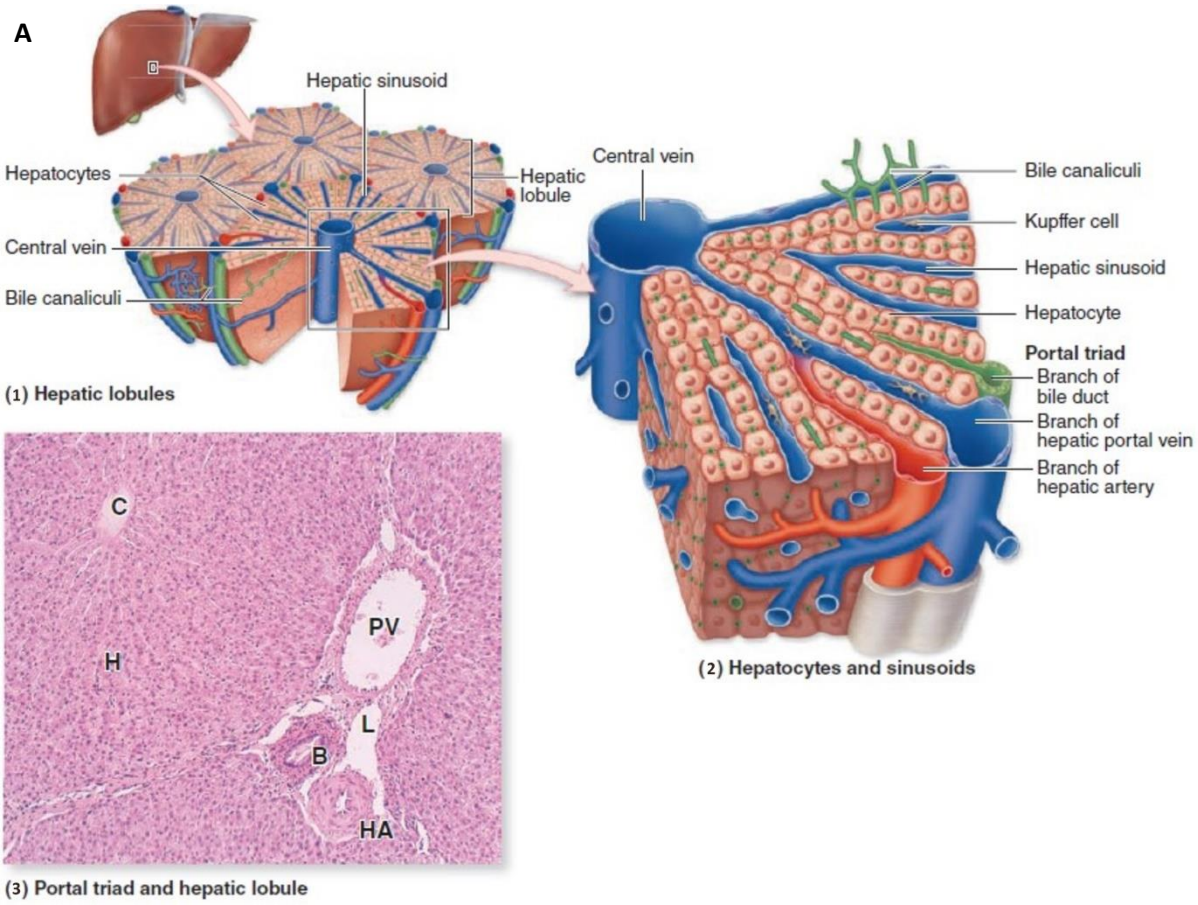
The liver has a dual blood supply, both from the hepatic artery and the portal vein. Although the hepatic artery provides about three fifths of the oxygen, it delivers only one fifth of the volume. The other four fifth is supplied by the portal vein, draining the largest part of the gastro-intestinal tract. At the porta hepatis, the hepatic artery enters the liver together with the portal vein and hepatic duct, after which they successively divide together[2].

The liver is enclosed by the serosa. Just beneath the serosa lays the tunica fibrosa, which encloses the liver parenchyma. At the porta, the tunica fibrosa enters the liver and leads the branches of the vessels inward. It divides together with the vessels, becoming thinner with every division. The thinnest divisions, the trabeculae, divide the liver into countlessly small hexagonal-shaped units, the classical hepatic lobules (Figure 2A) [2].

Hepatic lobule

The classical hepatic lobule consists of a central vein in the middle and a portal triad at three corners (Figure 2A). The portal triads contain tiny branches of the hepatic artery, portal vein, bile duct, and a lymph capillary. Blood flows from the portal triads to the central vein through small capillaries, so called sinusoids. They are located between rows of hepatocytes, the hepatic laminae. Often, there are interruptions within the laminae, creating a connected network of sinusoids. The endothelial lining of the sinusoids is separated from the hepatocyte surface by a perisinusoidal space, the space of Disse (Figure 2B). The endothelium is porous because of small pores in the cells and an interrupted basement membrane, resulting in a direct exchange of substances between blood and hepatocytes. The pores are too small for the passage of blood cells, but large molecules can easily cross. Highly phagocytic stellate macrophages (Kupffer cells) reside within the perisinusoidal space to take up foreign particles. Once arrived at the central vein, the blood flows through converging vessels into the final hepatic veins leaving the liver. They end in the caudal vena cava. The sinusoids, with their discontinuous epithelium, cause all substances taken up by the gastro-intestinal tract to first come in close contact with the liver cells before entering the actual circulation[1,2].

Small bile canaliculi are present between hepatocytes (Figure 2B). The bile pigment comes from bilirubin, which is absorbed by hepatocytes and conjugated. Together with bile salts, protein, and cholesterol it is secreted to the bile canaliculi. The canaliculi form an anastomosing network, ending in the hepatic duct branches of the portal triads.



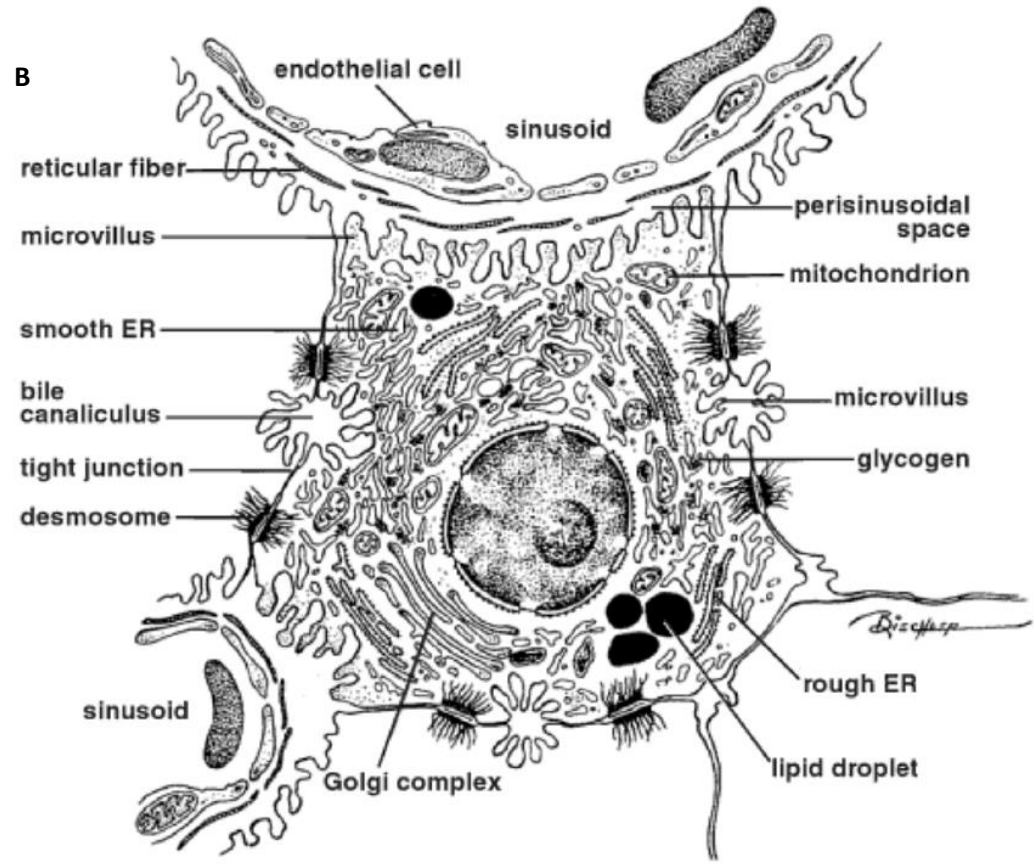


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Figure 2: Microanatomy of the liver.
(A) Liver anatomy at three levels[3]. (1) Hexagonal shaped hepatic lobules showing the central vein in the middle of a lobule. (2) Part of the hepatic lobule, enlarged, showing the portal triad and sinusoids. Blood from two sources (hepatic artery and portal vein) flows through the sinusoids to the central vein. (3) Microscopic image of the liver, showing the central vein (C), the hepatic laminae (H), and a portal triad containing mall branches of the portal vein (PV), hepatic artery (HA) and bile duct (B). Hematoxylin & eosin stain.
(B) Hepatocyte with neighboring structures, drawn from an electron microscopic image[1]. Three different surface types can be distinguished: (1) the apical membrane, which has microvilli projecting into the perisinusoidal space; (2) contact surfaces with other hepatocytes and (3) surfaces bordering the bile canaliculi. In this case, the hepatocyte has close contact with two sinusoids. Note the discontinuous sinusoidal epithelium. The cell is attached to other hepatocytes with tight junctions (not shown) and desmosomes.

These converge into hepatic ducts, entering the liver at the porta. Unlike the accompanying branches of the hepatic artery and portal vein, the flow is directed outward, leading the bile to the common hepatic duct. Bile flows into the gallbladder through a branch, the cystic duct. The common hepatic duct is called the bile duct or ductus choledochus distal to the origin of this branch. Under the influence of food, bile from the gallbladder is transported to the gut via the bile duct[2].

Functions of the liver

The liver has numerous functions. Although bile production may be the most apparent one, the metabolic functions are far more important[2]. Next to protein, carbohydrate and lipid metabolism, the liver metabolizes drugs and hemoglobin. In addition, it synthesizes fibrinogen, globulins, albumin and clotting factors, it stores lipids, vitamin A and B, and glycogen, and it phagocytes foreign particles[1]. Here, we will focus on the metabolism of pharmaceuticals.

The main goal of drug metabolism in the liver is excretion. In order to be excreted, hydrophilicity is needed. Most hydrophilic substances are directly excreted with the urine. However, most pharmaceuticals are lipophilic, so they must be transformed into hydrophilic metabolites by de liver or kidneys before they can be excreted. This process of chemically transforming either exogenous or endogenous substances is called biotransformation[4].

The liver is the main site of biotransformation, although this also occurs at other locations like the kidneys and the gastro-intestinal tract. Biotransformation is dependent on species, age, nutrition and co-exposition (inhibition or induction, competition), making it hard to predict the outcome[4].
The nature of the biotransformation reactions depends on certain structural elements of the substrate. Depending on these elements, a specific enzyme will recognize the substrate and convert it (substrate specificity). The variety of reactions that can occur are grouped into Phase I and Phase II reactions, based on the nature of the chemical transformation. Reactions of both phases can occur subsequently or simultaneously. It is also possible that only one of the two phases occurs. The product is usually less biologically active and more hydrophilic. However, in some cases (especially with Phase I biotransformation) the products are more active or even toxic, a process called bioactivation[4,5].

Phase I reactions generally add a small functional group or unmask an existing functional group, e.g. -OH, $-NH_2$ or -SH[6]. In addition to making the compound more hydrophilic, the functional group can be recognized by a Phase II enzyme. The most important enzyme to catalyze Phase I reactions is the Cytochrome P-450 (CYP450) family. Enzymes from this family mainly execute oxidation reactions, but reduction reactions are also possible. The main Phase

l reactions and enzymes are listed in Table 1[4].

Phase II biotransformation encompasses a variety of conjugation reactions, causing a decrease in activity and an increase in hydrophilicity, which facilitates excretion. The conjugating enzymes attach a body's own compound to the pharmaceutical or metabolite. The enzymes are specific for the conjugating compound (Table 2). Excretion can occur either through the kidney or with the bile. In case of large molecules, including the largest part of Phase II biotransformation products, the excretion route is mainly via the bile.

Table 1: Phase I enzymes per reaction, including main liver isoforms and cofactor requirements[7].

Reaction	Enzyme	Main Liver isoforms	Cofactor requirement
Oxidation	Cytochrome P450 enzyme family (CYP450)	CYP3A4, CYP2D6, CYP2C, CYP1A2, CYP2E1	O_2, NADPH
	Flavin-containing monooxygenase (FMO)	FMO3, FMO4, FMO5	O_2, NADPH
	Peroxidase		
	Monoamine oxidase	MAO-A, MAO-B	O_2, H_2O
	Alcohol dehydrogenase	ADH1A, ADH1B, ADH1C	NAD+
	Aldehyde dehydrogenase	ALDH1, ALDH2	NAD(P) +
	Aldehyde oxidase	AO	O_2, H_2O
	Xanthine oxidase	XO	O_2, H_2O
	Prostaglandin H synthases	PHS - 1, PHS - 2	O_2
Reduction	Nitro-reductase	P450, non-P450 enzymes	NADPH
	Azo-reductase	P450, non-P450 enzymes	NADPH
	Aldo-keto reductase	AKR1A1, AKR1B1, AKR1C1-4, AKR1D1	NADPH, NADH
	Quinone reductase	NQO1, P450 reductase	NAD(P)H, NADPH
Hydrolysis	Epoxide hydrolase	EPHX1 (mEH), EPHX2 (sEH)	H_2O
	Esterase	hCE1, hCE2	H_2O
	Peptidase	Aminopeptidase, carboxypeptidase, endopeptidase	H_2O
	Alkaline phosphatase		H_2O

Table 2: Phase II enzymes with their main liver isoforms and the accompanying compounds or functional groups, which are attached to the substrate during conjugation[4,6,7].

Enzyme	Main liver isoform	Accompanying compound/functional group
UDP-glucuronosyltransferases (UGTs)	UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A9, UGT2B	uridine-5´-diphospho-a-D-glucuronic acid (UDPGA)
sulfotransferases (SULTs)	SULT1A1, SULT1B1, SULT1E1, SULT2A1	3'-phosphoadenosine-5'-phosphosulphate (PAPS)
N-acetyltransferases (NATs)	NAT1, NAT2	acetyl group (from acetyl CoA)
glutathione S-transferases (GSTs)	GST A1-1, GST M1-1, GSTP1-1	reduced glutathione (GSH)
various methyltransferases, mainly thiopurine S-methyl transferase	catechol O-methyl transferase (COMT), thiopurine S-methyl	methyl group

(TPMT) and catechol O-methyl transferase (COMT))	transferase (TPMT), PNMT, etc.	
amino acid conjugation enzymes	Acyl - CoA synthetase, acyl - CoA: amino acid N - acyltransferase, etc.	ATP, acetyl CoA, amino acids

Drug Induced Liver Injury (DILI)

It is clear that the liver plays a vital role in metabolizing drugs and deactivating toxins, but what if a xenobiotic is toxic to the liver itself? Drug induced liver injury (DILI) is not an uncommon phenomenon. Above 900 drugs, toxins and herbs are known to result in liver injury[8]. DILI still occurs in clinical phases of drug development or post-marketing. In fact, DILI accounts for approximately 50 percent of all acute liver failures[9]. As liver failure is one of the most lethal diseases in the Western world[10], DILI is a serious health concern.

Today, we do not have an *in vitro* model that can sufficiently predict the toxicity of a drug on the human liver, which is the main reason for DILI being such a serious health problem. As a matter of fact, 40% of hepatotoxic compounds in humans fail to be detected preclinically. The current *in vitro* systems for the prediction of DILI (*e.g.* microsomes, immortalized cell lines, primary cell lines, precision cut liver slices) all have important disadvantages[5]. For instance the procurement of cells (ethics), culture handiness, stability, and the fast decrease or even complete absence of essential metabolic enzymes[5]. Animal testing (*in vivo* models) are used for drug testing after tests have been performed on *in vitro* models. Unfortunately, they cannot give a sufficient prediction of DILI either. The cause is a great variability in drug metabolism amongst even closely related species such as mammels[11]. For example, different species vary markedly in their expression and catalytic activities of enzymes from the CYP450 family[4,5]. Conjugation activity is also species dependent, and to a certain level even race dependent or individual dependent[4]. Dogs for example do not have acetylation capacity, cats hardly have glucuronidation capacity, and horses cannot conjugate salicylates with glycine[4,11]. Consequently, we cannot predict the efficacy or toxicity in humans based on animal studies[11]. The unpredictable pharmacokinetics and metabolism of humans are the main reasons for failure of a drug during clinical trials[12]. To give a better prediction of DILI in humans and decrease the clinical burden, a new model is needed with increased predicting capability.

In addition to a reduction of the clinical burden, having good prediction system of DILI would save large amounts of money. Instead of drug withdrawal at the end of the drug development process, the investigations could be stopped in an earlier stage. From 1992 to 2002, drug toxicity inflicted over 90 percent of the market withdrawals[13]. The major causes for two out of three drugs drawn back from the market were liver toxicity and cardiovascular toxicity[13]. A good working *in vitro* model, which can be used in an early stage of drug development, would likely decrease this number.

Bioprinting an *in vitro* 3D liver construct

The aim of this study is to develop a bioprinted *in vitro* model of the human liver to give a better prediction of DILI compared with a 2D hepatocyte culture. In our approach, we make use of state of the art additive manufacturing techniques combined with (adult) stem cells, to create

cell laden 3D construct. The 2D primary human hepatocyte culture is our negative control, as it is the gold standard for studying the metabolism and toxicity of drugs *in vitro*[14].

The advantage of 3D over 2D is the ability to mimic the original build-up of the tissue, as the complex 3D structure of the *in vivo* liver is not represented by a regular 2D hepatocyte culture[15]. By imitating the liver architecture with biofabrication techniques and liver specific cells, liver organ modules can be produced that have enhanced hepatic characteristics.

A main benefit of using a 3D-printing based technique is that once we have established an *in vitro* 3D liver model, it can be easily recreated. Moreover, a reproducible printing method allows to easily incorporate patient-specific stem cells, creating a patient-specific model.

Biofabrication techniques are already being explored as a Regenerative Medicine (RM) technique to recreate various tissues. For example, Korean researchers are investigating the printing of heart, adipose and cartilage tissue using a decellularized extracellular matrix hydrogel[16]. In Harvard, biofabrication techniques are explored as a method for the recreation of vascularization[17]. At Utrecht University, biofabrication of bone and cartilage is investigated, especially for regenerating (osteo)chondral defects[18,19]. The liver, being an organ with excellent self-regeneration capacity, should be a great candidate to rebuild using bioprinting. Until now, a functional bioprinted liver device has not been established.

We hypothesize that biofabrication technologies combined with (adult) stem cells can yield a functional liver bioreactor, which can be used for the *in vitro* prediction of DILI. In this study, we have established a method for bioprinting a 3D cell-laden liver construct. Flow perfusion chambers were custom designed and printed using stereolithography technology. Cytotoxicity measurements show that we are able to measure cell death in our model. These promising results implicate that we may have found a new way of testing drug toxicity.

Methods

Generation of a 3D printed liver construct

Cell types

To recreate liver tissue, several cell types are needed. The liver mainly consists of hepatocytes. The second most abundant cell type is the sinusoidal endothelial cell. Stellate cells are a third main cell type[20]. Of course there are other cells present in the liver, such as Kupffer cells, but to keep it manageable we focused on these three major cell types.

The first cell type we used for our cell-laden constructs is a human liver cell line (Huh7). This is an immortalized fast growing cell line, which made it an accessible cell type for the first printing experiments. The cells resemble hepatocytes, although they do not possess all hepatocyte characteristics. In the end, some constructs were printed with the main cell type of interest, human liver organoids. These are small organ buds consisting of Hepatic Progenitor Cells (HPCs). HPCs originate from bile ducts. Once activated, they can differentiate into either hepatocytes or cholangiocytes[21]. They were isolated in our research group[22] from a liver biopsy (courtesy of Luc van der Laan, Ersasmus MC, Rotterdam, the Netherlands).

Hydrogels

Gelatin methacrylamide (gelMA) was chosen as a cell-laden hydrogel. Gelatin, a denatured form of collagen, is a biocompatible and biodegradable material. On itself however it will become liquid above 30°C, which means that it would collapse at normal culture temperature (37°C). This issue is overcome by attaching crosslinkable methacrylamide side groups. The resulting gelMA hydrogel still becomes liquid at around 30°C, but after crosslinking using UV light it will keep its shape. In a previous study of Billiet et al., HEPG2 cells have been printed in gelMA with high viability[23]. In their study, 5-20% w/v gelMA was used. They were not able to print 5% w/v gelMA, because of the low viscosity. The highest cell viability (97%) was seen with the lowest percentage of gelMA (10% w/v). We used 5% w/v gelMA from batch BK01, synthesized by the orthopedics lab (curtesy of Jos Malda, University Medical Center Utrecht, Utrecht, the Netherlands). In order to print such a low percentage of gelMA, we made use of a second hydrogel as a support material.

The second hydrogel is pluronic F127 (also called lutrol). One reason for incorporating a support hydrogel was that it enables one to print a broader range of hydrogel types and concentrations[24]. In our case, it allowed us to print a low percentage of gelMA (5% w/v). Instead of an often used thermoplastic support material, we used a material that can be washed away after printing. In this way we were able to create a network of pores, allowing culture media to flow through the construct.

Photo initiator

To initiate gelMA crosslinking under influence of UV irradiation, a photo-initiator (PI) is needed. We used irgacure 2959 (Ciba Specialty Chemicals Inc.), which has several properties: (1) it is applicable in aqueous solutions, due to its reasonable solubility in water, (2) no significant cytotoxic effects are correlated with the use of the PI in absence of UV-A irradiation, and (3) due to its highly efficient radical formation, the cell survival levels after crosslinking are acceptable[23]. The PI is added to both gelMA and pluronic F127, to prevent it from diffusing from gelMA to pluronic F127 during/after printing, which would decrease the crosslinking capability.

Printing gelMA and pluronic F127

In order to print a construct of gelMA and pluronic F127, a model had to be designed and many parameters had to be optimized. The process of developing this printing method is given in Appendix II. Here, we will explain the final method of bioprinting a 3D liver construct.

Constructs were printed using the RegenHU 3D discovery® bioprinter at the printing facility (UMC). After sterilizing the cabinet and other preparations (see standard operating procedures (SOP) 1-7), constructs were printed. The print files were created and optimized (Appendix II) using Biocad 1.0 software (RegenHU, Biosystem Architects). Up to four constructs were created in one print, depending on the amount of gelMA and the experimental set-up.

After crosslinking and washing away pluronic F127 (SOP 8-9), the cell-laden constructs were taken back to the culture lab for further culture. To make the prints more comparable, washing times in HBSS were kept the same for every construct printed on that day (between days this time varied from 1.5 to 3.5 hours).
Depending on the experimental set-up, constructs were either placed in a well plate (static culture, SOP 10) or in a bioreactor (perfused culture, SOP 11).

Table 3: Printing parameters for bioprinting a 3D liver construct (after optimization)

Parameter	gelMA	pluronic F127
pressure (bar)	0.5	4.5
needle (inner diameter in mm)	0.30	0.25
feed rate (mm/s)	20	15
layer thickness (mm)	0.22	0.22
valve openingstime (µs)	280	n/a
dosing distance (mm)	0.7	n/a
z-offset	0.3	0

Printing a flow perfusion chamber using stereolithography technology

The flow perfusion chamber "Prometheus 2.1" was designed using Rhinoceros 4.0 software. With Magics 13 software (Envisiontec), labels were added and a support structure was generated. Perfactory RP 2.9 software was used to place the parts with their support on a digital platform and make copies. All stereolithography prints were manufactured using the Perfactory® 3 Digital Shell 3D Printer (DSP) (Envisiontec). Both PIC100 (Envisiontec) and Photopolymer R05 (Envisiontec) were used to print earlier versions of Prometheus 2.1 depending on availability (see Appendix VII for a more detailed description of the stereolithography materials). Because of the higher strength, the final version of Prometeus (Prometheus 2.1) was printed with Photopolymer R05. After every print job, support material was removed and prints were left immersed in 70% alcohol on a shaker for at least 10 minutes. They were then left to dry and harden for at least one day.

Assays

Alamar Blue assay

Constructs were incubated with a 5% Alamar Blue solution (Invitrogen) for two hours, after which fluorescence at 540/590 nm was measured at the TECAN plate reader (infinite M200) (see SOP 15 for protocol).

Cell count
Cells were counted using a BIO-RAD TC20 Automated Cell Counter, in technical triplicate (see SOP 16 for protocol).

ATP assay
The Molecular Probes® ATP Determination Kit was used for ATP measurements (SOP 17). For every read-out day, an ATP dilution series was prepared from the ATP stock solution by diluting 1:1 with demi water each step. All samples, including the dilution series and negative controls, were measured in triplicate. The negative controls consisted of fresh and Triton X-100 containing media or Advanced DMEM/F12, in case of cells or organoids, respectively. All data was processed using Excel. First, technical triplicates were averaged. Subsequently, a calibration graph was generated from the dilution series data, including a linear function. Using the function, the ATP concentrations of the samples were calculated. The appropriate controls, either fresh media or Triton X-100 containing media or Advanced DMEM/F12, were subtracted.

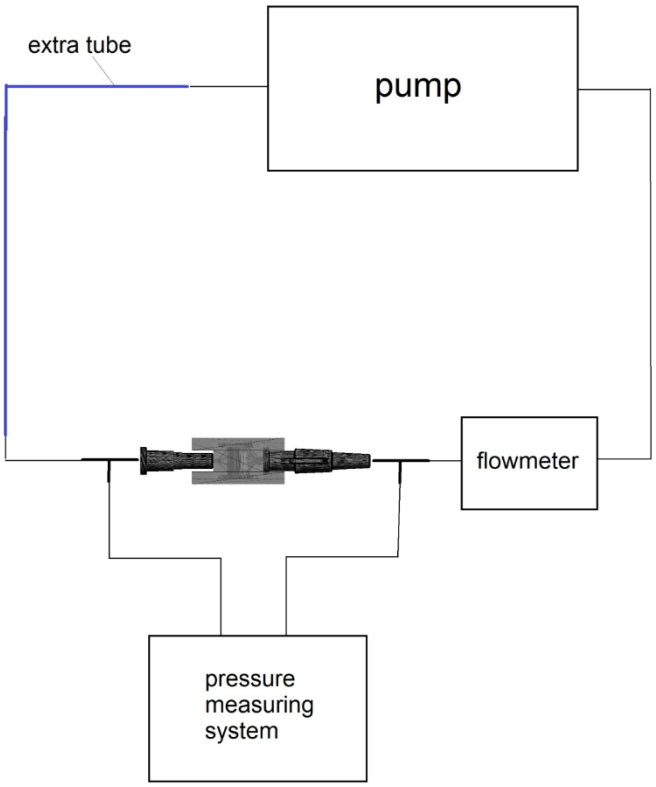
Flow rate and pressure tests

Flow rate and pressure measurements were performed at the Technical University of Eindhoven. An ibidi pump system was used for unidirectional flow. It pumps back and forth between two reservoirs. Two fourfold valves open/close at the moment of direction change in such a way that an unidirectional flow is created.
The ibidi pump system can be combined with several different perfusion sets, suitable for obtaining different ranges of flow rates. The available perfusion set (red) is suitable for generating flow rates of 2.0-42.3 mL min^{-1}, using the manufacturer's µ-slide. The flow rate we (initially) aimed for is 0.200 mL min^{-1}. In order to obtain this, the yellow (0.17-0.74 mL min^{-1}) or black (0.07-0.33 mL min^{-1}) perfusion set is needed, assuming our bioreactor has the same resistance as the manufacturers µ-slide. As a result, it was expected not to obtain the desired flow rate of 0.200 mL mL^{-1} using the available equipment.
The bioreactor consisted of a flow perfusion chamber (Prometheus 2.1) with a printed gelMA construct inside, without cells. Demi water was used as perfusion fluid. The experimental set up is shown in Figure 3.

Pump pressure was varied between 0 and 100 mbar. At every pressure value, the flow rate and the pressure over the construct were determined. Since demi water was pumped back and forth, measurements were performed for both directions separately. To assess variability of the measurements, flow rate was measured 5 times (technical quintuplicate) and pressure was measured 10 times (technical decuplicate). Flow rate values were corrected by setting the value at a pressure of 0 bar to 0 mL min^{-1}.

Cytotoxicity testing of stereolithography materials

First, an indirect cytotoxicity test was performed on photopolymer R05. Six 15 mL Falcon tubes were filled with 4 mL Huh7 medium (see Table 6 (SOP 20) for medium composition). Every day, one 5 mm printed cube consisting of photopolymer R05 was added to one tube, for five days in total. One tube was left empty. Three days after adding the last cube, Huh7 and LX2 cells were seeded on two separate 96 well plates (5,000 cells per well). After one day, the culture medium was replaced with incubated medium from the tubes (n=10 per tube). An MTT assay was performed as described in SOP 18.



Specifications:

Pump:
Ibidi pump system
(red perfusion set)

Flowmeter:
Transonic System Inc.
Team21 – Compatible
TS410
Transit time tubing flowmeter

Pressure measuring system:
PEEKEL
Instruments
Multichannel compact amplifier system
PICAS

Extra tube:
Combidyn-Druckslchlauch PE
150 cm, TRANSPARENT
Supplier: BRAUN
Ref. number: 5215027

Figure 3: Experimental set up of flow and pressure measurements.

Second, a more direct cytotoxicity test on both materials (R05 and PIC100) was performed. GelMA pucks containing Huh7 cells ($2 \cdot 10^6$ mL^{-1}) were produced as described by Mol[22]. They were evenly divided over 11 wells from two 6 well plates. Six wells contained stereolithography printed inserts, 3 consisting of photopolymer R05 and 3 consisting of PIC100. The pucks were kept in culture for two days. On day two the pucks were digested and counted (see SOP 13 and 16). Since the non-printed gelMA was more easily digested, a lower enzyme concentration was used than described in SOP 13 (2 mg of both enzymes per g gelMA).

Third, a test was performed which was most representative for the situation inside the bioreactor. GelMA containing $2 \cdot 10^6$ Huh7 cells per mL was printed together with pluronic F127 as described in SOP 8, with a 2.20 mm building height. Printing 2-3 constructs at a time, in total 14 constructs were printed. After crosslinking and washing away Pluronic F127 (SOP 8 and 9), the constructs were divided over 14 wells from three 6 well plates. Three wells containing Photopolymer R05 inserts and three wells containing PIC100 inserts. The three constructs from one print were distributed over the three conditions for a better comparison.
On day 1 and day 2 after printing, an Alamar Blue assay was performed (SOP 15). The incubation was performed on another well plate to prevent Alamar Blue from reacting with the stereolithography material.
The constructs were digested on day 2 (SOP 13). Because the digestion protocol still had to be optimized for printed gelMA constructs, 2 mg of collagenase XI and dispase were used per g gelMA, which is lower than the final concentration in the SOP. After 1 hour, no gelMA was visibly dissolved, so the amount of enzymes was increased four times. Another hour later, still nothing had visibly happened. The solution was collected and spun down. Surprisingly,

a cell pallet appeared, on which cell count was performed (SOP 16). Afterwards, the constructs were transferred to a 48 well plate, where they were washed with HBSS and subsequently submerged in 0.5 mL TrypLE (1x) (Gibco by life technologies). The constructs were not broken down by TrypLE after 105 minutes of incubation on a 37°C orbital shaker (Heidolph Titramax 1000), and no more cells were extracted from the constructs.

Pilot experiment: HUVECs in/on gelMA

A pilot experiment was performed to investigate how HUVECs (Human Umbillical Vein Endothelial Cells) react in gelMA. This was done because in the extent of this project one of the goals will be to recreate blood vessels. The plan is to seed HUVECs on the inner channel walls of the printed construct. HUVECs have shown to form vessel-like structures when seeded on matrigel. In fact, this 'sprouting assay' is a generally used method, for instance to show the effect of anti-angiogenetic peptides[25]. In this pilot experiment, we assessed the morphology of HUVECs in and on gelMA, with the sprouting assay on matrigel as a positive control.

Two GelMA pucks were created as described by Mol[22]. One without cells and one containing 50,000 HUVECs. The pucks were placed in a 96 well plate and Endothelial Cell Growth Medium (CELL Applications Inc.) was added. As a positive control, matrigel either with or without 50,000 HUVECs was plated out in two other wells. 50,000 HUVECs were seeded on top of the cell-free gelMA puck and matrigel droplet. Volumes of gelMA and matrigel were kept the same (56.5 µL). Microscopic pictures were taken after 6 hours and 4 days.

Results

Printing a 3D liver construct

The final model for the 3D liver construct is cylindric-shaped and permeable (Figure 4). It consists of cell-laden gelMA (5% w/v).
During printing, gelMA is kept at 37°C, since it has a predictable viscosity (liquid) around this temperature. To prevent gelMA from flowing out of the construct during printing, it is printed in between strands of supporting pluronic F127 (40% w/v). In addition, all layers contain two circles of pluronic F127 on the outside to prevent leaking of gelMA. The dimensions (height and diameter) of the printed construct are a bit larger than the cylindrical-shaped chamber of the Prometheus 2.1 (the bioreactor, which will be discussed in detail in the next section). The reason for this is that the construct always collapsed a little after printing and washing. For the construct to be space-filling, the digital model thus needs to be slightly bigger than the bioreactor chamber.
After printing, the construct has an almost perfect cylindrical shape (Figure 4C; 1,2). At this point, the construct cannot be handled. If one would touch it, it would immediately collapse because the pluronic F127 does not become hard enough and because gelMA is still liquid. The next steps are UV-crosslinking of gelMA (15 minutes, 365 nm) and washing away pluronic F127 (>1 hour in 4°C HBSS). The remaining porous gelMA construct has many tiny channels at the former location of the pluronic F127 strands (Figure 4B). These channels allow the media to flow through easily. The construct maintains its cylindrical shape very well after the crosslinking and washing steps (Figure C;3,4). It fills the entire space of the bioreactor chamber (Figure C,5).

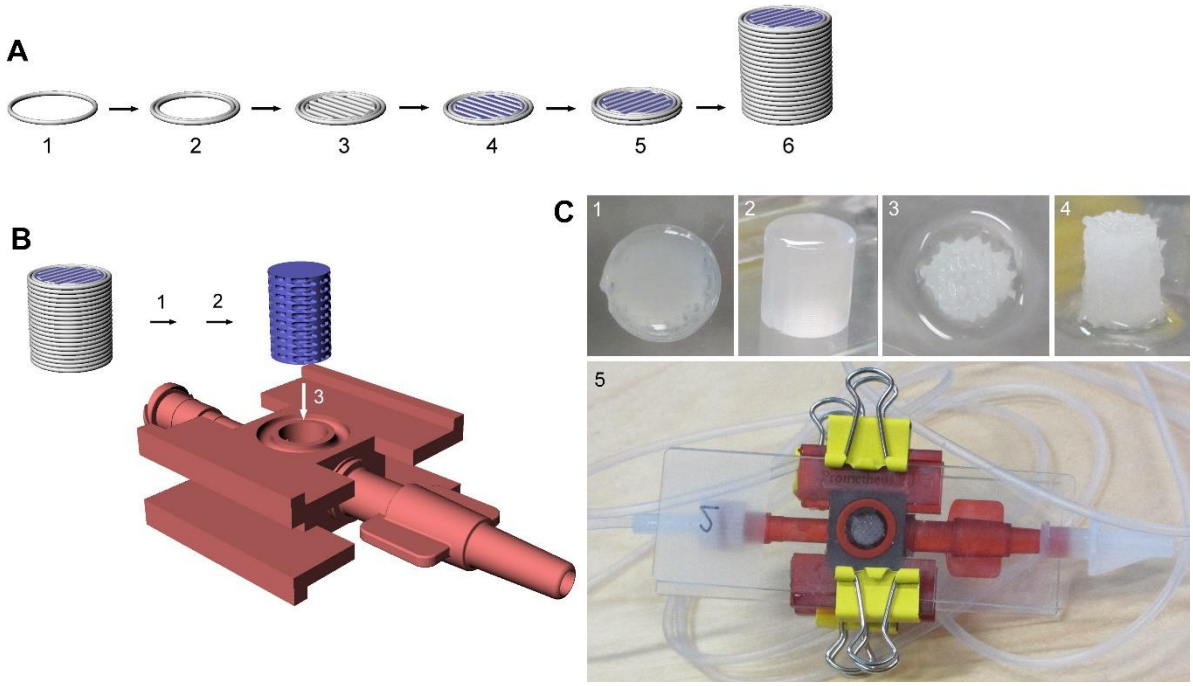


Figure 4. Creating a hydrogel construct
(A) Stepwise scheme of printing gelMA (5% w/v) and pluronic F127 (40% w/v) simultaneously: 1) A circle (r=5.25) of pluronic F127 (40% w/v) is printed clockwise; 2) A second circle (r=4.75) of pluronic F127 is printed counterclockwise, in order to prevent a leaking point at the beginning/end of the strand; 3)The inner circle is filled with continuous filling with 1 mm spacing; 4) GelMA (5% w/v) filling is laid down in strands, also with a spacing of 1 mm. Because 5% w/v gelMA has low viscosity, it fills the gaps in between the pluronic F127 filling entirely; 5) The second layer is exactly the same as the first, only rotated 90°; 6)Printing these two layers is repeated until a height of 11.88 mm is reached.

(B) Steps after printing: 1) GelMA is crosslinked by UV irradiation (365 nm) for 15 minutes; 2) Pluronic F127 is washed away in cold HBSS; 3) The construct is placed inside the bioreactor.
(C) Photos of the printed constructs: 1-2) Top and side view of printed construct; 3-4) Top and side view of construct after crosslinking and washing away pluronic F127; 5) Top view of construct inside Prometheus 2.1

Prometheus 2.1: description of features

The Prometheus 2.1 bioreactor was costum designed and printed using stereolithography technology. It has sereval features (Figure 5). To make it possible to observe the construct at any time, the top and bottem of the bioreactor are open. After the construct has been placed in the bioreactor, it is sealed with a glass slide on the top and bottom. To provide a water proof seal, an O-ring is placed in the groove around the chamber (Figure 5A;3). Both the O-rings and Prometheus 2.1 can be autoclaved, so the inside of bioreactor can be kept sterile. The platform is designed to hold the glass slide in place and secure it with clamps (Figure 5C). For perfusion, any kind of tubing can be connected to Prometheus 2.1 because of the luer lock connectors.
The flow is distributed over the height of the construct through four separate channels. As can be seen in Figure 5B, the diameter of the outlet channels is bigger compared to the inlet channels, to prevent build-up of pressure in the chamber. In fact, the area of each of the outlet channels is exactly five times larger then the area of the associated inlet channel (calculations shown in Appendix III).

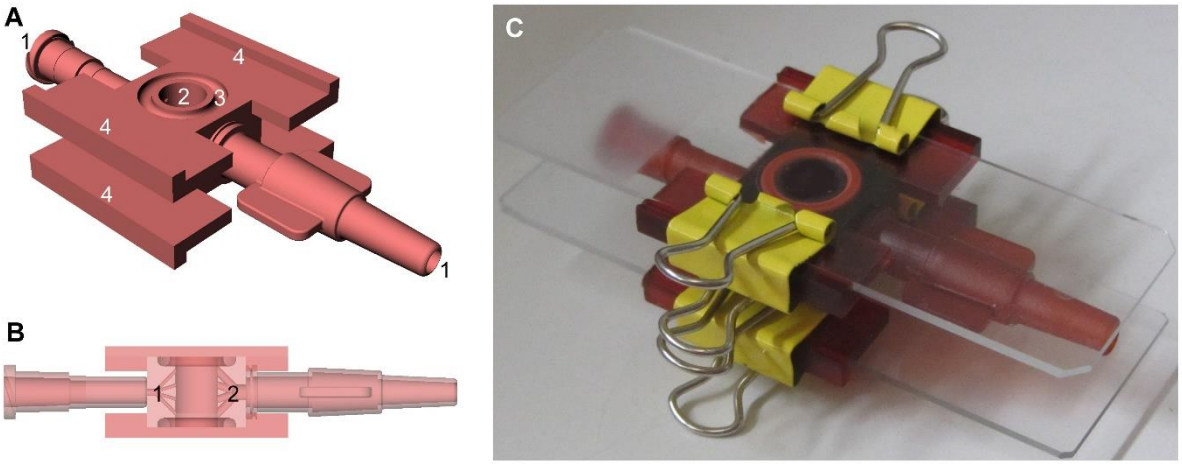


Figure 5: Prometheus 2.1
(A) Perspective view: 1) Luer lock connectors on inlet and outlet provide connection to conventional tubing; 2) A cylindric-shaped chamber, designed for a space filling gelMA construct; 3) A groove for the O-ring surrounds the chamber, providing a water proof seal; 4) Both on top and on the bottom there is a platform to secure the glass slide with clamps.
(B) Side view. Prometheus 2.1 is made transparend to give a better view of the channels. The outlet channels (2) have a bigger diameter then the inlet channels (1), to prevent pressure build-up inside the chamber.
(C) Photograph of assembled Prometheus 2.1 (without tubing).

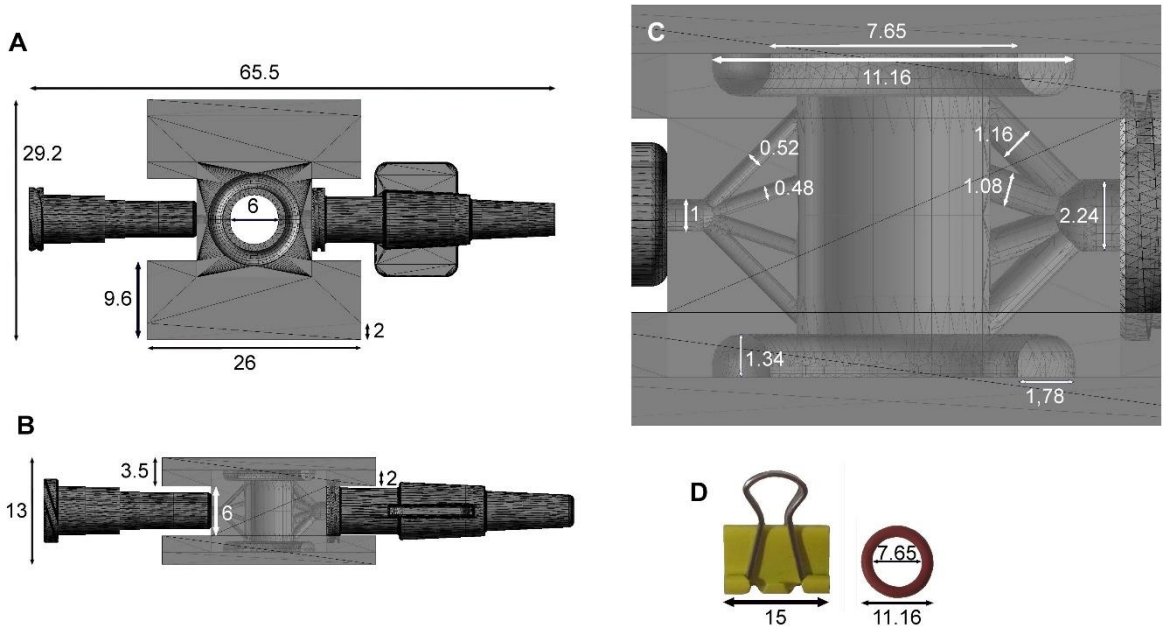


Figure 6: Dimensions of Prometheus 2.1 and accessories (in mm).
(A) Top view of Prometheus 2.1.
(B) Side view of Prometheus 2.1.
(C) Enlargement of side view.
(D) Accessories: Clamp (left) and O-ring (right).

Because the inlet channel devides into four separate channels at different angles (15° and 45°), the tube length is also different. To correct for this, the channels have different diameters (Figure 6C). These were calculated using the Hagan-Poiseuille equation (see Appendix III), keeping the flow rate constant.
By looking closely, one can see that the O-ring groove consists of two parts (Figure 6C). The deepest 2/3 of the groove has the shape of the O-ring itself, but with only half of the total height (by a cutoff horizontally in the middle). In the more superficial 1/3 of the groove, the edges go up vertically. The total height of the groove is 0.75 times the height of the O-ring itself, so that part of the O-ring will always protrude to make a water-proof seal.

Testing the bioreactor

Viability tests

In order to investigate the viability of the constructs over time, Alamar Blue assays and cell counts were performed on printed constructs containing Huh7 cells. From Figure 7A it is clear that the viability, measured by Alamar Blue, is reasonably stable over time, looking at day 1-4 after printing. The values change a little over time, but there is no clear decrease or increase. Between every two time points there is, however, a statistically significant difference ($p<0.05$).
A stable viability is also seen in the cell count results (Figure 7B). The percentage live cells seems to increase a bit, while the total amount of cells decreases. Overall, no significant differences were found in the cell count results.
It is interesting that the measured amount of cells per construct is lower than the theoretical amount ($1{,}5867 \cdot 10^5$). In fact only 18.1 +/- 7.8 % and 15.5 +/- 5.2 % of the theoretical amount of cells was actually counted on day 2 and 4, respectively. This may be due to cell loss during the digestion process. The theoretical amount was based on the cell concentration in gelMA ($2 \cdot 10^6$ cells mL^{-1}) and the weight of printed gelMA

per construct (79.3 mg), determined as described in SOP 8.

To find out whether human liver organoids in gelMA would show the same results, organoid-containing 3D liver constructs were printed and kept in culture for two days. As a control we used empty gelMA constructs. Unfortunately, the constructs looked less beautiful compared to all other printed constructs and it seemed like they contained less gelMA. Nevertheless, an Alamar Blue assay was performed on day 1 and 2 (Figure 7C). Only a small difference can be observed between organoid-containing constructs and empty gelMA constructs, which is not significant. This could be due to the smaller constructs. Note that the values are below zero, which means that they are lower than the control (only Alamar Blue working solution). This is probably a result of gelMA interacting with Alamar Blue, since the empty gelMA constructs also give a negative value.

The viability of Huh7-containing 3D constructs in our bioreactor (perfusion condition) was also assessed by Alamar Blue and cell count. As a control, part of the constructs were cultured static (n=3).

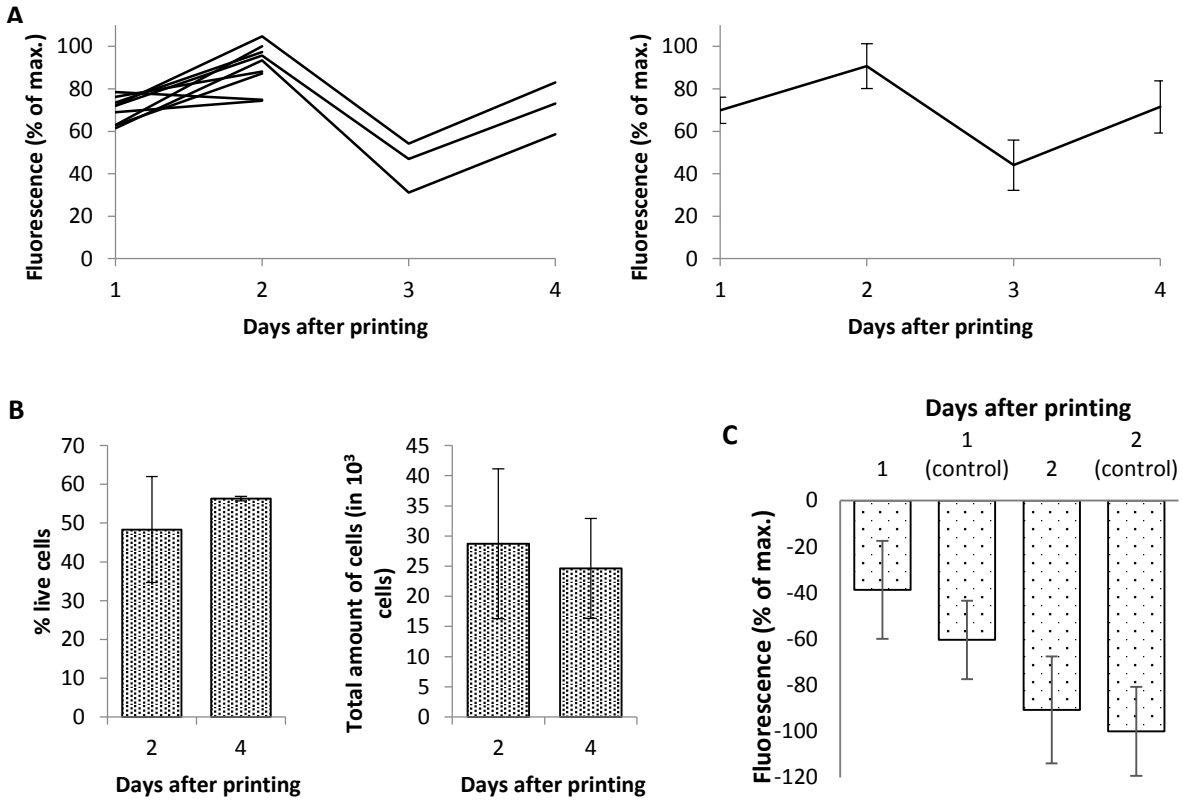


Figure 7: Viability of 3D printed liver constructs.

(A) Results of Alamar Blue assays on Huh7-containing printed gelMA constructs at day 1-4 after printing. Relative fluorescence (maximum average value is set to 100%) is presented on the vertical axis. On the left, samples are represented separately. Some of the samples were sacrificed to perform Cell Count on day 2. On the right, samples are averaged per time point. Error bars represent standard deviations.

(B) Results of cell count on Huh7-containing printed gelMA constructs at day 2 and 4 after printing (n=3). Because the constructs had to be digested in order to count the cells, the constructs on day 4 are not the same constructs as on day 2.

(C) Alamar Blue results of organoid-containing gelMA constructs at day 1 and 2 after printing (n=4). Empty gelMA constructs (n=2) are also shown for both days (control). All values are negative, probably due to interaction of gelMA with Alamar Blue.

For practical reasons, an Alamar Blue assay and cell count were only performed on day four. Unfortunately, all static controls for this experiment were lost due to infection, leaving us without a control.
The Alamar Blue assay on day four resulted in a fluorescence value of -499.3 +/- 54.2 (n=3). As before, this negative value could be a consequence of gelMA interacting with Alamar Blue.

One construct (n=1) was sacrificed for cell count measurement on day four. The total amount of cells was 2.95 · 10^5, of which 27.3 % was alive. This is 70.7% of the theoretical amount of cells per construct (4.17 · 10^5 cells). If we compare this to the earlier measured static condition, it is a higher yield, although the viability is lower.

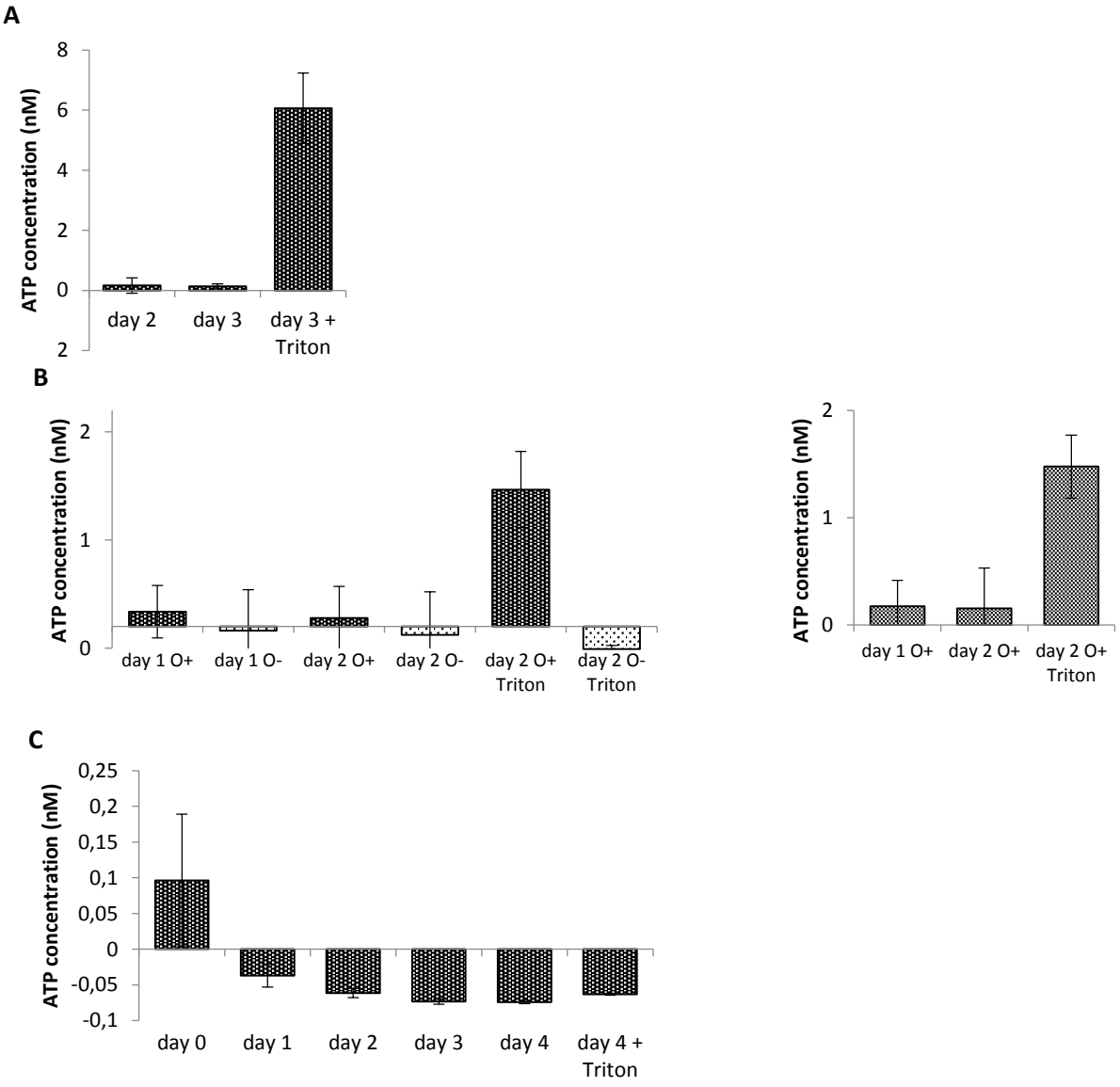


Figure 8: Cytotoxicity test results

(A) Results from ATP assay on Huh7-containing constructs. Addition of Triton X-100 increases the ATP-concentration in the media.

(B) Left: ATP measurement of organoid-containing constructs. In this experiment, also empty gelMA constructs were taken along as a control. In order to compare with (A) and to evaluate the influence of empty gelMA constructs, the outcomes are not controlled for the empty gelMA constructs here. Right: Same graph as on the left, but controlled for empty gelMA wells. "O+" represent organoid-containing constructs, "O-" are empty gelMA constructs.

(C) Results from ATP assay on perfusion condition (Huh7-containing constructs). Note that the maximum value on the y axis is 32 times smaller compared to the static condition (A).

Cytotoxicity tests

The ability to measure cytotoxicity in the gelMA constructs was evaluated by addition of a cell lysing agent, Triton X-100. As a result of cell death, the cellular ATP-content is released. In theory, the ATP diffuses to the media, which we should be able to measure. Because we aim to use our construct for drug toxicity testing, this experiment was a proof of principle. We intended to show whether we could measure cytotoxicity of cells in our construct from a media sample.

In Huh7-containing gelMA constructs, we are able to measure a convincing 44-fold increase in ATP-levels after addition of Triton X-100 ($p<0.05$) (Figure 8A). ATP-levels at day 2 and 3, before addition of Triton X-100, were close to zero (0.16 +/- 0.27 nM and 0.14 +/- 0.08 nM, respectively). This means almost no measurable cell death occurred during the culture period.

The cytotoxicity is less clear in the organoid-containing constructs. This could be due to the low amount of gelMA per construct (as described before for the viability tests). Still, we see a 16-fold increase in ATP-levels after addition of Triton X-100 ($p<0.05$). The empty gelMA constructs however show that gelMA causes a lower measurement outcome (Figure 8B, O-). Subtracting the gelMA controls gives a higher value for both before and after addition of Triton X-100 (Figure 8C), making the ATP increase lower (9-fold instead of 16-fold), but still significant ($p<0.05$). If empty gelMA constructs had also been taken along in the experiment with Huh7 cells in gelMA, the increase would probably be lower too.

ATP measurements of the perfusion condition unfortunately do not show an increase after Triton X-100 addition (Figure 8D). Moreover, the overall values are significantly lower compared with the static condition. Most values are even negative.

Flow rate and pressure tests

No significant differences in flow rates were found between the two pumping directions (Figure 9A).

At 0-9.8 mL min^{-1}, flow rate has a linear correlation with pump pressure ($R^2=0.9991$) (Figure 9B). At lower flow rates, the correlation is less clear ($R^2=9792$) (Figure 9B, right). In addition, there are relatively more fluctuations in the measurements at low flow rates, as represented by the error bars (probably also caused by a baseline error of the measuring device). Since the used perfusion set is suitable for creating a flow rate of 2.0-42.3 mL min^{-1} (using the manufacturer's µ-slide system) the flow rate has a beautiful linear correlation at the intended flow rate range. Consequently, changing the perfusion set to one suitable for our flow range would result in a linear correlation around 0.200 mL min^{-1} too. Flow rate can thus be predicted from pump pressure with high accuracy.

Between pressure over the construct and flow rate, a linear correlation was found too ($R^2=0.995$) (Figure 9C). As expected, pressure over the construct increases when flow rate increases. At lower flow rates, the pressure measurement is relatively less accurate, represented by the error bars (Figure 9C, right). At 0.200 mL min^{-1}, the pressure over the construct is around 0.40 mbar (calculated using the formula in Figure 9C).

In the final test set up for the bioreactor, the ibidi pump system was not used for practical reasons, meaning that the flow rate results were not applicable. Pressure build-up however is dependent on flow rate and will thus not change when using another pump. Consequently, results from pressure tests can be translated to the final system.

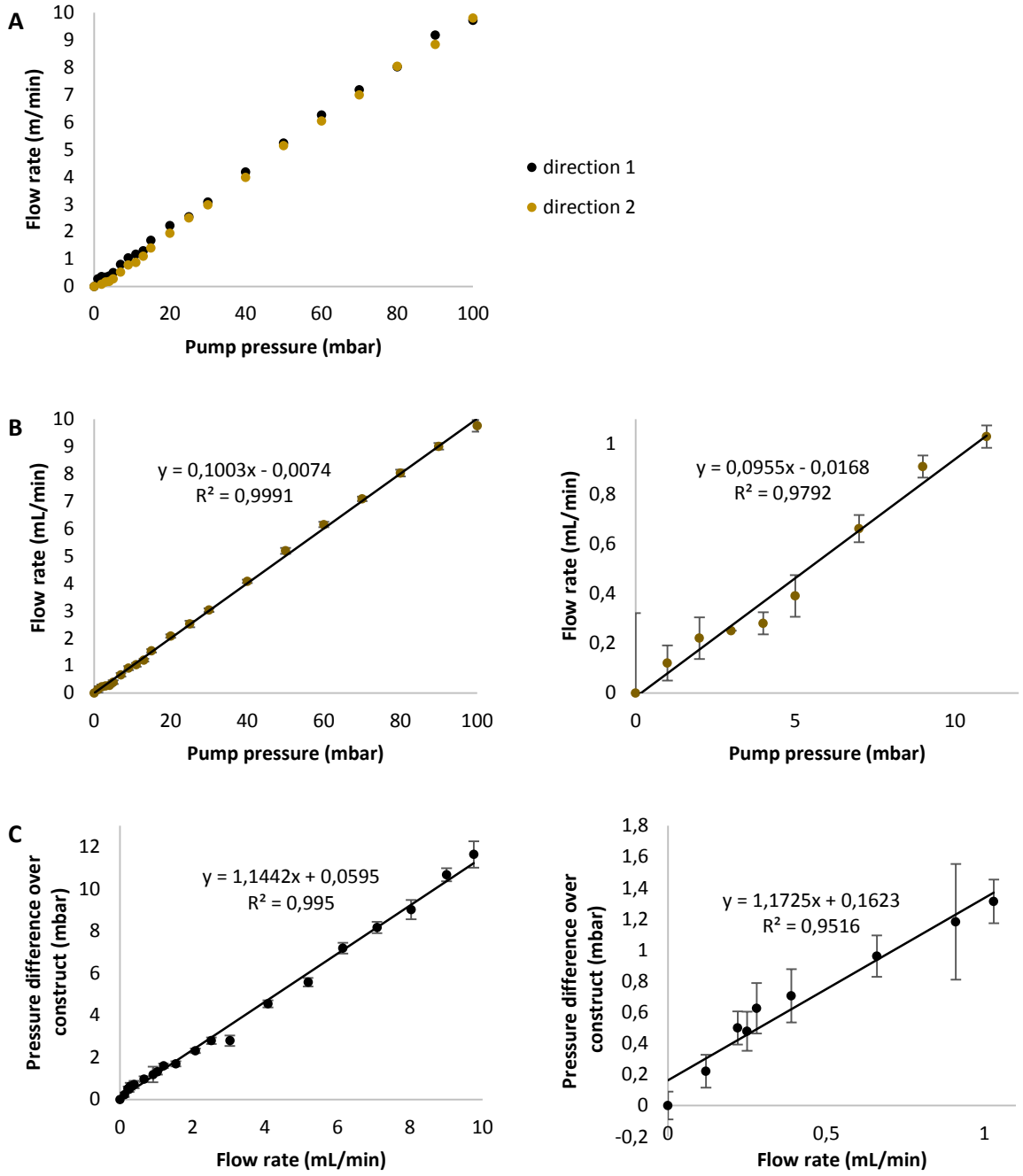


Figure 9: Flow rate and pressure test results.
(A) Flow rate versus pump pressure at the two different pumping directions.
(B) Flow rate versus pump pressure, including a linear trendline (left: flow rates from 0 to 9.8 mL min^{-1}; right: flow rates from 0 to 1 mL min^{-1}). Error bars represent standard deviations of technical quintuplicates.
(C) Pressure difference over the construct versus flow rate, including a trendline (left: flow rates from 0 to 9.8 mL min^{-1}; right: flow rates from 0 to 1 mL min^{-1}). Error bars represent standard deviations of technical decuplicates.

Cytotoxicity testing of stereolithography materials

The indirect cytotoxicity test is negative for both Huh7 and LX2 (Figure 10A). In the beginning, the viability in terms of MTT assay outcome drops, but this is not significant. The more direct test, in which gelMA pucks are cultured in wells printed with stereolithography technology, shows a clearer drop in viability (Figure 10B). Comparing either one of the

stereolithography materials with the control, the difference is significant ($p<0.05$). The percentage of live cells in pucks cultured in R05 wells is slightly lower compared with PIC100 wells, although not significant. These data show that both PIC100 and photopolymer R05 have a negative effect on cell viability.

The final cytotoxicity test assesses the effect of culturing bioprinted cell-laden gelMA constructs inside a stereolithography printed well. Looking at the Alamar Blue results (Figure 10C), constructs cultured in R05 wells already show a significant drop in viability on day 1, and both materials show a significant drop on day 2. The spaghetti plot from both days (Figure 10C, right) shows an interesting outcome, apart from cytotoxicity. The viability of the control constructs does not drop. In fact, most control samples show an increased viability on day 2. In the PIC100 wells, 2 of the 3 constructs show a decrease in viability and in the R05 wells all have decreased.

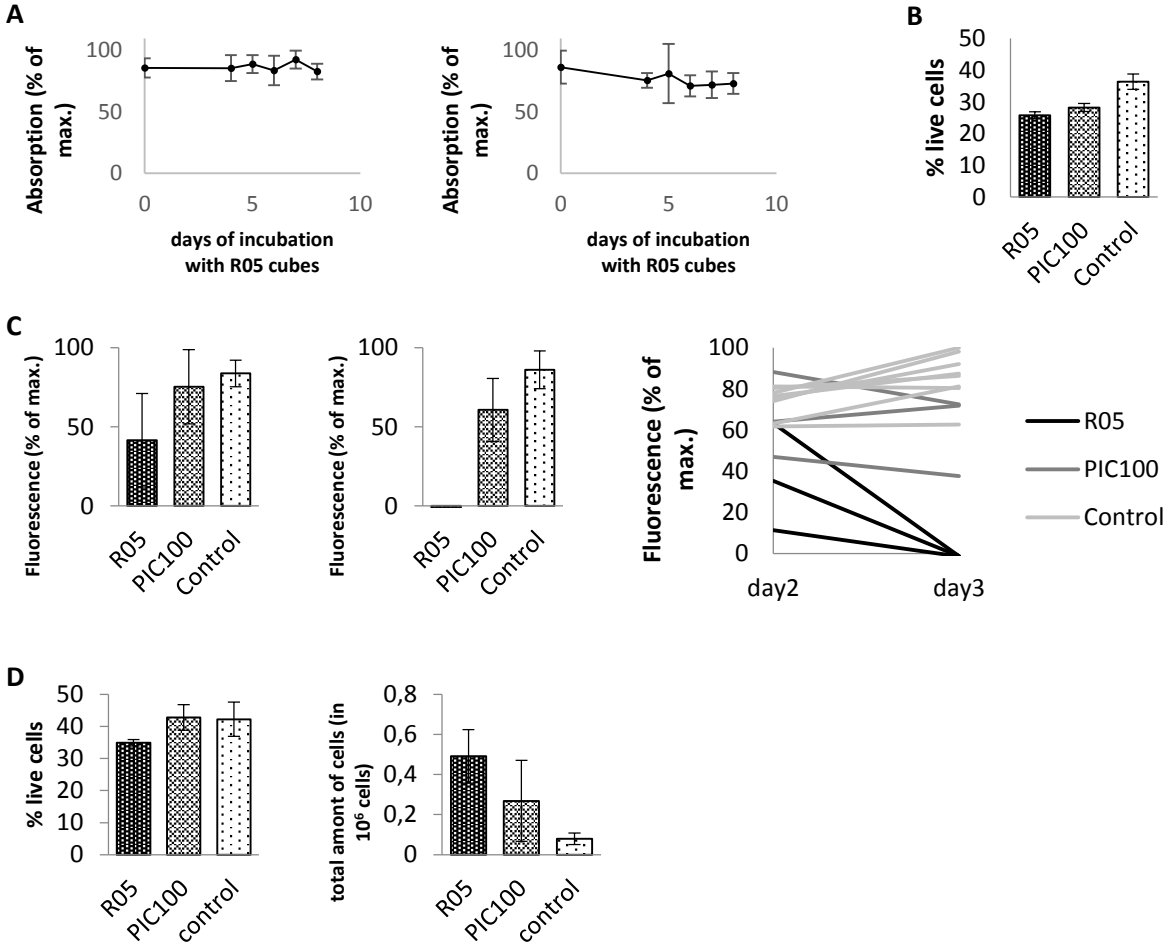


Figure 10: Results of cytotoxicity tests on stereolithography materials.

(A) MTT assay results of indirect cytotoxicity test. Left: Huh7; Right: LX2.

(B) Cell count result of cytotoxicity test with gelMA pucks in stereolithography printed wells.

(C) Alamar Blue results of bioprinted constructs in stereolithography printed wells. Day 1 (left), day 2 (middle) and both days represented in a spaghetti-plot(right).

(D) Cell count results of bioprinted constructs in stereolithography printed wells, showing the % live cells (left) and the total amount of cells (right).

Error bars represent standard deviations.

Surprisingly, cell count results on day 2 (Figure 10D) show only a significant viability decrease in the R05 wells. This contradicts the results from the experiment with gelMA pucks (Figure 10B), which did show a negative effect of PIC100 on cell count outcomes. Another unexpected result is the high amount of cells in the construct cultured in PIC100 and especially in R05, compared to the control constructs (Figure 10D, right). If we compare this with the viability results from the second experiment (Figure 10B) and the Alamar Blue results of the third experiment (Figure 10C, left and middle), it shows an opposite trend.

In conclusion, cell count results have detected significant negative effects of R05. The percentage live cells after two days in culture drops with around 10%. Cell count results on pucks/constructs cultured in PIC100 wells are inconclusive, as in the final experiment the percentage live cells does not differ from the control. Alamar Blue results show a clear drop in viability for both R05 and PIC100.

Pilot experiment: HUVECs in/on gelMA

Figure 11 shows that after six hours, nice vessel-like structures have already been formed on the matrigel. Although on gelMA no tubes have emerged, the cells do possess a more stretched morphology when compared with the HUVECs in gelMA. The rather rounded morphology seen with the HUVECs in gelMA is also visible when the HUVECs are in matrigel. Since endothelial cells *in vivo* are quite stretched out, the real life situation of being spread over the inner wall of a blood vessel appears to be morphologically best represented by HUVECs on gelMA.

After four days, HUVECs in gelMA or matrigel look the same as before. Yet, because of the round morphology it is not clear whether they are still alive. Additionally, if dead, they cannot detach because they are stuck inside the gelMA/matrigel. The HUVECs on gelMA on the other hand are able to detach, but we only see a minor decrease in cell number. In fact, the attached HUVECs on gelMA after four days are bigger in number and show a more stretched morphology when compared with HUVECs on matrigel. The results of this pilot experiment are very promising, since our idea for the future is to seed the tubes of are constructs with HUVECs. The stretched morphology implies attachment and spreading out over the inner walls of the tubes. The image of HUVECs on gelMA after 4 days suggests that this endothelial lining will be stable over time.

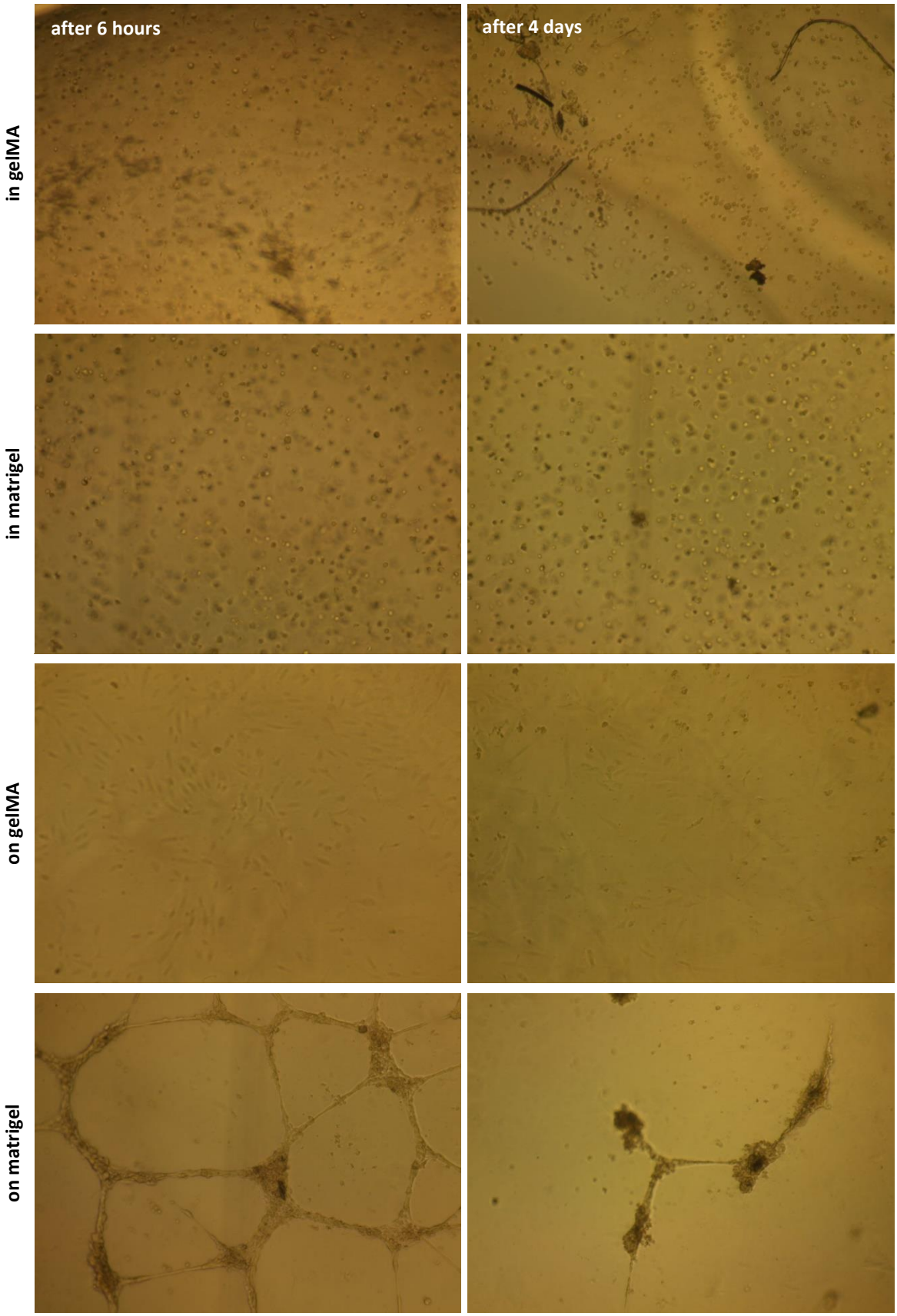


Figure 11: Microscopic images of HUVECs seeded in and on gelMA/matrigel.
Images were taken at 6 hours (left) and 4 days (right) after seeding. Different conditions are shown in vertical direction on the left.

Discussion

Our results show that we are able to print a cell-laden 3D liver construct with stable shape and viability. Cells from an immortalized liver cell line (Huh7-cells) as well as hepatic progenitor cells (human liver organoids) can successfully be printed and subsequently cultured. We are able to measure cytotoxicity in our construct from a simple medium sample, opening new avenues for drug toxicity testing. Staying viable for at least four days allows for acute toxicity measurements. Our 3D-printing based technique enables us to easily reproduce the constructs as well as the custom designed flow perfusion chamber (Prometheus 2.1), and even to expand the number, to improve statistical power without any ethics issues. Using human cells, our construct has great potential to beat animal models when it comes to the prediction of drug toxicity in humans. We are not there yet, but this system is a good first step in creating a model that can sufficiently predict DILI and decrease the clinical burden.

In our study, we managed to print an interconnected pore network using 5% w/v gelMA. Such a network was printed before, but only for a concentration range of 10-20% w/v[23]. We were able to print the low viscous 5% w/v gelMA because of our combined bioprinting approach with pluronic F127 as a support structure. This is mainly due to the double outer pluronic F127 circles, preventing gelMA from flowing out of the construct. Pluronic F127 is an easily printable material, which can be deposited much more precisely then gelMA. Consequently, the shape of the final gelMA construct is better controllable compared with printing gelMA alone.

Viability of the printed liver constructs

Viability in the perfused condition

For the perfused constructs, unfortunately we were not able to obtain positive results for the Alamar Blue assay as well as the ATP assay. A probable cause is the cells being washed out of the construct caused by the flow. The cell count however did result in a reasonable total amount of cells (70.7% of the theoretical amount). Nevertheless, the actual amount of cells could be lower. The automated cell counter could have confused gelMA fibers with dead cells, which could explain the low percentage of live cells (27.3%). In order to see whether cells were washed out by the flow, cell count was performed on the flow through during one of the experiments. The 50 mL Falcon tube was replaced every time. The results indicate that occasionally a small amount of cells was counted using the automated cell counter (Supplementary Figure 12). With the last two positive counts however, a manual count was also performed, which gave only debris as a result (probably small gelMA fibers). This means the previous counts presumably did not contain any cells either, merely debris. This outcome shows that possibly some cells are washed away, but not enough to cause the low assay results.

Nevertheless, the flow rate may have an effect on the assay results, especially since the static cultures did give positive results. If not the flow rate, pressure could also damage cells. We tried to prevent pressure build-up by increasing the diameter of the outgoing channels (see Appendix III). The short-term pressure build-up was low (see Results section), but long-term pressure build-up should be further assessed. Another possible cause for the low assay outcomes is the material of Promethues 2.1,

Photopolymer R05. Our results show a decrease in viability when static constructs are cultured in wells made out of Photopolymer R05. However, this is only a minor decrease and cannot explain the difference in assay outcomes between the static and perfusion condition.

Cell count outcomes

The cell count outcomes (total amount of cells) from all conditions are lower compared to the theoretical amount of cells. There are 3 possible explanations: (1) less cells are printed; (2) cells are lost during the culture period; or (3) cells are lost during the digestion process.

The first explanation does not seem plausible. We would even expect the opposite (more cells per construct), because cells will sink to the bottom of the cartridge, causing more cells to come out at the beginning of printing. To test whether such a process was happening, cell counts of the constructs were arranged by print number. The resulting bar graph (Supplementary Figure 13) shows that the number of cells is not decreasing with later prints. In other words, the amount of cells per construct does not depend on the print number.

The second explanation was only experimentally assessed in the perfusion condition, as mentioned before (Supplementary Figure 12). There, some cells were possibly lost during the culture period. This could be a result of flow, but there is also a possibility that cells detach from the construct independent of flow. To find out, cell count should be performed on the media from the static culture every time it is refreshed.

The third explanation may be the most feasible, because during the digestion process, enzymes are used that can cause damage to the cells if exposed too long. The digestion protocol should be further optimized to minimize this effect. For instance, a higher enzyme concentration combined with a lower exposure time may affect the cell count outcome.

From the former it is clear that the low cell count outcomes are probably caused by losses during the culture period or during the digestion procedure. The printing process seems to have no effect. To learn the actual cause and improve the cell count outcome, we need to perform extra cell counts on the static culture and further optimize the digestion protocol, respectively.

In addition to the low total cell count, the percentage of live cells found in the gelMA constructs is also lower than expected. After digestion, only around 50-55% of the cells are alive, while another study found 98% live cells in printed gelMA constructs containing liver cells (HEPG2 in 10% gelMA)[23]. Our low outcome could again be due to the printing process, the culture period or de digestion procedure.

The culture period may be of influence because the Huh7 cells could not proliferate, which they normally do with a rather high rate. If this is the case differentiated organoids have an advantage over Huh7 cells, as they barely proliferate.

Reasons for the printing process and the digestion procedure to be a cause of cell death are explained before. One way to assess whether the digestion procedure plays a role is by performing the digestion procedure on 2D cultured Huh7 cells too. These cells have high viability (90-100%) when cultured in 2D, so any decrease in viability due to the enzyme exposure could be easily detected.

Printing parameters versus viability

Although printing parameters such as pressure and feed rate were optimized for the printing process, they were not optimized with regard to cell viability. Printing pressure, needle diameter and needle shape have shown to influence cell viability, as shown by Billiet et al.[23]. They

found that the highest viability of HEPG2 cells is obtained with a pressure of <1 bar, a needle diameter of 200 µm and a conical shaped needle. In our case, two of these conditions were met. The printing pressure was 0.5 bar and the needle diameter was 300 µm. The shape of the needle (straight) however was not optimal, because in our case a microvalve was involved in the printing process. The necessity of a microvalve comes from using a low percentage of gelMA (5% w/v). The microvalve makes sure that gelMA is precisely deposited at the programmed places and does not leak after the pressure is turned off.
Altogether, the printing conditions as they are should not significantly decrease the viability. Yet, the influence of printing parameters on cell viability should be kept in mind in future experiments, especially if one wants to change them.

Influence of Photo Initiator on viability
Another factor influencing cell viability is the photo initiator. In this study, we used irgacure 2959, because of availability. However, another photo initiator, VA-086, has shown to have a higher biocompatibility[23]. Irgacure 2959 is nontoxic until it is irradiated with UV-light. After UV-A irradiation, toxic radicals form, which have shown to cause a drop in viability to 70% in chondrocytes[26]. In contrast, the same study obtained cell viabilities of >85% using VA-086. If available, it is certainly worth to test whether the choice of PI has an effect on viability in our constructs.

Influence of stereolithography materials on viability
As shown in our results, the stereolithography material photopolymer R05 does have an effect on cell viability. The advantage of manufacturing using 3D printing techniques is that the material can easily be changed. The other production steps can be kept exactly the same. Testing other stereolithography materials for their influence on cell viability is a promising way to increase viability of our constructs. The final cytotoxicity test that we performed, using printed constructs in stereolithography printed wells, can be repeated using these new materials to show superiority.

Future experiments

Increasing hepatic function
To enhance hepatic function, liver organoids will be differentiated into hepatocyte like cells in our future model. The differentiation protocol of Huch et al.[27] has already been successfully performed by our research group[22] and can thus easily be implemented in our study.

Another promising way of improving hepatic function is to increase cell-cell contact and incorporate other cell types. In the current study, just one cell type was used per construct, either a human hepatocyte cell line (Huh7) or human liver organoids. The Huh7 cells were printed as single cells in gelMA. In this way they were exposed to ECM-like components of gelMA, but direct contact with other cells was not yet established.
Cell-cell contact was found to be highly important for hepatocyte function. Not only the contact between hepatocytes improves their function, fibroblast in close vicinity (no more than 3-4 cells away) increases the functionality even further[28]. In addition, the presence of endothelial cell sheet on top and bottom of a hepatocyte cell sheet increases the maintenance of a hepatic phenotype, compared to a hepatocyte cell sheet alone[29]. In another study, the addition of mesenchymal stromal cells to primary rat hepatocytes increased albumin secretion and CYP1A1 activity[30]. These studies stress the importance of cell-cell contact and co-cultures for the functionality of hepatocytes.

However, some studies suggest the opposite. A decreased albumin production was seen when a human hepatocyte cell line was co-cultured with mouse endothelial and stellate cells[30]. Another study was performed by our lab, to investigate the influence of co-cultures in gelMA, with bioprinting in mind[22]. Hepatocyte like cells (HLCs), derived from organoids, were co-cultured with liver mesenchymal stromal cells (LMSCs) as aggregates in non-printed gelMA. The aggregates were obtained using Agarose stamp 4.3 (see Appendix IV). The reason for using aggregates was to enable cell-cell contact. Unexpectedly, the co-culture caused a decrease in the expression of several early and late hepatic markers, compared with the control containing only HLCs. In contrast, albumin expression increased slightly, although the overall outcomes of albumin were low and therefore unreliable[22]. Although the data is inconclusive, plenty of literature studies show a positive influence of cell-cell contact as well as co-cultures with other cell types on hepatic function. Taking this into account, co-cultures of HLCs and LMSCs are clearly worth further investigation.

The incorporation of other cell types also aids in a better replication of the liver build-up *in vivo*. Moreover, the target cells for DILI, although mainly hepatocytes, can be other liver cell types such as sinusoidal endothelial cells[31] or duct cells[32]. This stresses the importance of a multicellular approach.

Mimicking vascularization

Since our 3D liver construct already contains small canals, the incorporation of endothelial cells is a logical next step. HUVECs have shown to attach to the inner surface of tubes from gelMA construct[17]. A HUVEC-suspension was injected in the tubes, followed by gentle rocking. After 48 hours, they form a nearly uninterrupted layer with 95% confluency[17]. This strategy can be resembled to create a vasculature network lined by endothelial cells.

One pilot experiment has already been performed in our study. We found that HUVECs attach to a gelMA surface already after 6 hours, showing a stretched morphology and remain stretched for at least 4 days after seeding. Endothelial cell seeding thus promises to generate great results.

To visualize the endothelial lining in our constructs, Green Fluorescent HUVECs (GFP-HUVECs) have already been purchased for the continuation of this research. Unlike in non-printed cell-laden gelMA constructs, normal cells are not microscopically visible in printed gelMA construct. The Green Fluorescent Protein will overcome this problem, enabling visualization of HUVEC attachment, morphology and growth.

Bioprinting and cell-seeding can be combined with angiogenesis methods to induce microvasculature formation. The optimal way of combining these two techniques is being investigated for bioprinted constructs in general[17] and may result in new ideas to mimic vascularization in our *in vitro* model.

Additional Viability Assays

In the continuation of this project, we would like to include more assays. A live dead stain was already performed on non-printed gelMA pucks containing a c0-culture of organoids and LMSCs[22]. This assay can be further optimized to obtain images of live and dead cells in our printed constructs. It will give an indication of the viability of the cells inside the constructs, without digesting the constructs. In addition, this stain shows the location of live and dead cells, providing more information about for instance the homogeneity.

Another promising read out is micro RNA 122 (miR-122) measurement. A study in which relative expression of miR-122 of different cell types was assessed showed a significantly higher expression in hepatic

cells compared with other cell types. In fact, Huh7 appeared to have the highest expression[33] (Supplementary Figure 14). MiR-122 is thus an excellent candidate for measuring viability as well as hepatic function.

Assays measuring hepatic function

Concerning drugs and drug toxicity, the main function of the liver is biotransformation. Some xenobiotics first have to be biotransformed to become toxic (bioactivation)[4,5]. As a result, the presence of biotransformation is essential for a good prediction of DILI. Several ways to increase hepatic function, including biotransformation, have already been proposed (differentiation towards HLCs, co-culture with other cell types). To assess functionality, several assays can be used.

A general applied assay to evaluate hepatic function is albumin measurement in the medium. This assay was performed before by our research group[22]. In addition, a qPCR was performed for several early and late hepatic markers, ductal markers, stem cell markers and a mesenchymal marker. Although these assays will provide useful information about hepatic function and differentiation, they are not specific for determining biotransformation activity.

The main enzymes involved in Phase I biotransformation reactions are from the CYP450 family. A CYP450 assay is thus a valuable addition to our set of assays. It has already been performed on non-printed gelMA constructs[22]. This assay determines the metabolic activity by measuring the conversion of a substrate (luciferin-PFBE) by CYP3A4. This assay however still has to be optimized, because the controls resulted in a higher outcome than the samples[22].

Phase II enzymes should also be taken into account. UDP-glucuronyltransferase activity can be assessed using a method described by Seo et al.[34]. In this assay, 6 different substrates were used, and the resulting glucuronyl conjugates were analyzed in a single liquid chromatography-tandem mass spectrometry (LC-MS/MS) run.

For cellular acetyltransferase activity analysis, radio-labeled sodium acetate can be used as a substrate, as described by the manufacturers protocol[35].

Glutathione S-transferase (GST) activities can be assessed using the GST Fluorometric Activity Assay Kit. It is based on the reaction of the conversion of two substrates (monochlorobimane (MCB) and glutathione) to a fluorescent product, catalyzed by GST[36].

Several other assays exist measuring the direct enzyme content, whereas the former described ones determine activity by conversion of a (labeled) substrate. Since we are interested in biotransformation activity, the activity assays are most applicable. They are a promising addition to increase the predicting capability of our constructs.

Testing chronic toxicity:

In the future we will aim to extend the culture period. Most cytotoxic drugs, such as acetaminophen, cause acute liver toxicity. Yet, these drugs are also the ones generally screened out by conventional pre-clinical research. Besides subacute and chronic toxicity, in some cases drugs show delayed toxicity (e.g. augmentin or erythromycins). In these cases, cytotoxicity can manifest up to 3-4 weeks after a treatment has been ended[37].

We have shown that our constructs have stable viability the first 4 days after printing. For practical reasons, we have not yet tried to extend this period. Establishing a method for long term culture would increase the value of our model substantially.

Multiple drug testing

The variety of drugs tested on our construct will also be expanded. At this moment, we have already shown that we are able to measure cytotoxicity caused by Triton X-100. In the future, we can start with testing convenient drugs, such as acetaminophen.

Eventually, drugs causing a cytotoxicity which cannot be detected in other *in vitro* systems or animal models should be tested, to show the superiority of our model. EC_{50} values of those drugs can then be compared with *in vivo* values to determine sensitivity.
Moreover, we will simultaneously test cytotoxicity of multiple drugs to investigate cross reaction. Use of concomitant drugs can increase chances of obtaining DILI[37]. Substances heightening the risk of DILI, such as ethanol[37], should be assessed together with the drug of interest, to determine their influences on cytotoxicity of the drug. In addition, cross reactions of medicines that are administered together, for instance because they both treat a certain disease, should be investigated simultaneously.

Possibilities of the printed liver construct

The end product, an *in vitro* model with superior DILI prediction capability, can have multiple applications. It can be used as in drug development to detect DILI in an early stage. Consequently, it will decrease the chances of DILI occurring on human subjects and it will save money. Additionally, DILI tests on animal models will be replaced, decreasing the amount of test animals.
The *in vitro* printed liver model can also be used in personalized medicine. Since drug metabolism does not only differ between species, but also between individuals[4,5,11], we eventually may want to predict drug cytotoxicity in individuals or groups of individuals. Risk factors for specific groups are alcohol use, age, sex, underlying diseases like HIV or diabetes, and genetic factors[38,39]. Cells of individuals or representative groups of individuals could be cultured and printed to create a more personal *in vitro* model.

In the far future, the project could be extended to aim for a transplantable device. Liver transplantation is today still the only therapy for liver failure patients[30]. There is however a shortage of donor livers, which stresses the need for new treatments[30]. A functional transplantable device would overcome this problem.
Making a construct for transplantation is however much more challenging than creating an *in vitro* device. All used materials, including cells, must be approved for transplantation. Probably even more challenging, the device should be a fully functional liver substitute, with the same capacity of a normal human liver. This seems more of an application in the future, but with the current rate of increasing technology it may be possible someday.

Conclusion

Concluding, promising results have been obtained during this study. We have developed an *in vitro* liver model with reasonable stable viability, in which we can measure cytotoxicity. Our results open the way for further research to improve the model. We already have some ideas for increasing viability and function. Other future goals are long term culture and testing of multiple drugs. The final model for DILI prediction will decrease the clinical burden, save money, and replace animal testing.

References

1. Frappier BL. Liver. In: *Dellman's Textbook of Veterinary Histology*. Vol 6th ed. Blackwell Publishing; 2006:201-206.

2. Dyce KM, Sack WO, Wensing CJG. The liver. In: *Textbook of Veterinary Anatomy*. Vol 4th ed. Saunders, Elsevier; 2010:135-139.

3. Mescher AL. *Junqueira's Basic Histology*. Vol 13th ed. Mc Graw Hill Education; 2013.

4. *Syllabus Inleiding in de Veterinaire Farmacologie*. Utrecht University, Faculty of Veterinary Medicine; 2015. https://uu.blackboard.com/webapps/blackboard/execute/content/file?cmd=view&content_id=_2132233_1&course_id=_87664_1.

5. Coecke S, Ahr H, Blaauboer BJ, et al. Metabolism: A bottleneck in in vitro toxicological test development. *ATLA Altern to Lab Anim*. 2006;34(1):49-84.

6. Jancova P, Anzenbacher P, Anzenbacherova E. Phase II drug metabolizing enzymes. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub*. 2010;154(2):103-116.

7. Penner N, Woodward C, Prakash C. Drug Metabolizing Enzymes and Biotransformation Reactions. In: Zhang D, Surapaneni S, eds. *Enabling Technologies in Drug Design and Development*. Vol 1st ed. John Wiley & Sons; 2012:545-565.

8. Mehta, N. (North Shore University Hospital, Long Island Jewish Medical Center NYHQ, Ozick, L.A. (Leumit Health Clinic I, Gbadehan E (Columbia UC of P and S. Drug-Induced Hepatotoxicity. 2015:1-11.

9. Tuschl G, Lauer B, Mueller SO. Primary hepatocytes as a model to analyze species-specific toxicity and drug metabolism. *Expert Opin Drug Metab Toxicol*. 2008;4(7):855-870.

10. Van de Bovenkamp M, Groothuis GMM, Meijer DKF, Olinga P. Liver fibrosis in vitro: Cell culture models and precision-cut liver slices. *Toxicol Vitr*. 2007;21(4):545-557.

11. Walsh CT, Schwartz-Bloom RD. Interspecies variation. In: *Pharmacology: Drug Actions and Reactions*. Vol 7th ed. CRC Press; 2004:285.

12. Kola I, Landis J. Can the pharmaceutical industry reduce attrition rates? *Nat Rev Drug Discov*. 2004;3(8):711-715.

13. Schuster D, Laggner C, Langer T. Why Drugs Fail - A Study on Side Effects in New Chemical Entities. *Curr Pharm Des*. 2005;11(27):3545-3559.

14. LeCluyse EL. Human hepatocyte culture systems for the in vitro evaluation of cytochrome P450 expression and regulation. *Eur J Pharm Sci*. 2001;13(4):343-368.

15. Gunness P, Mueller D, Shevchenko V, Heinzle E, Ingelman-Sundberg M, Noor F. 3D organotypic cultures of human heparg cells: A tool for in vitro toxicity studies. *Toxicol Sci*. 2013;133(1):67-78.

16. Pati F, Jang J, Ha D-H, et al. Printing three-dimensional tissue analogues with decellularized extracellular matrix bioink. *Nat Commun*. 2014;5:3935.

17. Kolesky DB, Truby RL, Gladman a. S, Busbee T a., Homan K a., Lewis J a. 3D bioprinting of vascularized, heterogeneous cell-laden tissue constructs. *Adv Mater*. 2014;26(19):3124-3130.

18. Malda J, Visser J, Melchels FP, et al. 25th anniversary article: Engineering hydrogels for biofabrication. *Adv Mater*. 2013;25(36):5011-5028.

19. Schuurman W, Levett P a., Pot MW, et al. Gelatin-methacrylamide hydrogels as potential biomaterials for fabrication of tissue-engineered cartilage constructs. *Macromol Biosci*. 2013;13(5):551-561.

20. Keulen D Van, Spee B, Malda J. Printing a liver tissue construct. 2014.

21. Kruitwagen HS, Spee B, Schotanus B a. Hepatic progenitor cells in canine and feline medicine: potential for regenerative strategies. *BMC Vet Res*. 2014;10(1):137.

22. Mol M. Co-cultures for creating a bioprinted liver construct. 2015.

23. Billiet T, Gevaert E, De Schryver T, Cornelissen M, Dubruel P. The 3D printing of gelatin methacrylamide cell-laden tissue-engineered constructs with high cell viability. *Biomaterials*. 2014;35(1):49-62.

24. Schuurman W, Khristov V, Pot MW, van Weeren PR, Dhert WJ a, Malda J. Bioprinting of hybrid tissue constructs with tailorable mechanical properties. *Biofabrication*. 2011;3(2):021001.

25. Chadha KC, Nair B, Godoy A, et al. Anti-angiogenic activity of PSA-derived peptides. *Prostate*. 2015;75(12):1285-1299.

26. Rouillard AD, Berglund CM, Lee JY, et al. Methods for Photocrosslinking Alginate Hydrogel Scaffolds with High Cell Viability. 2011;17(2).

27. Huch M, Gehart H, van Boxtel R, et al. Long-Term Culture of Genome-Stable Bipotent Stem Cells from Adult Human Liver. *Cell*. December 2014:1-14.

28. Bhatia SN, Balis UJ, Yarmush ML, Toner M. Effect of cell-cell interactions in preservation of cellular phenotype: cocultivation of hepatocytes and nonparenchymal cells. *FASEB J*. 1999;13(14):1883-1900.

29. Hirose M, Yamato M, Kwon OH, et al. Temperature-Responsive surface for novel co-culture systems of hepatocytes with endothelial cells: 2-D patterned and double layered co-cultures. *Yonsei Med J*. 2000;41(6):803-813.

30. Nibourg GA, Chamuleau RA, van Gulik TM, Hoekstra R. Proliferative human cell sources applied as biocomponent in bioartificial livers: a review. *Expert Opin Biol Ther*. 2012;12(7):905-921.

31. DeLeve LD, Wang X, Kuhlenkamp JF, Kaplowitz N. Toxicity of azathioprine and monocrotaline in murine sinusoidal endothelial cells and hepatocytes: the role of glutathione and relevance to hepatic venoocclusive disease. *Hepatology*. 1996;23(3):589-599.

32. Odin J a., Huebert RC, Casciola-Rosen L, LaRusso NF, Rosen A. Bcl-2-dependent oxidation of pyruvate dehydrogenase-E2, a primary biliary cirrhosis autoantigen, during apoptosis. *J Clin Invest*. 2001;108(2):223-232.

33. Fukuhara T, Kambara H, Shiokawa M, et al. Expression of MicroRNA miR-122 Facilitates an Efficient Replication in Nonhepatic Cells upon Infection with Hepatitis C Virus. *J Virol*. 2012;86(15):7918-7933.

34. Seo K, Kim H, Jeong ES, Abdalla N, Choi C, Kim D. In Vitro Assay of Six UDP-Glucuronosyltransferase Isoforms in Human Liver Microsomes , Using Cocktails of Probe Substrates and Liquid Chromatography – Tandem Mass Spectrometry s. 2014.

35. Acetylation assays using Acetyl CoA. *PerkinElmer*. http://www.perkinelmer.com/Resources/TechnicalResources/ApplicationSupportKnowledgebase/radiometric/acetylation.xhtml.

36. GST Fluorometric Activity Assay Kit. Highly sensitive in vitro assay. *BioVision*. http://www.biovision.com/gst-fluorometric-activity-assay-kit-2818.html.

37. Kaplowitz N. Drug-Induced Liver Injury. 2011;90033(Suppl 2):44-48.

38. DeLeve L, Kaplowitz N. Prevention and therapy of drug-induced hepatic injury. In: *Therapy of Digestive Disorders*. Vol Philadelphia: WB Saunders, Harcourt, Brace; 2000:334-348.

39. Zimmerman HJ. Drug-induced liver disease. *Clin Liver Dis*. 2000;4.1:73-96.

Appendices

Appendix I: Supplementary Figures

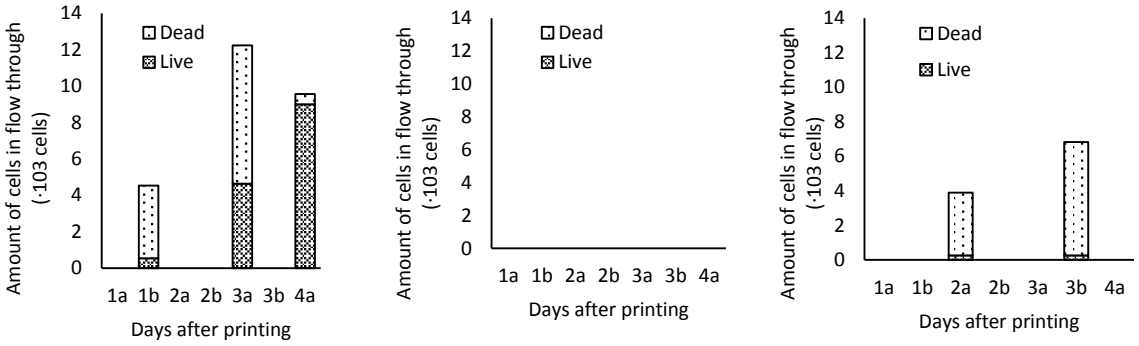


Figure 12: Cell count results of flow through.
The three graphs represent the three individual constructs (n=3). Tubes were replaced twice a day, in the morning (a) and the afternoon (b).

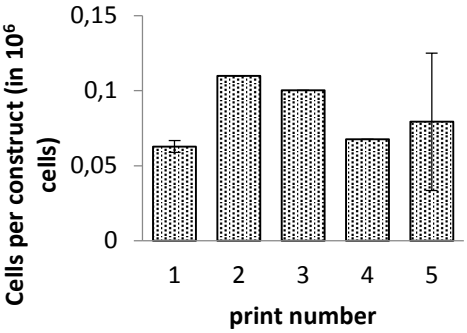


Figure 13: Cell count arranged by print number.
Print 1 consisted of 2 constructs and print 5 consisted of 3 construct. Print 2-4 consisted of 3 constructs, but only one was taken along, because the other two constructs were cultured in a different condition.

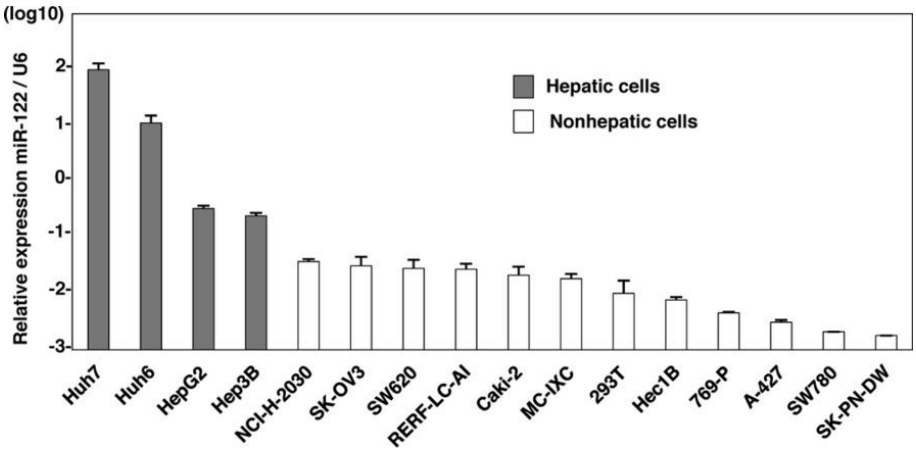


Figure 14: Significantly higher endogenous expression of miR-122 in Huh7 compared with other hepatic and non-hepatic cell lines[33].

Appendix II: Developing a bioprinted gelMA construct

The development of our bioprinted gelMA construct involved changing several printing and non-printing parameters. Some parameters (pressure and temperature) will be discussed below. Others such as feed rate and layer thickness were also optimized, but do not require additional explanation. The design (digital printing file) of the construct has also developed over time, as explained below.

Constuct design

The first idea was to create a cube-shaped construct of gelMA and pluronic F127 (Figure 16A). After crosslinking, we planned to use a 6mm biopsy punch to make the construct perfectly round and precisely fit in the cylindrically shaped chamber of Prometheus 2.1.
During printing, we ran into the issue of gelMA flowing out of the construct. To prevent this, a square of pluronic F127 was printed on the outside (Figure 16B). This resulted in a printable construct.
Next, a method for crosslinking had to be established. At the first trial, the construct was not entirely crosslinked. A cause could be that the photo initiator, needed to initiate crosslinking, diffused from gelMA to pluronic F127. We decided to add photo initiator to pluronic F127, as shown in SOP 4. Additionally, a microscope slide was placed on top of the construct, supported by two piles of microscope slides on the sides (Figure 23). The final method worked well and resulted in a crosslinked construct from top till bottom.
The punching procedure however did not work (Figure 15). The cause was that gelMA was squeezed to the side, resulting in an odd shaped final construct (Figure 15C,D).

We decided to skip the stamping procedure. Due to the outer pluronic F127 layer, the shape of the final gelMA construct was much more controllable, enabling us to print a cylindric-shaped construct right away.
A double layer of pluronic F127 was printed, because pluronic F127 starts flowing out of the printhead around a mm after it should, leaving a tiny space open. To prevent gelMA from flowing out, the second layer was printed in opposite direction, so the openings in the two pluronic F127 rings were located on different places.
The diameter and height of the cylindrically shaped construct were optimized, because it slightly collapses during the manufacturing process. With a diameter of 7.5 mm and a height of 11.88 mm, the end product fitted best.
The final model (Figure 16C) is also shown in the Results section.

Figure 15: Punching of gelMA construct.
(A) Second design of gelMA construct, just after printing.
(B) Construct after crosslinking and washing away pluronic F127.
(C) Just after punching (punched part on the right).
(D) Just after punching, top view (punched part on the right).

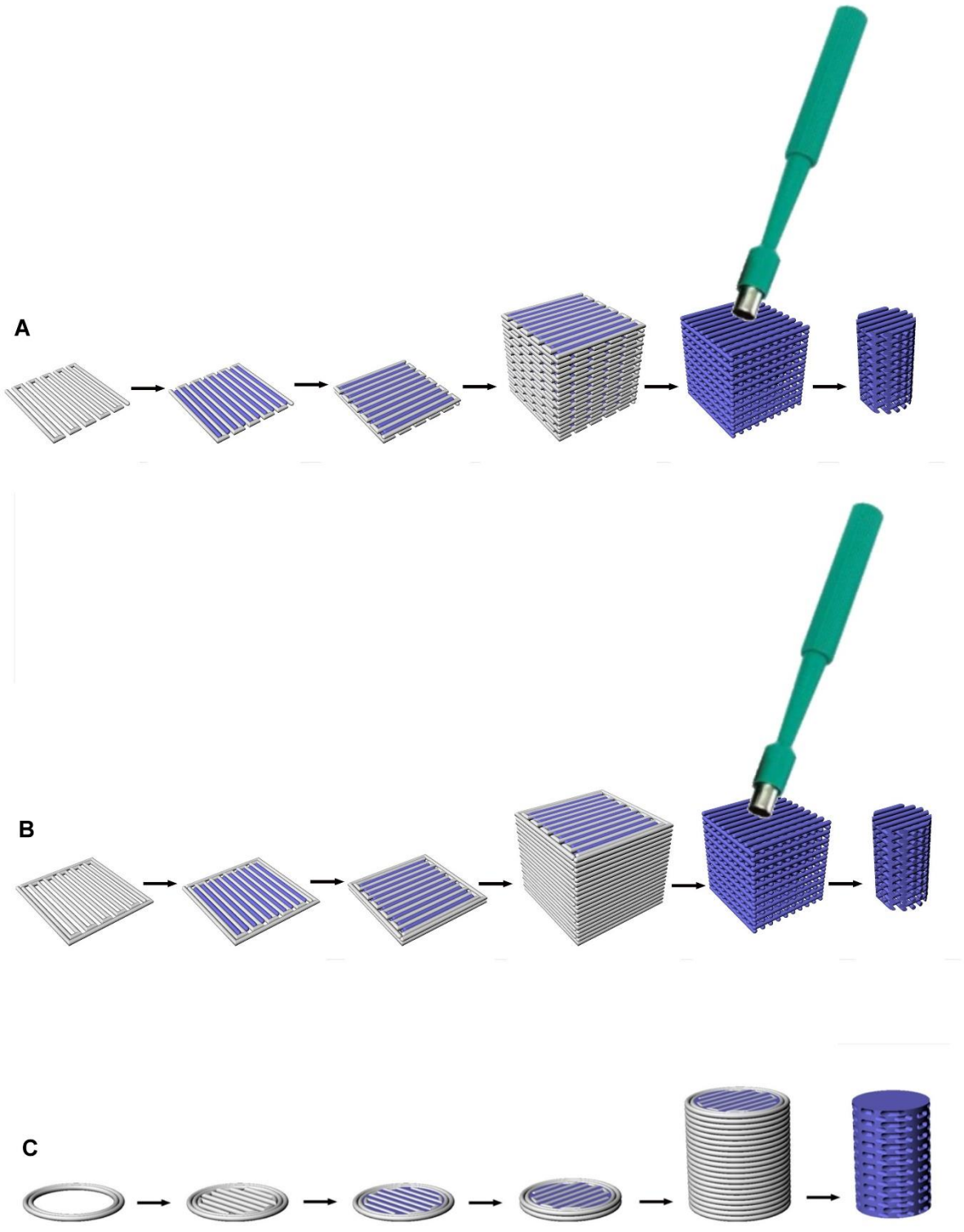


Figure 16: Development of gelMA construct design.
(A) Initial design. A cube consisting alternating gelMA and pluronic F127 strands. The pluronic F127 line is continuous because it cannot be deposited with high precision on the beginning and the end of a line. This has nothing to do with pluronic F127 itself, which has very nice printing properties, but merely with the absence of a microvalve.
(B) Second design. A square of pluronic F127 is printed on the outside to prevent gelMA from leaking out during printing.
(C) Final design, cylindrically shaped.

GelMA pressure

The initial used pressure was low, around 0.1 bar. Such a low pressure fluctuates more, because of the inaccuracy of the pressure regulator. The final pressure was set at 0.5 bar, causing less fluctuations. To prevent too much gelMA to flow out, the dosing distance and valve openingstime were adjusted. The microvalve is not continuously open, it opens for a certain time (valve openingstime), and after it has traveled a certain distance (dosing distance) it will open again. By decreasing the valve openingstime and increasing the dosing distance, less gelMA comes out of the printhead per time unit. This enabled us to increase the pressure to 0.5 bar, at which the pressure can be regulated more accurately.

Pluronic F127 pressure

Initially, the pressure of pluronic F127 was set at 3 bar. However, using a thinner needle (25 gauge) and a higher pressure (4.5 bar) resulted in a more precisely deposition.

Printing temperature of gelMA

At room temperature, gelMA is hardened. For this reason, we initially started printing gelMA at 30°C. At this temperature, gelMA is more or less liquid. The temperature was however not precisely controllable, resulting in a fluctuating gelMA viscosity. For this reason, we decided to increase the temperature to 37°C, at which gelMA is completely liquid. Small temperature fluctuations at this temperature do not change the viscosity of gelMA, resulting in a constant and predictable gelMA viscosity.

Appendix III: Designing Prometheus 2.1

The first model of our custom designed bioreactor was called Prometheus 1.0. The final version was very much alike, but some details had changed.

Platform

We started with a platform of 30x29.6x1.5 mm (Figure 17C,E). The outer 2 mm edges were 1 mm thicker (Figure 17C), so the glass slide could be secured. After printing, the platform seemed to bend a little when the clamps were attached. Also, the space for the microscope slide turned out to be a bit too large. For these reasons, the platform was made 0.5 mm thicker (Figure 17C,D) and 0.4 mm less wide (Figure 17E,F). Because the first model contained more than enough space for the clamps, the platform was also made 4 mm shorter (Figure 17E,F).

O-ring groove

The O-ring groove was first designed by creating a donut-shaped groove, cut horizontally at half of the total height (Figure 18B). The width of the groove was chosen 0.5 mm larger than that of the O-ring itself (Figure 18B,C). This was done to leave some space for the O-ring to protrude in horizontal direction when it is pressed down by the microscope slide. In the final version, a different O-ring was used, because of possible toxicity of the former one. The material of the first O-ring was not known, so we decided to use a silicon-rubber O-ring (Polymax, Article number: BS011SR70) (Figure 18D). The advantages of this O-ring is that the material is known to be not toxic to the cells and that it is autoclavable. Its dimensions are a bit different from the former used O-ring. The new groove was not only adapted to these other dimensions, but it also had a different, more conventional, shape. The width was exactly the same as the width of the O-ring. To create space for the O-ring to protrude, the groove was made deeper, with a total depth of 0.75 times the height of the O-ring (Figure 18D,E).

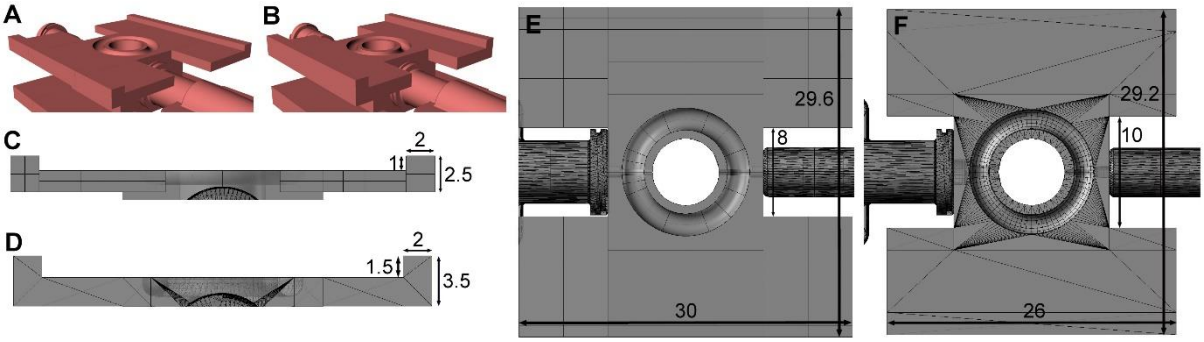


Figure 17: Dimensions of platform of Prometheus 1.0 and Prometheus 2.1.
(A) and (B) Perspective view of platform of Prometheus 1.0 and Prometheus 2.1, respectively.
(C) and (D) Front view of platform of Prometheus 1.0 and Prometheus 2.1, respectively, with dimensions in mm.
(E) and (F) Same as (C) and (D), but top view instead of front view.

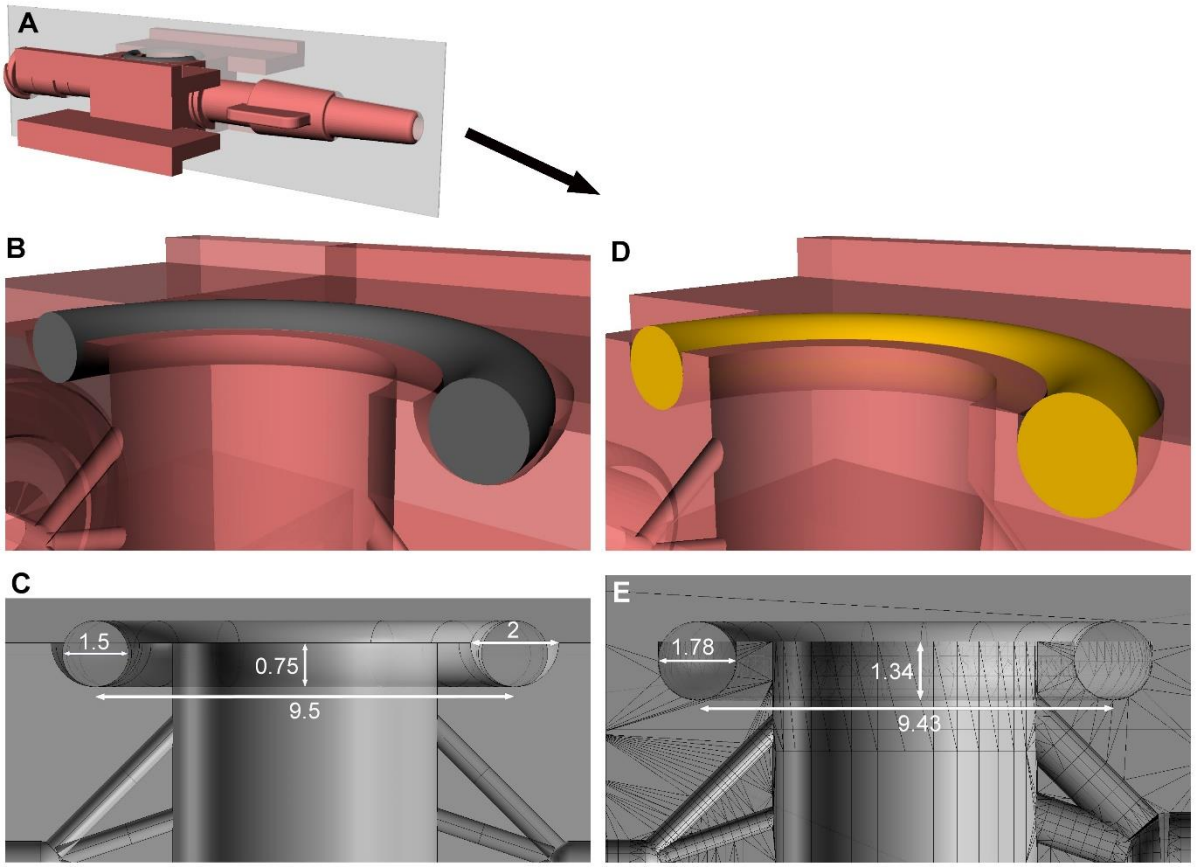


Figure 18: Dimensions of O-ring groove of Prometheus 1.0 and Prometheus 2.1.
(A) Plane showing the cross section.
(B) Cross section of Prometheus 1.0, perspective view.
(C) Cross section of Prometheus 1.0, side view, with dimensions of the O-ring and O-ring groove.
(D) and (E) Same as (B) and (C), but for Prometheus 2.1. Note that the O-ring is made from another material (represented by the different color) and has different dimensions. Additionally, the groove is deeper and less wide.

Calculations of tube length and diameter

The inlet tube (Figure 19A; tube 1) divides into four separate tubes (Figure 19A; tube 2a, 3a, 3b and 2b). Because these tubes make a different angle with tube 1, they have different lengths. According to the *Hagan-Poiseuille equation*, flow rate depends on the tube length:

$$\Delta Q = \frac{\pi P d^4}{128 \eta l}$$

ΔQ = flow
P = pressure difference
d = diameter of tube
η = viscosity
l = length of tube

Because we wanted to keep the flow rate the same for all tubes, we used this equation to calculate the radiuses of the tubes.

Taking P and η as a constant, the following formula was obtained:

$$\Delta Q = \frac{c \cdot d^4}{l}$$

c = undefined constant

in which c is a constant.

The area (A) depends on d^2: $A = \pi r^2 = \pi (\frac{1}{2} d)^2 = \frac{1}{4} \pi d^2$

So, we were able to express the flow rate in terms of the area:

$$\Delta Q = \frac{c \cdot A^2}{l}$$

A = area

By placing the area in front of the equal-sign, we obtained the following equation:

$$A^2 = c \cdot \Delta Q \cdot l$$

Apparently, A^2 had to be proportional to l when we want the flow rate to be the same. To make life easier, $c \cdot \Delta Q$ was replaced with a single constant (C):

$$A^2 = C \cdot l$$

We kept the total area all four channels together the same as the area of tube 1. By doing this, we obtained the following equations:

$$A_1 = A_{2a} + A_{2b} + A_{3a} + A_{3b} = 2 \cdot A_{2a} + 2 \cdot A_{3a}$$

A_1 = area of tube 1, etc.

We then filled in $A = \sqrt{C \cdot l}$ and grouped the variables together:

$$A_1 = 2 \cdot \sqrt{C \cdot l_{2a}} + 2 \cdot \sqrt{C \cdot l_{3a}} = 2 \cdot \sqrt{C} \cdot \sqrt{l_{2a}} + 2 \cdot \sqrt{C} \cdot \sqrt{l_{3a}} = 2 \cdot \sqrt{C} \cdot (\sqrt{l_{2a}} + \sqrt{l_{3a}})$$

l_1 = length of tube 1, etc.

The variables A_1, l_{2a} and l_{3a} were filled in to obtain the value of C:

$$0.785375 = 2 \cdot \sqrt{C} \cdot (\sqrt{4.242641} + \sqrt{3.162278})$$
$$\sqrt{C} = \frac{0.785375}{2 \cdot (\sqrt{4.242641} + \sqrt{3.162278})}$$
$$\sqrt{C} = 0{,}10234$$
$$C = 0.10234^2$$
$$C = 0.010468$$

Having obtained the value of C, we were able to calculate the area of tube 2a, 2b, 3a and 3b:

$$A_{2a} = \sqrt{C \cdot l_{2a}} = \sqrt{0.010468 \cdot 4.242641} = 0.210744$$

$$A_{3a} = \sqrt{C \cdot l_{3a}} = \sqrt{0.010468 \cdot 3.162278} = 0.181944$$

Radiuses were calculated from the area using $A = \pi r^2$:

$$A = \pi r^2$$
$$r = \sqrt{\frac{A}{\pi}}$$
$$r_{2a} = \sqrt{\frac{0.210744}{\pi}}$$
$$r_{2a} = 0.259006$$
$$r_{3a} = \sqrt{\frac{0.181944}{\pi}}$$
$$r_{3a} = 0.240658$$

The tube lengths, radiuses and areas are summarized in Table 4.

Initially, the radiuses of the inlet and outlet tubes were the same (Figure 19A). In the final model, the outlet tubes are much larger (Figure 19B) to prevent build-up of pressure in the chamber. The radiuses of the outlet tubes were calculated by multiplying the area of the inlet tubes with a factor 5 (Table 4).

Table 4: Lengths, radiuses and areas of the tubes in Prometheus 2.1 (in mm).

tube	length	radius	area
1	6	0.5	0.785375
2a and 2b	4.242641	0.259006	0.210744
3a and 3b	3.162278	0.240658	0.181944

tube	length	radius	area*
6	6	1.118034	3,926875
4a and 4b	4.242641	0.538128	1,05372
5a and 5b	3.162278	0.579154	0,90972

***** = 5 · area of inlet tubes

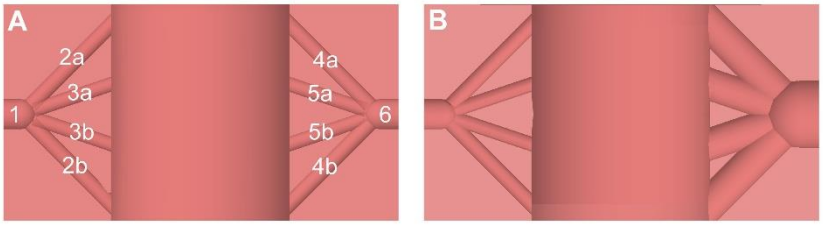


Figure 19: Tubes of Prometheus 1.0 and Prometheus 2.1, numbered.
(A) Tubes of Prometheus 1.0, numbered.
(B) Tubes of Prometheus 2.1. Note that tube 4a-6 are larger compared to the first version.

Appendix IV: Obtaining agarose microwells

Cell aggregates in gelMA were tested by a coworker[22]. The agarose microwells in which the aggregates were formed, were created by the author. The designing process will be described below. Subsequently, the features of the final model will be discussed, and at the end of this section the stamping procedure will be provided.

Designing Agarose Stamp 4.3

In order to create microwells using agarose, a stamp was designed and 3D printed using stereolithography technology (see methods section "Printing a flow perfusion chamber using stereolithography technology" for printing process). Several versions were created, each with one or a few adaptations. Not only the models of the agarose stamp were adapted during the process, we also had to optimize the stamping procedure.

We started with Agarose Stamp 1.0, a simple model created for a 48 well plate (Figure 20, Agarose stamp 1.0). It was printed from top to bottom, because we did not want to have any support structure on the stamping surface. In order to make the printing process more easy, the lower part of the model contains a cone. This cone makes an angle of >45° with the xy-plane, so no support material is needed there (the Perfactory® 3 Digital Shell 3D Printer (DSP) can print without support if the angle with the xy-plane is >45°). The only place where support material is needed is on top of the upper cylinder. To make the stamp fit precisely into a well from a 48 well plate (Greiner Bio-One), the inside diameter of the well was measured using a caliper. Since the wall of the well is not exactly vertical and because the stamp does not touch the ground, the diameter could not be precisely measured. Therefore we printed 4 stamps with different diameters: 10.7, 10.9, 11.1 and 11.3 mm. We chose the one which fitted best (diameter = 11.1 mm). By trying to stamp agarose using Agarose Stamp 1.0, a problem was encountered: an air bubble develops in the middle of the agarose when the stamp is pushed down.

In the next model (Agarose Stamp 2.0), we incorporated a tube to release the air (Figure 20, Agarose stamp 2.0). Using a syringe, the air bubble in the middle could be sucked away through the tube.

Stamping agarose using Agarose stamp 2.0 resulted in tiny microwells. However, when a cell suspension (50-200 cells/microwell) was added, only a few cells fell into the microwells and formed aggregates. The remaining cells formed large clusters on top of the agarose. This was probably caused by the small size of the microwells (200 µm). Because only a small amount of cells can be used per well, too many wells are needed to obtain a sufficient amount of aggregates.

To overcome this problem, we switched to a 12 well plate and 400 µm pyramids by creating Agarose Stamp 3.0 (Figure 20, Agarose stamp 3.0). Apart from a larger well diameter and larger pyramids, a few other adaptations were made. On top of the model, a connection to a syringe was created, to make it easier to suck away the air bubble. The stabilizer was expanded, because in a larger well more stabilization in the xy-plane is needed. In addition, the air tube was made thinner. This was done because at the lower inlet of the air tube (in the middle of the agarose surface), no microwells are created. This area thus needs to be as small as possible to have maximum efficiency.

Trying out Agarose Stamp 3.0, we still came across some issues. Air bubbles were still hard to get rid of, because in the 12 well plate they developed on several places instead of only in the middle. Additionally, the agarose tends to break when the stamp is taken off. This could also be a consequence of the larger well area of the 12 well plate compared with the 48 well plate. Lastly, the stamp has a weak point, just above the stabilizer. During cleaning or transportation, some of the stamps broke at this point.

Agarose stamp 4.0 (Figure 20, Agarose stamp 4.0) had several adjustments. To overcome the air bubbles issue, the air tube diameter was increased again. Additionally,an extra tube was created for release of the vacuum at the moment of stamp removal. At the time of stamping, the vacuum release tube is sealed at the upper side with a plug, so no agarose will get into the tube. The plug is removed after the agarose has hardened. This creates an opening for the air to flow between the agarose surface and the stamping surface, thus releasing the vacuum. The weak point was strengthened by the plug entrance.

Even with the new vacuum release system, the agarose in around half of the wells broke when the stamp was taken off. This was an improvement, but the situation was still not ideal. In addition, the stamp had

another weak point, just above the stabilizer.

We decided to try a different approach: directly printed microwells (Figure 20, R05 microwells). We called them 'R05 microwells', because of the stereolithography material photopolymer R05. Both a version with 200 µm microwells and one with 400 µm microwells was created. Coincidently, the R05 microwells fitted nicely into a 6 well plate, making the printed lid unnecessary. This model contained several benefits. Stamping in agarose was no longer needed, saving a lot of work and money. In addition, the material could be reused and autoclaved. On the other hand, the cells were not visible under the microscope, because one cannot look through the bottom of the well. The reason for rejecting this model was that the cells didn't form aggregates. Moreover, half of the cells got stuck on the surface, and half of the cells that were taken out of the well were dead.

To improve the outcomes of the Agarose stamp, we increased the percentage of agarose. Both 2% and 4% agarose gave excellent results compared to the former used 1%, only 10-20% of the wells broke. This outcome gave new hope for the Agarose stamp.

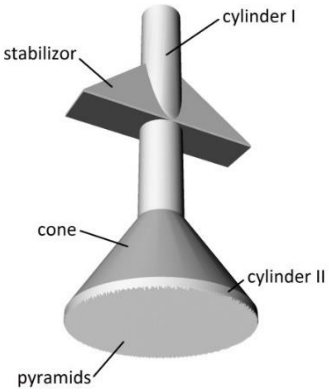
Using a different way of stamping, air bubbles could be prevented without needing any air tubes. The stamp is wetted with agarose first. Then, it is placed in an approximately 45° angle on the well and gently pushed horizontally. With these new techniques, Agarose stamp 4.1 was made much simpler. All tubes were removed and the core cylinder was made broader to provide more strength (Figure 20, Agarose stamp 4.1).

For a more sterile procedure, the stamp was adapted to enable closing the lid of the well plate, simply by cutting of the top part of the stamp (Figure 20, Agarose stamp 4.2). Unfortunately, this also resulted in a weaker stabilizer. It was easily broken during handling, especially since the only available material at this time was PIC100, which is less strong compared with photopolymer R05.

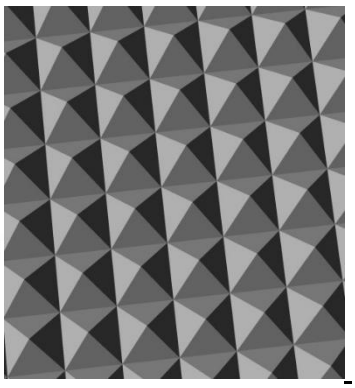
Agarose stamp 4.3 contained some extra stabilization (Figure 20, Agarose stamp 4.3). On one half, the space between the stabilizer and the lower cone needed to be kept open to be able to keep the stamp in a 45° angle during stamping. For this reason, the extra stabilization was only present on the other half. The extra stabilization was sufficient to keep the stabilizer from breaking.

The final model, Agarose stamp 4.3, is an efficient, reusable and reproducible stamp. It is suitable for the generation of agarose microwells, to obtain similar-sized cell aggregates, optionally consisting of multiple cell types [22].

Agarose stamp 1.0



10x zoom of pyramid surface



Dimensions:

in general:
Radius: 5.55
Height: 25

cylinder I
Radius: 1.5

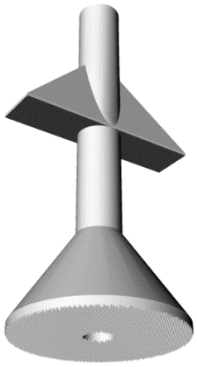
stabilizer:
Length: 13
Width: 3
Height: 7

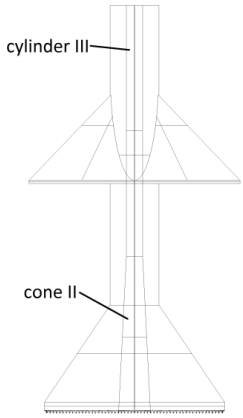
cone:
Height: 6

cylinder II:
Height: 0.500

pyramids:
Length: 0.200
Width: 0.200
Height: 0.200

Agarose stamp 2.0



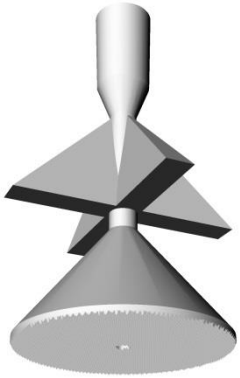


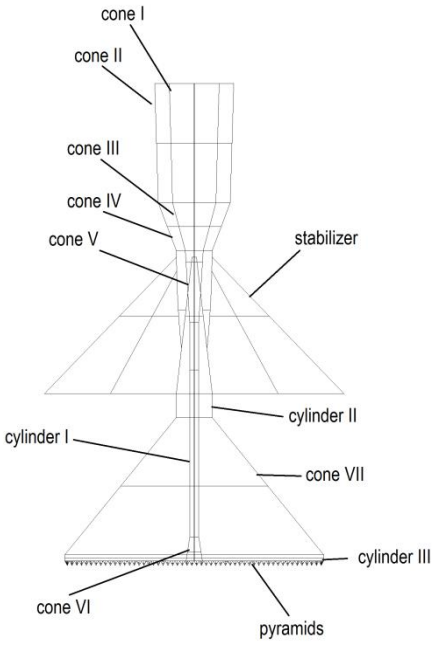
Dimensions:

cylinder III
radius: 0.5
height: 15

cone II
bottom r: 1
top r: 0.5
height: 10

Agarose stamp 3.0





Dimensions:

Cone I
top r: 2.080
bottom r: 1.807
height: 10

Cone II
top r: 3.25
bottom r: 3
height: 10

Cone III
top r: 1.807
bottom r: 0.75
height: 4

Cone IV:
top r: 3
bottom r: 1.5
height: 4

Cone V:
top r: 0.75
bottom r: 0.375
height: 6

Cone VI:
top r: 0.375
bottom r: 0.75
height: 2

Cone VII
top r: 1.5
bottom r: 10.8
height: 11.5

Cylinder I
radius: 0.375
height: 18

Cylinder II
radius: 1.5
height: 14

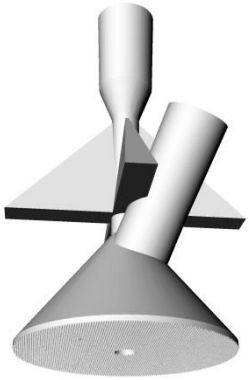
Cylinder III
radius: 10.8
height: 0.5

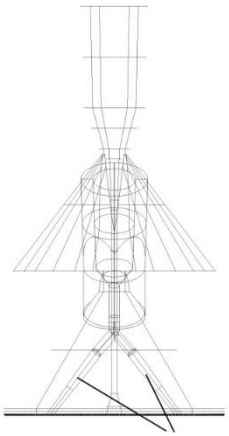
Stabilizer:
width: 12.5
height: 13

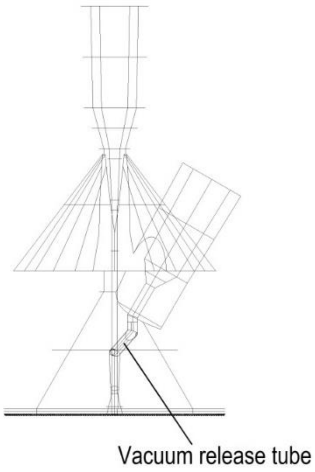
Pyramids:
Length/width: 0.4
height: 0.4

Figure 20: Agarose stamp models, in chronological order. (1)
On the right, the dimensions are given in mm.

Agarose stamp 4.0







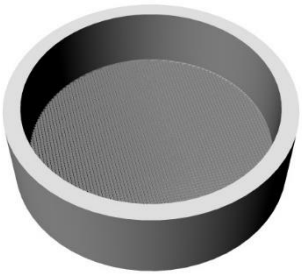
Dimensions:

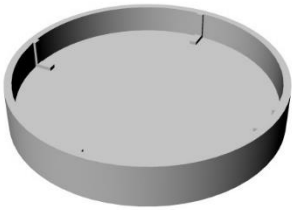
Air tube
r at bottom ≈ *1*

Air tube (vacuum release):
r at bottom ≈ *0.25*

Pyramids:
Length/width = *0.2*
Height = *0.2*

R05 microwells

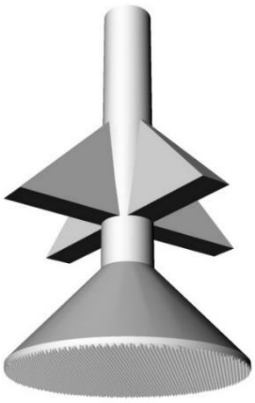

well

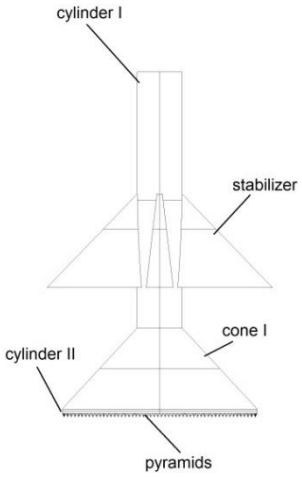

lid

Dimensions:

Well:		Lid:	
Inner r:	*15*	*Inner r:*	*17.5*
Outer r:	*17*	*Outer r:*	*18.5*
Thickness of base:	*2*	*Thickness of base:*	*2*
Height:	*10*	*Height:*	*8*

Agarose stamp 4.1





Dimensions:

Cylinder I
radius: *2.5*
height: *28.5*

Cylinder II
radius: *10.8*
height: *0.5*

Cone I
top r: *2.5*
bottom r: *10.8*
height: *9*

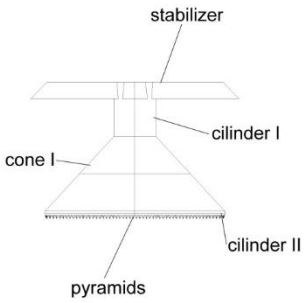
Pyramids
width: *0.4*
height: *0.4*

Cone I
top r: *2.080*
bottom r: *1.807*

Total height: *38*

Figure 20: Agarose stamp models, in chronological order. (2)

Agarose stamp 4.2



Agarose stamp 4.3

Dimensions:

Stabilizer		Cone I	
outer r:	*12.5*	*top r:*	*2.5*
inner r:	*10.6*	*bottom r:*	*10.8*
height:	*2*	*height:*	*9*
Cylinder I		**Pyramids**	
radius:	*2.5*	*width:*	*0.4*
height:	*6.5*	*height:*	*0.4*
Cylinder II		*Total height: 16.4*	
radius:	*10.8*		
height:	*0.5*		

Figure 20: Agarose stamp models, in chronological order. (3)

Features of Agarose stamp 4.3

Agarose stamp 4.3 has several features, which will be discussed from top to bottom. The cross-shaped part on top of the model (the stabilizer) keeps the stamping surface horizontal when the stamp is in the well. It also standardizes the height, so that it always leaves a 2 mm thick agarose layer. The stabilizer is only 2 mm in height and has a flat upper surface to allow the well plate to be closed when the agarose is solidifying (see stamping procedure). To keep the stamps stronger, also during the 3D printing process, two arms of the stabilizer are strengthened (Figure 21A,B; left two arms of the stabilizer). The other two arms are not strengthened, to keep space to rotate the stamp during the stamping procedure. The stamping surface is covered with tiny pyramids. They are printed with high resolution (50 µm layer thickness), as can be seen on the microscopic images (Figure 21C,D). The ground surface of each pyramid is 400x400 µm and the height is also 400 µm (Figure 21E).

After stamping, an agarose surface is left, containing 2290 microwells. It can be stored up to one week, covered in Hank's Balanced Salt Solution (HBSS; Gibco by life technologies). After adding a cell suspension, cells are evenly distributed and form aggregates overnight[22]. The aggregates can easily be removed with ice-cold Advanced DMEM/F12 (Gibco by life technologies).

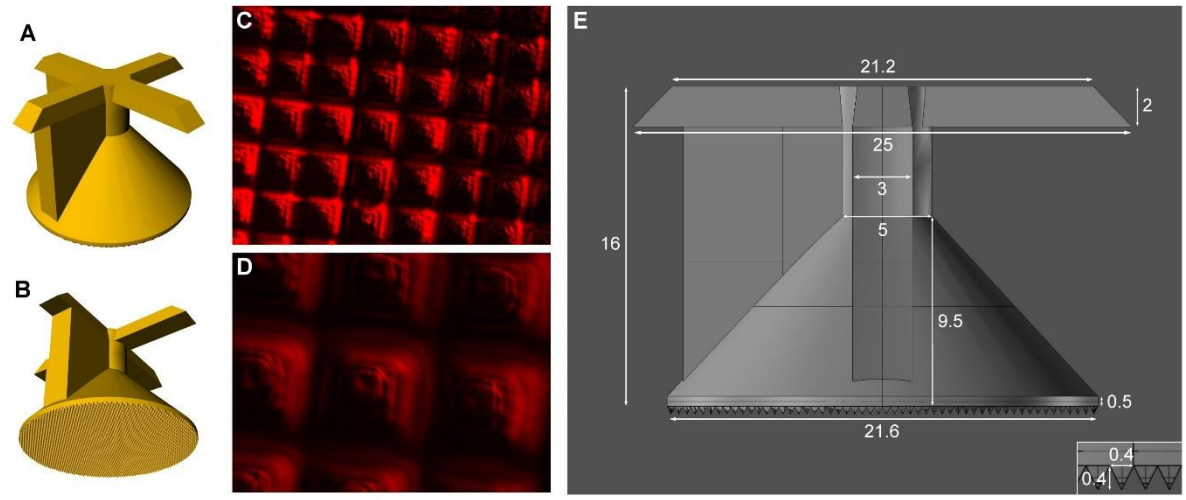


Figure 21: Agarose stamp 4.3
(A) and (B) Perspective view of Agarose stamp 4.3.
(C) and (D) Microscopic images of the pyramid surface (40x and 100x magnification, respectively)
(E) Dimensions (mm) of Agarose stamp 4.3, with a magnification of the pyramids in the lower right corner.

Stamping procedure

A 4% agarose solution (Sigma-Aldrich) was diluted 1:1 with HBSS to obtain a 2% solution. It was placed into a 70°C water bath until it had completely become liquid.

The stamps were placed on a paper tissue and thoroughly sprayed with alcohol. They were put inside the flow cabinet to dry for >5 minutes.

1.2 mL of 2% agarose was pipetted into 6-7 wells of a 12 well plate per time, in order to make sure the agarose was not yet hardened at the moment of stamping. 1.8 mL 2% agarose was pipetted into a well from a 6 well plate. Directly after pipetting the agarose, the stamps were put onto the wells containing agarose as follows: (1) the surface of the stamp was wetted by placing it into the well of the 6 well plate containing agarose; (2) the stamp was placed onto a well from the 12 well plate containing agarose. It was a bit rotated at the moment of touching the agarose and then gently turned horizontal, in order to prevent air bubbles to develop (Figure 22A).

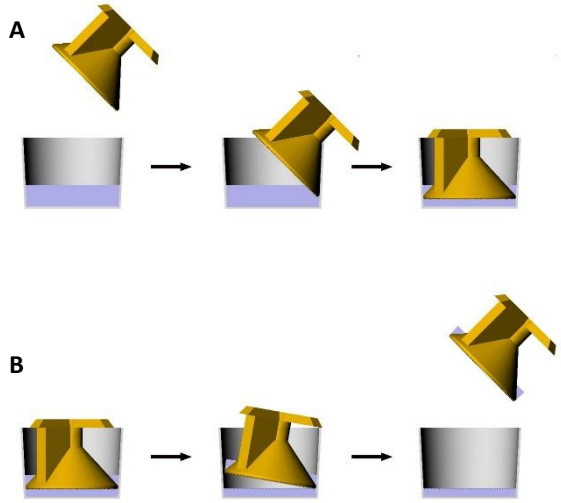


Figure 22: Stamping procedure
(A) The agarose stamp was rotated when it touched the agarose surface to prevent development of air bubbles.
(B) After hardening of the agarose, the stamp was gently taken off by first rotating it and subsequently pulling it up, using only minimal force.

This process was repeated until no stamps were left. The lid was put on the well plate again and it was left at room temperature. After 30 minutes, the stamps were taken off. First, the agarose above the stamp was broken on several placed (around 3) using a blue pipette tip in order to make space for the air, so it could release the vacuum. In order to prevent damage of the agarose surface, the stamp was gently taken off by first rotating it slightly and then pulling it op (Figure 22B). Only minimal force was used. The remaining agarose on the stamp and the 6 well plate was collected for reuse with a blue pipette tip. The procedure was repeated until the desired amount of agarose microwells was obtained.

Appendix V: Standard Operating Procedures (SOPs)

SOP 1: Preparing 6.25% w/v gelMA

Objective: Preparing a sterile 6.25% w/v gelMA hydrogel, which can be combined with a cell suspension to create a 5% w/v cell-laden gelMA hydrogel.

Materials

Equipment

- Flow cabinet
- Fridge (4°C)
- Water bath at 72°C
- Analytical scale

Supplies

- Nitrile gloves
- 2 15 mL Falcon tubes
- 1 50 mL Falcon tube
- Electronic pipette controller (Pipetboy)
- 2 sterile 10 mL pipette tips
- Weighing paper
- 0.22 µm syringe filter
  Syringe-driven filter unit
  Durapore® PVDF Membrane
  0.22 µm
  Supplier: Millex®-GV
  REF: SLGV033RS
- 10 mL syringe
  Supplier: Braun Omnifix®
  REF: 4616103V
- Scalpel blade no. 22
- Tin foil

Media and Chemicals

- Irgacure 2959 (PI)
  Supplier: Ciba Specialty Chemicals Inc.
  Item: 0298913AB
- Hanks' Balanced Salt Solution (1X) (HBSS)
  Supplier: Gibco by life technologies
  Ref. number: 14025-050
- Gelatin methacrylamide (gelMA), freeze dried
  Synthesized by orthopedics lab, UMC (Batch BK01)

Method

1. Weigh 12.5 mg photo initiator (PI) powder (= irgacure 2959) on a weighing paper using an analytical scale. Transfer it to a 15 mL Falcon tube.

2. Add 10 mL HBSS and let it dissolve in a 72°C water bath to obtain a concentration of 1.25 mg mL^{-1}. It takes around 15 minutes to completely dissolve.
3. Filter sterilize the solution using a 10 mL syringe and a 0.22 µm filter.
4. Weigh an empty 50 mL Falcon tube .
5. Cut around 350 mg freeze dried gelMA from the -20°C stock and weigh it at an analytical scale.
6. Calculate the amount of PI-supplemented HBSS as follows:

$$\text{Amount of PI-supplemented HBSS (mL)} = 16 \cdot \text{gelMA weight (g)}$$

7. Add the calculated amount of sterilized PI-supplemented HBSS.
8. Leave the tube wrapped in tin foil in the fridge (4°C) overnight.

SOP 2: Preparing 5% w/v cell-laden gelMA

Objective: Preparing a cell-laden 5% w/v gelMA hydrogel, operational for bioprinting or the production of gelMA pucks.

Materials

Equipment

- Flow cabinet
- Tabletop centrifuge
  Rotofix 32A
  Hettich Zentrifugen
- Water bath at 37°C

Supplies

- Nitrile gloves
- Gilson pipette with tips (2-20 µL and 100-1000 µL)

Media and Chemicals

- 6.25% w/v sterile gelMA as prepared in SOP1
- Cell suspension or organoid suspension in a 15 mL Falcon tube
- Advanced DMEM/F12 in case of organoids
  Supplier: Gibco by life technologies
  Cat. number: 12634-010
- Huh7 media in case of Huh7 cells (see Table 6 (SOP 20))
- Trypan Blue
  Supplier: Bio-Rad
  Cat. number: 145-0013

Method

1. Transfer 6.25% w/v gelMA from the fridge to the 37°C water bath.
2. Spin down the cell suspension at 1500 rpm (347 rcf).

3. Remove the supernatant and add 1 mL of 37°C media (in case of cells) or Advanced DMEM/F12 (in case of organoids).
4. Perform cell count as described in SOP 16 (starting with step 3, not in triplicate).
5. Spin down again at 1500 rpm (347 rcf).
6. Calculate the amount of media (in case of cells) or Advanced DMEM/F12 (in case of organoids):

$$\text{Amount of media or Adv. DMEM/F12 (mL)} = \text{amount of live cells} \cdot 10^{-7}$$

7. Add the calculated amount of media or Advanced DMEM/F12 and homogenize by pipetting up and down several times.
8. Add liquid 6.25% w/v gelMA from the water bath in a 4:1 ratio (gelMA : cell suspension) and homogenize by pipetting up and down several times.
9. Transfer the tube to the 37°C and keep it at 37°C until use.

SOP 3: Preparing 5% w/v gelMA (without cells)

Objective: Preparing a 5 % w/v gelMA hydrogel for use as a control or for printing trials.

Note:
If 6.25% w/v gelMA is already present, simply add HBSS in a 1:4 ratio (HBSS : gelMA).
If only freeze dried gelMA available, use the protocol below.

Materials

Equipment

- Flow cabinet
- Fridge (4°C)
- Water bath at 72°C
- Analytical scale

Supplies

- Nitrile gloves
- 2 15 mL Falcon tubes
- 1 50 mL Falcon tube
- Electronic pipette controller (Pipetboy)
- 2 sterile 10 mL pipette tips
- Weighing paper
- 0.22 µm syringe filter
  Syringe-driven filter unit
  Durapore® PVDF Membrane
  0.22 µm
  Supplier: Millex®-GV
  REF: SLGV033RS
- 10 mL syringe
  Supplier: Braun Omnifix®

REF: 4616103V
- Scalpel blade no. 22
- Tin foil

Media and Chemicals
- Irgacure 2959 (PI)
  Supplier: Ciba Specialty Chemicals Inc.
  Item: 0298913AB
- Hanks' Balanced Salt Solution (1X) (HBSS)
  Supplier: Gibco by life technologies
  Ref. number: 14025-050
- Gelatin methacrylamide (gelMA), freeze dried
  Synthesized by orthopedics lab, UMC

Method

1. Weigh 10 mg photo initiator (PI) powder (= irgacure 2959) on a weighing paper using an analytical scale. Transfer it to a 15 mL Falcon tube.
2. Add 10 mL HBSS and let it dissolve in a 72°C water bath to obtain a concentration of 1 mg mL^{-1}. It takes around 15 minutes to completely dissolve.
3. Filter sterilize the solution using a 10 mL syringe and a 0.22 µm filter.
4. Weigh an empty 50 mL Falcon tube .
5. Cut around 350 mg freeze dried gelMA from the -20°C stock and weigh it at an analytical scale.
6. Calculate the amount of PI-supplemented HBSS as follows:

$$\text{Amount of PI-supplemented HBSS (mL)} = 20 \cdot \text{gelMA weight (g)}$$

7. Add the calculated amount of sterilized PI-supplemented HBSS.
8. Leave the tube wrapped in tin foil in the fridge (4°C) overnight.

SOP 4: Preparing 40% w/v pluronic F127

Objective: Preparing a sterile 40% w/v pluronic F127 hydrogel for bioprinting.

Materials

Equipment
- Fridge (4°C)
- Water bath at 72°C
- Analytical scale

Supplies
- 2 50 mL Falcon tubes
- Electronic pipette controller (Pipetboy)
- 2 sterile 25 mL pipette tips

- Weighing paper
- 0.22 µm syringe filter
  Syringe-driven filter unit
  Durapore® PVDF Membrane
  0.22 µm
  Supplier: Millex®-GV
  REF: SLGV033RS
- 10 mL syringe
  Supplier: Braun Omnifix®
  REF: 4616103V
- Tin foil

Media and Chemicals

- Irgacure 2959 (PI)
  Supplier: Ciba Specialty Chemicals Inc.
  Item: 0298913AB
- Hanks' Balanced Salt Solution (1X) (HBSS)
  Supplier: Gibco by life technologies
  Ref. number: 14025-050
- Pluronic F127
  Supplier: Sigma-Aldrich
  Product number: P2443-250G
  Cas number: 9003-11-6

Method

1. Weigh 32 mg photo initiator (PI) powder (= irgacure 2959) on a weighing paper using an analytical scale. Transfer it to a 50 mL Falcon tube.
2. Add 32 mL HBSS and let it dissolve in a 72°C water bath to obtain a concentration of 1 mg mL^{-1}. It takes around 15 minutes to completely dissolve.
3. Weigh 12 g pluronic F127 in a 50 mL Falcon tube using an analytical scale.
4. Add 30 mL PI-supplemented HBSS. Keep at 4°C and shake thoroughly around every hour until completely dissolved (this will take at around a day).
5. Weigh the tube with content using an analytical scale.
6. Autoclave with the lid left slightly open, using the liquid program.
7. Weigh again and add the evaporated amount of water as filter-sterilized MilliQ.
8. Wrap in tin foil and keep at 4°C until use.

SOP 5: Sterilizing the bioprint cabinet and equipment.

Objective: To sterilize the bioprinter and bioprint cabinet, and to gather and sterilize equipment.

Materials

Equipment

- Autoclave
- Flow cabinet
- Bioprinter in flow cabinet
  - 3D Discovery
  - Supplier: RegenHU
- Water bath (bring from another lab)
- UV lamp BVL-4.LC (borrow from the orthopedics lab, contact: Vivian Mouser (v.h.m.mouser@umcutrecht.nl))
  - Wavelength: 245 or 365 nm
  - Supplier: Boom laboratory supplier
  - Cat. number: LLG9971917

Supplies

- Nitrile gloves
- Box of tissues
- Spray bottle for 70% ethanol
- Microvalve (CF100, MVC03-006)
  - Supplier: RegenHU
    - For contact dispensing
    - Nozzle diameter: 0.3 mm
    - Stroke: 0.06 mm
- Needle (CF300, N03G)
  - Supplier: RegenHU
    - Inner diameter: 0.3 mm
    - External diameter: 0.5 mm
    - Typ G
- Adapter (10-32, UNF G, conical)
  - Supplier: RegenHU
- Luer-Lock Adapter
  - Luer Ad
  - Supplier: RegenHU
  - For UNF 10-32 conical
- Cartridge heater
  - Supplier: RegenHU
- 2 10 mL cartridges
  - Supplier RegenHU
- 25 gauge needle (clear)
  - Supplier: RegenHU
- 1 piston
  - Supplier: RegenHU
- 1 box of microscope slides
- 4 small graduated beakers or petri dishes
- Around 7 50 mL Falcon tubes (depending on the amount of prints one intends to make)
- Tray for 50 mL falcon tubes
- Clean lab coat
- Extension cord (for UV lamp)
- Big shopper

Media and Chemicals

- 70% ethanol
- Hank's Balanced Salt Solution (1x) (HBSS)
  Supplier: Gibco by life technologies
  Ref. number: 14025-050
- 40% w/v pluronic F127 (prepared as described in SOP 4)

Method

At the laboratory of Veterinary medicine:

1. Autoclave the microvalve, together with its needle and adapter, inside a 50 mL Falcon tube using the 'solid' programme.
2. Fill the other 50 mL tubes with at least 35 mL sterile HBSS in a sterile flow cabinet. The amount of tubes depends on the amount of prints one intends to make.
3. Fill the spray bottle with 70% ethanol.
4. Bring the following materials to the printing facility (use a big shopper):
   - Autoclaved microvalve (with accessories), sterile in a 50 mL Falcon tube.
   - Around 6 50 mL tubes filled with sterile HBSS.
   - Tray for 50 mL Falcon tubes
   - Clean lab coat
   - UV lamp
   - Extension cord
   - Water bath
   - Nitrile gloves
   - Box of tissues
   - Box of microscope slides
   - 40% w/v pluronic F127
   - Spray bottle with 70% ethanol

At the printing facility:

5. Clean the cabinet of the bioprinter and spray it with 70% ethanol.
6. Spray all equipment listed below with ethanol and let it dry inside the bioprint cabinet:
   - Falcon tube containing sterilized microvalve with accessories
   - 2 10 mL cartridges
   - Luer lock adapter
   - 25 gauge needle
   - 1 piston
   - 4 small graduated beakers or petri dishes to collect flow through
   - All but one of the 50 mL Falcon tubes filled with HBSS
   - Around 30 microscope slides (number depends on the amount and height of the prints)
     Note: The microscope slides can be placed on a folded tissue (sprayed with 70% ethanol) at the edge of the bioprinter platform. In this way, they will not stick together and the ethanol will be evaporated sooner.
7. Put the cartridge heater in place (in cartridge 4). Do this before turning on the flow, because the safety hood needs to be entirely open to install the cartridge heater.
8. Pre-warm print head 4 by turning on the print head heater and cartridge heater at 37°C.
9. Turn on the air flow and UV-lamp, leave for 20-30 minutes.

10. Turn on the water bath at 37°C for when the cell-laden gelMA arrives. Put the tray for 50 mL Falcon tubes in the water bath and place one tube filled with sterile HBSS in the tray.

SOP 6: Transporting cell-laden gelMA to the printing facility

Objective: To safely transport cell-laden gelMA to the bioprinter, keeping it at 37°C until the hydrogel comes out of the print head.

Materials

Equipment

- Autoclave

Supplies

- Autoclavable jar (around 0.7 L)
- polystyrene box

Media and Chemicals

- demi-water
- cell-laden gelMA (prepared as described in SOP 2; inside a 15 mL Falcon tube, in the water bath)

Method

1. Fill a jar with demi water. Leave a few cm of air on top of the water level.
2. Autoclave the jar using the liquid program.
3. Place the jar in a 37°C water bath and leave it for at least 30 minutes, so that all the content is at the right temperature (37°C).
4. Just before transport, place the 15 mL tube containing cell-laden gelMA in the jar and place the jar in the polystyrene box.
5. Once arrived at the printing facility with the box, immediately transfer the 15 mL falcon tube to the 37°C water bath. The water bath should already have reached the right temperature at arrival (see SOP 5).

SOP 7: Filling cartridges and adjusting parameters

Objective: Filling the cartridges and adjusting all parameters to prepare for bioprinting, after carrying out this protocol the prints can be started.

Materials

Equipment

- Bioprinter
  3D Discovery
  Supplier: RegenHU
- Water bath (bring from another lab, see SOP 5)

Supplies

- Nitrile gloves
- Box of tissues
- Clean lab coat
- Spray bottle with 70% ethanol
- Autoclaved:
  - Microvalve (CF100, MVC03-006)
    Supplier: RegenHU
    For contact dispensing
    Nozzle diameter: 0.3 mm
  - Needle (CF300, N03G)
    Supplier: RegenHU
    Inner diameter: 0.3 mm
    External diameter: 0.5 mm
    Typ G
  - Adapter (10-32, UNF G, conical)
    Supplier: RegenHU
- Luer-Lock Adapter
  Luer Ad
  Supplier: RegenHU
  For UNF 10-32 conical
- 2 10 mL cartridges
  Supplier: RegenHU
- 1 piston
  Supplier: RegenHU
- 25 gauge needle (clear)
  Supplier: RegenHU
- 1 piston
  Supplier: RegenHU
- microscope slides
- 4 small graduated beakers or petri dishes

Media and Chemicals

- 70% ethanol
- 40% w/v pluronic F127 (prepared as described in SOP 4)
- cell-laden gelMA (prepared as described in SOP 1; inside a 15 mL Falcon tube, in the water bath)

Method

1. Place the microvalve, adapter, O-ring, and needle in print head 4 according to the 3D discovery® manual.
2. Attach the luer lock adapter and a 10 mL cartridge. Fill with 70% ethanol and flush at a pressure of 3 bar. Collect the flow through in a small graduated beaker or petri dish.

3. Do the same with around 10 mL 37°C HBSS from the water bath to remove any remaining ethanol.
4. Attach a 25 gauge needle to another 10 mL cartridge and fill with sterile 40% w/v pluronic F127. Place a piston on top.
5. Put the cartridge in place (printhead 1).
6. Flush until all air bubbles are removed (around 10 seconds) at 4.5 bar.
7. Perform needle length measurement for both print heads as described in the 3D discovery® manual.
8. Adjust printing parameters according to Table 5.
9. Transfer the cell-laden gelMA to the cartridge of print head 4.
10. Optimize XY-offset by printing two strands of pluronic F127 perpendicular to each other, and printing gelMA strands in the exact same way on top. Manually adjust any deviations in XY-direction in the software. Repeat until the lines are printed exactly on top of each other.

Table 5: Printing parameters for bioprinting a 3D liver construct (after optimization).

Parameter	gelMA	pluronic F127
pressure (bar)	0.5	4.5
needle (inner diameter in mm)	0.30	0.25
feed rate (mm/s)	20	15
layer thickness (mm)	0.22	0.22
valve openingstime (µs)	280	n/a
dosing distance (mm)	0.7	n/a
z-offset	0.3	0

SOP 8: Sterile bioprinting of cell-laden gelMA and pluronic F127

Objective: Printing a sterile cell-laden gelMA/pluronic F127 construct, which can be cultured after UV irradiation and washing away pluronic F127.

Materials

Equipment

- Bioprinter in flow cabinet
  3D Discovery
  Supplier: RegenHU
- Analytical scale
- All set up as described in SOP 5-7.

Method

1. Load the printing file, use one of these files from the folder "Mariette en Monique":

Height (mm)	Amount of constructs per print	File
11.88	1	2015-06-08 gelma + lutrol_04x3_7.5mm_clockwise-counterclockwise 1 construct

	2	2015-06-08 gelma + lutrol_04x3_7.5mm_clockwise-counterclockwise 2 constructs
	3	2015-06-08 gelma + lutrol_04x3_7.5mm_clockwise-counterclockwise
2.2	1	2015-07-01 gelma + lutrol_1 construct_2.2mm
	2	2015-07-01 gelma + lutrol_2 constructs_2.2mm
	4	2015-07-01 gelma + lutrol_4 constructs_2.2mm

Note: 11.88 mm constructs can be used for either static or perfused culture, 2.2 mm constructs can only be used for static culture.

2. Initiate printing. Keep watching to see whether everything goes according to plan. If necessary, abort the print and adjust parameters.
3. After a print has been finished, crosslink it inside the flow cabinet (SOP 9).
4. Check whether the microvalve is not obstructed by flushing it for only a second. Be careful not to waste too much gelMA. Flow through can be collected in a small graduated beaker or petri dish.
5. Repeat steps 1-3 until the desired amount of constructs is printed.
6. Determine the exact gelMA weight per print by firs weighing a microscope slide and subsequently printing a construct without pluronic F127. The construct should be low (around 2 mm) to save gelMA.
7. Weigh the microscope slide with the printed gelMA and subtract the weight of the microscope slide alone.
8. Calculate the amount of gelMA per construct using the exact height of the gelMA construct and the height of the other printed constructs.

SOP 9: UV crosslinking and washing away pluronic F127

Objective: Crosslinking gelMA, allowing it to maintain its structure at 37°C, and washing away pluronic F27.

Materials

Equipment

- UV lamp BVL-4.LC (borrow from the orthopedics lab, contact: Vivian Mouser (v.h.m.mouser@umcutrecht.nl))
  Wavelength: 245 or 365 nm
  Supplier: Boom laboratory supplier
  Cat. number: LLG9971917
- Fridge (4°C)

Supplies

- Glass slides
- Blue pipette tips
- Extension cord

Media and Chemicals

- 50 mL Falcon tubes containing sterile HBSS (see SOP 5 for preparation)

Method

1. Directly after a print is finished, put a glass slide on top of the constructs as shown in Figure 23, to prevent contact with oxygen. Oxygen namely prevents crosslinking by trapping the radicals formed by UV-irradiation.
2. Attach an extension cord to the UV lamp and clean it UV with a 70% ethanol sprayed tissue. Place the lamp inside the safety cabinet, on top of the glass slide covered print (Figure 23).
3. Irradiate constructs with 365 nm UV light (UV-A) for 15 minutes.
4. Gently remove top glass slide and transfer the bottom glass slide including the constructs to one of the 50 mL tubes containing HBSS.
5. Write down the time on the 50 mL tube and place it in the fridge.
6. After all constructs are printed and crosslinked, take the tubes back to the culture lab and place them directly in the fridge (4°C).
7. Inside the flow cabinet, take the constructs out one by one, keeping the washing times equal between prints. Blue pipette tips can be used to handle the constructs without touching. They can be cultured either static (SOP 10) or perfused (SOP 11). When using both conditions, divide the constructs from one print over the two conditions as much as possible, to make the conditions more comparable.

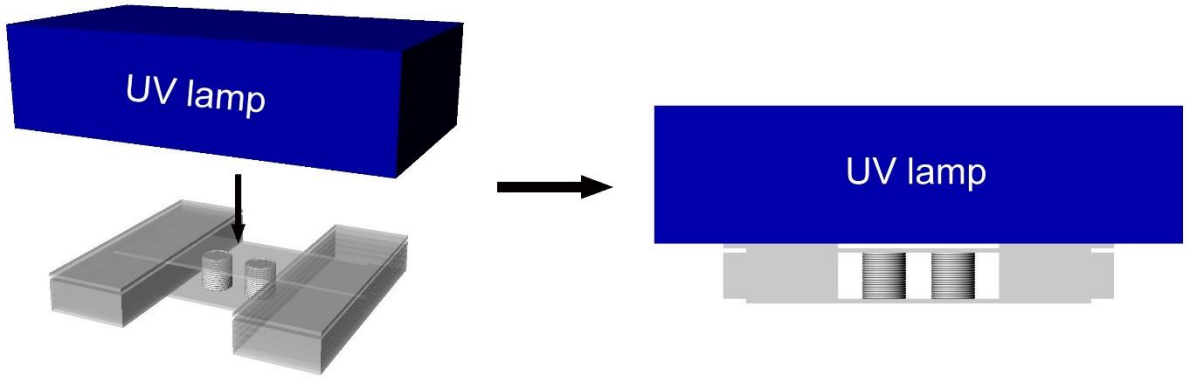


Figure 23: UV irradiation of constructs.
Left: Perspective view. Two microscope slide stacks were placed on the long ends of the printing slide. Another glass slide was placed on top, just touching the construct surface, thus preventing contact of gelMA with air. At the long ends, another two glass slides were placed to stabilize the UV lamp.
Right: Side view. The UV lamp is placed on top. The shining area of the lamp is in close to the constructs, increasing the crosslinking capacity.

SOP 10: Static culture of bioprinted cell-laden gelMA constructs

Objective: Culturing cell-laden gelMA constructs to perform several assays on them.

Materials

Equipment

- Flow cabinet
- Freezer (-20°C)
- CO_2 cell culture incubator

Supplies

- Nitrile gloves
- 24 well plate (Greiner Bio-One)
- Blue pipette tips (100-1000 µL)

Media and Chemicals

- Culture media (see Table 6 (SOP 20))
- Printed cell-laden gelMA constructs (see SOP 1-9)

Method

1. Transfer the constructs to a well 24 well plate using blue pipette tips (1 construct per well).
2. Add 1.5 mL culture media.
3. Refresh media daily as follows:
    - In case of only static culture: Refresh culture media daily and store the old media at -20°C for later use (ATP assay).
    - In case of comparison with a perfused culture: discard the old media and leave the new media on the constructs for 30 minutes. Take the media off and store it at -20°C. Add fresh media to continue culture.
      Note: The media is left on the constructs for only 30 minutes, because this is exactly the same amount of time in which 1.5 mL media passes through the construct in the perfused condition (with a flow rate of 3 mL $hour^{-1}$).

SOP 11: Perfused culture of bioprinted cell-laden gelMA constructs

Objective: Culturing cell-laden gelMA constructs in a perfused chamber and performing several assays on the constructs.

Materials

Equipment

- Flow cabinet
- Freezer (-20°C)
- Autoclave
- Water bath at 37°C
- Syringe pumps (1 per construct)
  Graseby™ 3500 infusion pump

Supplies

- Nitrile gloves
- Blue pipette tips
- 50 mL syringes (sterile) (1 per construct)
  Supplier: Braun OMNIFIX
- Clamps (4 per construct)

Supplier: Action (a Dutch store)
- Tubing (2 per construct)
  Combidyn-Druckslchlauch PE
  150 cm, TRANSPARENT
  Supplier: BRAUN
  Ref. number: 5215027
- Prometheus 2.1 (printed with stereolithography technology)
- Sillicon-rubber O-rings (2 per construct)
  Properties: Inner diameter = 7.65mm; cross section = 1.78mm
  Supplier: Polymax
  Article number: BS011SR70
- Tape
- Eppendorf tubes, not sterile (8 per construct, for 4 culture days)
- 24 well plate (Greiner Bio-One)

Media and Chemicals
- Culture media (see Table 6 (SOP 20))
- Printed cell-laden gelMA constructs (see SOP 1-9)

Method

1. Sterilize the desired amount of flow perfusion chambers (Prometheus 2.1) and O-rings using the autoclave (solid program).
2. Spray all other parts of the bioreactor set up (clamps, tubing, 60 mL syringes) thoroughly with 70% ethanol and let them dry inside the flow cabinet. (Tubing and 60 mL syringes are sterile packaged.)
3. Assemble Prometheus 2.1, with also the inlet tubing and outlet tubing attached.
4. Fill the 50 mL syringe with media and attach it to the inlet tubing.
5. Fill the chamber with media.
6. Take off the top microscope slide and place the bioprinted construct inside the cylindrical shaped chamber using blue pipette tips.
7. Put the second microscope slide back on top and secure it with clamps.
8. Take everything (only when fully attached!) out of the flow cabinet and built the set up as shown in Figure 24. Make sure the inlet tubing passes through the water of the water bath just before reaching Prometheus 2.1, thus reaching a temperature of 37°C. Attach the outlet tubing to the collection tube using tape, to make sure that it will maintain its place when leaving it.
9. Turn on the syringe pumps at 3 mL $hour^{-1}$.
10. Fill syringes and replace the collection tubes twice a day. From the collection tubes, store 1 mL in an Eppendorf tube each time and discard the rest.
11. At the final culture day, transfer the constructs to a 24 well plate to perform read outs.

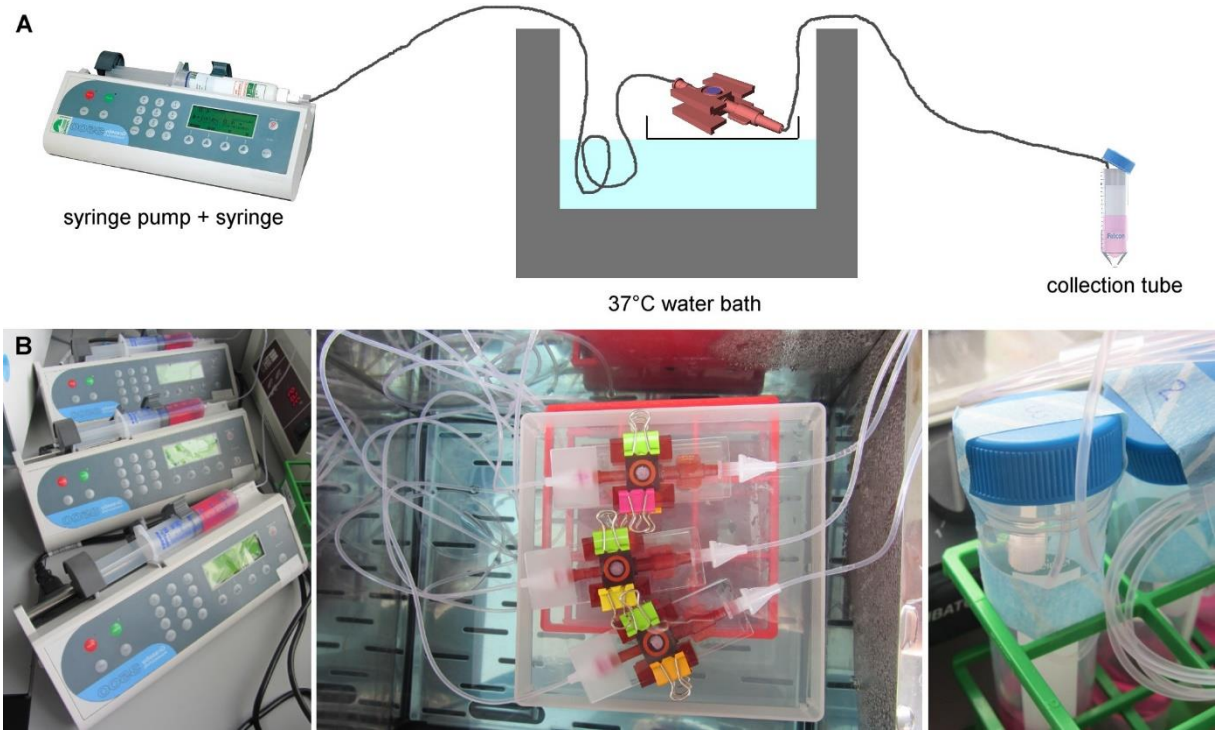


Figure 24: Set up for perfusion condition.
(A) Schematic set up of the perfusion experiment. The syringe is slowly emptied by the infusion pump (Graseby™ 3500). Media is heated by the water bath to 37°C before reaching the construct. After flowing through the construct, it is collected in a 50 mL Falcon tube (collection tube). The inside of the syringe, inlet tube and Prometheus 2.1 is kept sterile. The outlet tube however ends in a non-sterile Falcon tube.
(B) Photos of the actual experiment (n=3).

SOP 12: Pump testing

Objective: To test the accuracy of a syringe pump

Materials

Equipment

- 1 syringe pump
  Graseby™ 3500 infusion pump

Supplies

- 1 50 mL syringe
  Supplier: Braun OMNIFIX
- 1 Tube
  Combidyn-Druckslchlauch PE
  150 cm, TRANSPARENT
  Supplier: BRAUN
  Ref. number: 5215027
- 1 25 mL graduated cylinder
- Tape

Media and Chemicals

- Tap water

Method

1. Fill a 60 mL syringe with tap water and attach the tube to it.
2. Place the syringe into the syringe pump and place the end of the tubing into a 25 mL graduated cylinder. Secure it on top using tape.
3. Turn on the flow at 12 mL $hour^{-1}$ (=200 µL min^{-1}) and write down the starting time.
4. After at least one hour, stop the flow. Write down the time and the amount of water in the graduated cylinder.
5. Repeat step 3 and 4 two more times.
6. Calculate the flow of all 3 measurements, the mean, and the standard deviation using Excel.

SOP 13: Digestion of printed gelMA constructs

Objective: Digesting gelMA constructs to obtain a cell suspension suitable for cell count and/or other assays.

Materials

Equipment

- Orbital shaker
  Heidolph Titramax 1000

Supplies

- 15 mL Falcon tubes (amount of constructs + 1)

Media and Chemicals

- DMEM/Glutamax
  Supplier: Gibco
  Cat. number: 31966-021
- Enzymes in powder form or as 20 mg mL^{-1} stock solution at -20°C.
  Powder form:
  - Collagenase XI (powder)
    Collagenase from Clostridium histolyticum
    Supplier: Sigma-Aldrich
    CAS number: 9001-12-1
    KU-Pack Size: C9407-100MG
  - Dispase (powder)
    Supplier: Gibco
    Ref. number: 17105-041

Method

1. Turn on the heater of the orbital shaker (37°C).
2. If necessary, make a new stock solution of Collagenase XI and/or Dispase:
    a. Weigh 80 mg of Collagenase XI or Dispase.
    b. Dissolve it in 4 mL DMEM/Glutamax.
    c. Divide over 4 Eppendorf tubes using a blue pipette.
    d. Label the tubes and store them at -20°C.
    e. Every time a tube is taken out of the freezer and put back in, draw a small stripe on the lid.
3. Calculate the amount of Collagenase XI- and Dispase-solution to be used from the amount of gelMA per construct (measured at the end of printing). Use 28.4 mg of both enzymes per g gelMA. Make some excess by using the amount of constructs + 1. Calculation example:

8 constructs 90 mg gelMA/construct We need (8+1) * 0.090 * 28.4 = 23.0 mg of both enzymes This equals 23.0/20 = 1.15 mL stock solution Total volume = (8+1) * 1.5 mL = 13.5 mL So, we need:
11.2 mL DMEM/Glutamax 1.15 mL Collagenase XI stock solution 1.15 mL Dispase stock solution

4. Add 1.5 mL of the CollagenaseXI- and Dispase-containing DMEM/Glutamax to each well.
5. Transfer the well plate to the 37°C orbital shaker and turn it on. Leave for 20 minutes.
6. Look whether gelMA has dissolved yet. If not, check every 10 minutes. If after an hour nothing has happened, increase Collagenase XI and Dispase concentrations.
7. When all gelMA has visibly dissolved, transfer the content of each well to a 15 mL Falcon tube.
8. Wash the wells 2 times with cold Advanced DMEM/F12 to leave no cells behind.
9. Spin down at 1500 rpm (= 347 rcf) for 5 minutes and perform cell count as described in SOP 16 (starting from step 2).

SOP 14: Incubation with Triton X-100

Objective: To lyse cells in the printed construct by incubation with Triton X-100, for ATP measurement.

Materials

Equipment

- Flow cabinet
- Orbital shaker
    Heidolph Titramax 1000

Supplies

- Nitrile gloves
- Gilson pipette with tips (100-1000 µL)
- Eppendorf tubes (1 for every construct + 1)

Media and Chemicals

- Triton X-100
  Supplier: Sigma-Aldrich
  Ref. number: 9002-93-1
- Huh7 medium (see Table 6 (SOP 20)) in case of Huh7 cells
- Advanced DMEM/F12 in case of organoids
  Supplier: Gibco by life technologies
  Cat. number: 12634-010
- Bioprinted constructs or gelMA pucks in 24 well plate (see SOP 10/11)

Method

1. Turn on the heater of the orbital shaker at 37°C.
2. Make a solution of 1.25 mg mL^{-1} Triton X-100 in media (in case of Huh7 cells) or advanced DMEM/F12 (in case of organoids).
3. Discard the media from the constructs.
4. Add 1.5 mL of the Triton X-100 solution per well, including one empty well (control).
5. Place the plate on the orbital shaker and turn it on. Leave for 5 minutes.
6. In each well homogenize the solution by pipetting up and down around 3 times. Collect 1 mL in an Eppendorf tube for ATP measurements.
7. Discard well plate with constructs.

SOP 15: Alamar Blue assay

Objective: Measuring the viability in terms of the conversion of Alamar Blue by the cells.

Materials

Equipment

- Flow cabinet
- CO_2 cell culture incubator
- TECAN plate reader
  infinite M200

Supplies

- Nitrile gloves
- Gilson pipette with tips (100-1000 µL)
- 10 mL syringe
  Supplier: Braun Omnifix®
  REF: 4616103V

- 0.22 µm syringe filter
  Syringe-driven filter unit
  Durapore® PVDF Membrane
  0.22 µm
  Supplier: Millex®-GV
  REF: SLGV033RS
- 24 well plate (Greiner Bio-One)

Media and Chemicals

- Alamar Blue
  Supplier: Invitrogen
  Ref. number : DAL1025
- Hanks' Balanced Salt Solution (1X) (HBSS)
  Supplier: Gibco by life technologies
  Ref. number: 14025-050

Method

1. Turn of the light of the cabinet when workin with Alamar Blue.
2. Prepare Alamar Blue working solution (5% Alamar Blue in HBSS).
3. Filter sterilize the working solution.
4. Transfer the medium from each well separately to a well from a 24 well plate.
5. Wash cells with 1.5 mL HBSS.
6. Add 1.5 mL Alamar Blue working solution to each well, including one empty well.
7. Incubate cells for 2 hours in dark at 37°C.
8. Transfer 1 mL from each well to a well from a 24 well plate.
9. Wash the constructs with 1.5 mL HBSS.
10. Put the media back on the constructs/cells to continue culture.
11. Take the 24 well plate to the TECAN plate reader and measure fluorescence at 540/590nm for Alamar Blue.

SOP 16: Cell Count

Objective: Counting cells from a cell suspension, live and dead.

Materials

Equipment

- Automated Cell Counter (BIO-RAD TC20)

Supplies

- Gilson pipettes with tips (2-20 µL and other, depending on cell pallet size)

Media and Chemicals

- Huh7 medium (see Table 6 (SOP 20)) in case of Huh7 cells
- Advanced DMEM/F12 in case of organoids

Supplier: Gibco by life technologies
Cat. number: 12634-010

- Trypan Blue
  Supplier: Bio-Rad
  Cat. number: 145-0013

Method

1. Spin down the cell suspension and remove the supernatant.
2. Resuspend the cell pallet in an amount of media or Advanced DMEM/F12 ranging from 25-4000 µL, depending on the size of the pallet.
3. Transfer 3 x 10 µL to separate Eppendorf tubes (technical triplo).
4. Per Eppendorf tube, add 10 µL Trypan Blue and mix by pipetting up and down 10 times.
5. Load 10 µL of the suspension on a cell counter slide.
6. After 1 minute of incubation, count cells using the Automated Cell Counter.

SOP 17: ATP assay

Objective: Measuring the ATP content of a medium sample.

Materials

Equipment

- Luminometer
  LUMIstar OPTIMA
  BMG Labtech

Supplies

- 2 15 mL Falcon tubes
- Gilson pipettes (not sterile; 0.5-10 µL, 20-200 µL and 100-1000 µL)
- Multipipette with tip (volume: 10 mL)
  Electronic pipette / single-channel
  1 µl - 50 ml | Multipette® stream, Xstream
  Supplier: Eppendorf
- Polystyrene box with ice

Media and Chemicals

- Molecular Probes® ATP Determination Kit
  Supplier: Life technologies
  Cat. number: A22066
- Demi water
- Samples and controls

Method

1. Fill 1 15 mL Falcon tube with demi water.
2. Prepare a Standard Reaction Solution (SRS) in the other 15 mL Falcon tube, as described in the manufacturers' protocol (see Appendix VI ATP manufacturers' protocol). Keep protected from light as much as possible.
3. Wrap the tube with SRS in tin foil and leave on ice until use (no more than a few hours).
4. Prepare an ATP stock solution from the ATP stock solution in a cheap 96 well plate (without lid), diluting 1:1 with demi water each step. Set the highest concentration at 62.5-250 nM, depending on the expected ATP content in the samples. Make 9 dilutions and 1 well with only demi water. The volume per well should be above 40 µL (usually 75-150 µL).
5. Transfer 3 x 11.1 µL from each dilution to a white well plate (technical triplicate).
6. On the same well plate pipette 3 x 11.1 µL from each sample, including controls. Leave at least two wells empty as a second negative control.
7. Take the well plate with samples and ATP dilutions and the SRS together in a polystyrene box with ice to the luminometer. Also bring the multipipette with tips.
8. Add 100 µL SRS to each well using the multipipette. Leave 5 minutes at RT in the dark (already in the machine), to incubate before measuring luminescence.

SOP 18: MTT assay

Objective: Performing an MTT assay to determine cell viability.

Materials

Equipment

- Flow cabinet
- CO_2 cell culture incubator
- Orbital shaker
  Heidolph Titramax 1000
- Beckman plate reader
  Beckman Coulter® DTX 880
  Multimode Detector

Supplies

- Nitrilie gloves
- Cells with culture media on 96 well plate
- Tissues
- Tin foil

Media and Chemicals

- Thiazolyl Blue Tetrazolium Bromide
  Supplier: Sigma-Aldrich
  CAS number: 298-93-1
- Hanks' Balanced Salt Solution (1X) (HBSS)
  Supplier: Gibco by life technologies

Ref. number: 14025-050
- Culture media (see Table 6 (SOP 20))
- Dimethyl sulfoxide (DMSO)
  Supplier: Merck
  CAS number: 67-68-5

Method

1. Prepare a solution of 5 mg mL^{-1} Thiazolyl Blue Tetrazolium Bromide in HBSS (24 µL per well, including a set of wells with only media (control)).
2. Filter sterilize the solution.
3. Add 20 µL of the solution per well, including one set of wells containing only media (without cells; control).
4. Incubate 2 hours in the 37°C incubator, protected from light.
5. Carefully remove the media by decanting on a big layer of tissues.
6. Add 50 µl DMSO (depends on amount of formazan, use 100 µl DMSO when high).
7. Cover with tinfoil and agitate cells on orbital shaker for 15 min.
8. Read absorbance at 590 nm with a reference filter of 620 nm using the Beckman plate reader.

SOP 19: Thawing and plating cells

Objective: Thawing and plating cells for culture.

Materials

Equipment
- CO_2 cell culture incubator
- Water bath at 37°C
- Centrifuge (Rotofix 32A, Hettich Zentrifugen)
- Flow cabinet
- Automated Cell Counter (BIO-RAD TC20)

Supplies
- Polystyrene box with ice
- Large pincer
- Container for liquid nitrogen
- Protective gloves
- Protective glasses
- Nitrile gloves
- 15 mL Falcon tube
- Gilson pipette with tips (2-20 µL and 100-1000 µL)
- 1 box of pre-cooled green Gilson pipette tips (20-200 µL)
- Pipetting controller (Sartorius Midi Plus™ 710931)
- Sterile 10 mL pipettes
- Eppendorf tube

- T75 culture flasks (number depends on cell count)

Media and Chemicals

- Liquid nitrogen
- Culture medium in the water bath (37°C) (see Table 6 (SOP 20))
- Advanced DMEM/F12 (on ice)
  Supplier: Gibco by life technologies
  Cat. number: 12634-010
- Trypan Blue

Method

1. Pre-warm medium in water bath at 37°C.
2. Take out the vial from the -180°C freezer in liquid nitrogen.
   Safety note: Always wear protective gloves and glasses when handling liquid nitrogen.
3. Quickly defrost the cells by putting the tube in the 37°C water bath using a large pincer. Within a short time only a small piece of ice is visible, this is the moment the cells can be transferred to a 15 ml tube. Add up to 10-12 ml cold advanced DMEM/F12 to the tube and suspend the cells.
4. Spin down for 5 minutes at 1500 rpm.
5. Discard the supernatant. Resuspend the pellet of cells in 1 mL medium.
6. Count cells using the automated cell counter. Mix 10 µL of cell suspension and 10 µL of Trypan blue together in an Eppendorf tube and transfer 10 µL of this suspension to a cell counter slide. Count cells, write down the number of live cells and the percentage of live cells.
7. Depending on cell count, fill several T75 culture flasks with 10 mL medium.
8. Plate out between $2.5 \cdot 10^5$ and $5.0 \cdot 10^5$ cells per flask by pipetting the appropriate amount of cell suspension on top of the medium.
9. Place the culture flasks in the 37°C incubator for culture.

SOP 20: Passaging cells

Objective: Passaging cells to continue culture.

Materials

Equipment

- CO_2 cell culture incubator
- Flow cabinet
- Water bath at 37°C
- Centrifuge
  Rotofix 32A
  Hettich Zentrifugen
- Automated Cell Counter (BIO-RAD TC20)

Supplies

- Nitrile gloves
- Culture flask(s)
- Gilson pipettes with tips (2-20 µL, 20-200 µL and 100-1000 µL)
- Pipetting controller (Sartorius Midi Plus™ 710931)
- 10-25 mL pipettes (depending on size of culture flasks)
- 15 mL Falcon tubes (50 mL Falcon tubes in case of T175 culture flasks)

Media and Chemicals

- Hanks' Balanced Salt Solution (1X) (HBSS)
  Supplier: Gibco by life technologies
  Ref. number: 14025-050
- TrypLE (1x)
  Supplier: Gibco by life technologies
  Cat. number: 12604-013
- Trypan Blue
  Supplier: Bio-Rad
  Cat. number: 145-0013
- Culture media (see Table 6 for medium components)

Table 6: Medium components of cells

Cell type	Medium
Huh7 and LX2	89% Advanced DMEM/F12 Supplier: Gibco by life technologies Cat. number: 12634-010 10% FBS Supplier: Gibco by life technologies Cat. number: 16000-044 1% pen strep Supplier: Gibcy by life technologies Cat. number: 15140-122
HUVEC	Endothelial Cell Growth Medium Supplier: CELL Applications Inc.

Method

1. Pre-warm HBSS, TrypLE and culture media (Table 6) in the water bath.
2. Take the culture flask out of the incubator and into the flow cabinet.
3. Discard media.
4. Wash with 5, 10 or 30 mL pre-warmed HBSS (in case of a T25, T75 or T175 culture flask, respectively).
5. Discard HBSS and add pre-warmed TrypLE (Table 7).

Table 7: Amount of pre-warmed TrypLE to be added for detaching of cells (mL).

	T25	T75	T175
Huh7	0.5	1	3
LX2	1	2	6
HUVECs	0.5	1	3

6. Leave 5 (HUVECs) or 20 (Huh7 and LX2) minutes in the incubator to detach.

7. Observe cells under the microscope to see whether they are detached. If not, incubate a little longer and check every 5 minutes.
8. Take culture flask back to the cabinet.
9. Add 5, 10 or 30 mL pre-warmed media (in case of a T25, T75 or T175 culture flask, respectively).
10. Pipet up and down 5 times to collect cells.
11. Transfer cell suspension to a 15 mL Falcon tube (50 mL Falcon tube in case of T175).
12. Spin down 5 minutes at 1500 rpm (347 rcf).
13. Discard supernatant.
14. Dissolve cell pallet in 1 mL culture media. In case of a very large cell pallet, use 2 or 4 mL.
15. Perform cell count as described in (starting with step 3, not n triplicate).
16. Depending on cell count, plate out cells ($9 \cdot 10^4$ - $1.5 \cdot 10^5$ cells in T25; $2.5 \cdot 10^5$ - $5 \cdot 10^5$ cells in T75; and $7 \cdot 10^5$ - $1.5 \cdot 10^6$ cells in T175):
    a. Fill culture flask(s) with media (5 mL in T25, 10 mL in T75 and 30 mL in T175).
    b. Homogenize cell suspension by pipetting up at least 5 times.
    c. Pipet part of the cell suspension into the culture flask(s)
    d. Place flask(s) in incubator to continue culture.

SOP 21: Thawing and plating organoids

Objective: Thawing and plating organoids for culture.

Materials

Equipment

- CO_2 cell culture incubator
- Water bath at 37°C
- Centrifuge with temperature regulation (Eppendorf Centrifuge 5804R)
- Flow cabinet

Supplies

- 48 well plate (Greiner Bio-One)
- Polystyrene box with ice
- Large pincer
- Container for liquid nitrogen
- Protective gloves
- Protective glasses
- Nitrile gloves
- 15 mL Falcon tube
- Gilson pipette with tips (20-200 µL)
- 1 box of pre-cooled green Gilson pipette tips (20-200 µL)
- Pipetting controller (Sartorius Midi Plus™ 710931)
- Sterile 10 mL pipettes

Media and Chemicals

- Matrigel
  Supplier: BD Biosciences
  Cat. number: BD 356231
- Liquid nitrogen
- Culture medium components (see Table 8 (SOP 22))
- Advanced DMEM/F12 (on ice)
  Supplier: Gibco by life technologies
  Cat. number: 12634-010
- Hanks' Balanced Salt Solution (1X)
  Supplier: Gibco by life technologies
  Ref. number: 14025-050

Method

1. Prepare media no more than 6 hours before use (see Table 8 for medium components). Thaw components at 4°C (fridge) and keep on ice when outside the fridge.
2. Pre-warm 48-wells plate in incubator (37°C).
3. Pre-cool centrifuge to 4°C.
4. Put matrigel on ice.
5. Pre-warm medium in water bath at 37°C.
6. Take out the vial from the -180°C freezer in liquid nitrogen.
   Safety note: Always wear protective gloves and glasses when handling liquid nitrogen.
7. Quickly defrost the cells by putting the tube in the 37°C water bath using a large pincer. Within a short time only a small piece of ice is visible, this is the moment the cells can be transferred to a 15 mL tube. Add up to 10-12 mL cold advanced DMEM/F12 to the tube and suspend the cells.
8. Spin down for 5 minutes at 800-1000 rpm at 4°C.
9. Discard the supernatant. Resuspend the pellet of cells in 75 µL MatriGel (for 3 wells).
10. Use cold tips: put 25 µL matrigel-cell mixture like a drop in a pre-warmed 48-wells plate and transfer the plate to the incubator at 37°C for 10 minutes, to let the MG solidify.
11. Add 250 µL medium to each well.
12. Put around 250 µL HBSS in the surrounding wells to prevent drying of the medium.
13. Transfer the plate to the incubator and refeed every other day.

SOP 22: Passaging organoids

Objective: Passaging organoids to continue culture

Materials

Equipment

- CO_2 cell culture incubator
- Flow cabinet
- Water bath at 37°C

- Centrifuge with temperature regulation
  Eppendorf
  Centrifuge 5804R

Supplies

- Nitrile gloves
- 24/48 well plate
- Gilson pipettes with tips (2-20 µL, 20-200 µL and 100-1000 µL)
- Pipetting controller (Sartorius Midi Plus™ 710931)
- 10 mL pipettes
- 15 mL Falcon tubes

Media and Chemicals

- Advanced DMEM/F12 (on ice)
  Supplier: Gibco by life technologies
  Cat. number: 12634-010
- Matrigel
  Supplier: BD Biosciences
  Cat. number: BD 356231
- Culture medium components (see Table 8)

Table 8: Composition of expansion medium for human liver organoids.

constituent	Ordering information	% of total	storage
2x media	see Table 9	50	4°C, max. 1 week
Advanced DMEM/F12	Supplier: Gibco by life technologies Cat. number: 12634-010	44	4°C
R-spondin-conditioned medium	Provided by Calvin J Kuo	5,0	-20°C
n-Acetylcysteine (NAC)*	n-Acetylcysteine (NAC) Supplier: Invitrogen Cat. number: A9165-5G	0,25	-20°C
diluted A83**	Supplier: Tocris Bioscience Cat. number: 2939	0,5	4°C, max. 1 day
Forskolin	Supplier: Sigma-Aldrich	0,1	-20°C
FGF10***	Supplier: Peprotech Cat. number: 100-26	0,1	-20°C
Gastrin (GAS)#	Supplier: Sigma-Aldrich Cat. number: G9145	0,10	-20°C
EGF##	Supplier: Invitrogen	0,05	-20°C
HGF###	Supplier: Peprotech Cat. number: 100-39	0,05	-20°C

*NAC represents a 500 mM solution of *N*-Acetyl-L-Cysteine in milliQ
** diluted A83 was prepared by diluting A83-01 (from -20°C) 100x in Advanced DMEM/F12
***FGF10 represents a 100 µg mL^{-1} stock solution of FGF10 in PBS + 0.1% Bovine Serum Albumin (BSA)
#GAS represents a 10 µM stock solution of gastrin in PBS + 0.1% BSA
##EGF represents a 100 µg mL^{-1} stock solution of EGF in PBS + 0.1% BSA
###HGF represents a 50 µg mL^{-1} stock solution of HGF in PBS + 0.1% BSA

Table 9: Composition of 2x media.

constituent	Ordering information	% of total

Advanced DMEM/F12	Supplier: Gibco by life technologies Cat. number: 12634-010	86
Glutamax (x50*)	Supplier: Invitrogen Cat. number: 35050-061	2
Pen strep (x50)	Supplier: Gibcy by life technologies Cat. number: 15140-122	2
Hepes (x50)	Supplier: Invitrogen Cat. number: 15630-056	2
B27 supplement no vit. A (x25)	Supplier: Invitrogen Cat. number: 12587-010	4
N2 supplement (x50)	Supplier: Invitrogen Cat. number : 17502-048	2
Nicotinamide (NIC) (x50)**	Supplier: Sigma-Aldrich Cat. number: N0636-100G	2

*x50 means 50 times concentrated
**NIC (x50) represents a 1 M stock solution of Nicotinamide in milliQ

Method

Preparation:

1. Prepare media no more than 6 hours before use (see Table 8 for medium components). Thaw components at 4°C (fridge) and keep on ice when outside the fridge.
2. Pre-warm medium in water bath.
3. Cool centrifuge to 4°C.
4. Thaw matrigel at 4°C.
5. Pre-warm 24/48 well plates.
6. Pre-cool green pipette tips in freezer.
7. Pre-cool 15 mL Falcon tubes to collect the organoids in matrigel on ice.

Passage of organoids:

8. Discard culture media.
9. Add 0.5 or 1 mL ice-cold Advanced DMEM/F12 (for a 48 or 24 well plate, respectively). Break up matrigel by scraping and pipetting up/down around 3 times. Work fast to prevent warming up. Transfer up to max 4 to 2 wells (for a 48 or 24 well plate, respectively) to an ice-cold 15 mL Falcon tube.
10. Add cold Advanced DMEM/F12 up to max 10-12 mL to the tube and spin down 5 minutes at 800-1000 rpm (80-100 rcf).
11. Carefully discard supernatant.
12. Add 200 µL of new cold Advanced DMEM/F12. Break up the organoids by pipetting up and down using green pipette tips. Always keep cold!
13. Transfer a part (generally 1/6 to 1/8) to a new ice-cold 15 mL Falcon tube. If one wants to plate out more wells to expand the number of organoids, transfer a bigger part or leave everything in.
14. Add cold Advanced DMEM/F12 to max. 5 mL and spin down 5 minutes at 1500 rpm (407 rcf). Keep cold.
    Note: The amount of Advanced DMEM/F12 used in step 2 and 3 depends on the success of matrigel removal. The more matrigel is removed, the less medium can be used.
15. Discard as much supernatant as possible.

16. Resuspend pellet with matrigel (25 or 50 µL/well, for a 48 or 24 well plate, respectively). Put the matrigel-cell mixture in a "drop" in a pre-warmed 48/24 well plate.
17. Transfer the plate to the incubator and leave for 10 minutes.
18. Add 250 or 500 µL medium to each well, for a 48 or 24 well plate, respectively.
19. Put around the same amount of HBSS in the surrounding wells to prevent drying of the medium.
20. Transfer the plate to the incubator and refeed every other day.

Appendix VI: Manual of ATP determination kit

(see next 3 pages)

Molecular Probes™
invitrogen detection technologies

Product Information
Revised: 29–November–2005

ATP Determination Kit (A22066)

Quick Facts

Storage upon receipt:
- ≤–20°C
- Protect from light

Number of assays: 200–1000

Introduction

Molecular Probes' ATP Determination Kit (A22066) offers a convenient bioluminescence assay for quantitative determination of ATP with recombinant firefly luciferase and its substrate D-luciferin (Figure 1). The assay is based on luciferase's requirement for ATP in producing light (emission maximum ~560 nm at pH 7.8) from the reaction:

$$\text{luciferin} + \text{ATP} + O_2 \xrightarrow[\text{luciferase}]{Mg^{2+}} \text{oxyluciferin} + \text{AMP} + \text{pyrophosphate} + CO_2 + \text{light}$$

This assay is extremely sensitive; most luminometers can detect as little as 0.1 picomole of pre-existing ATP, or ATP as it is being formed in kinetic systems.[1] This sensitivity has led to numerous applications for detecting ATP production in various enzymatic reactions, including ATPase [2] and NADPH oxidase,[3] as well as for detecting low-level bacterial contamination in samples such as blood, milk, urine, soil and sludge.[4-8] The luciferin–luciferase bioluminescence assay has also been used successfully to study the effects of antibiotics on bacterial populations [9] and to distinguish cytostatic versus cytocidal potential of anticancer drugs on malignant cell growth.[10]

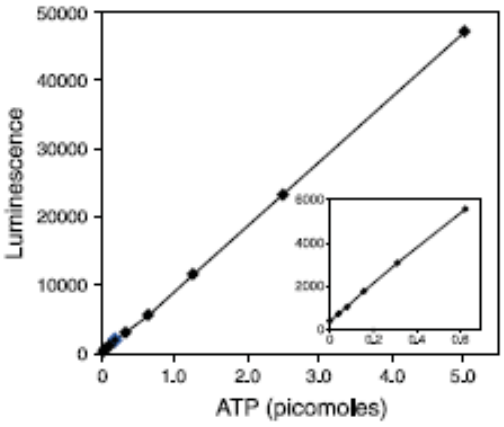


Figure 1. Detection of ATP using the ATP Determination Kit. Each reaction contained 1.25 µg/mL of firefly luciferase, 50 µM D-luciferin and 1 mM DTT in 1X Reaction Buffer. After a 15 minute incubation, luminescence was measured (arbitrary units).

Molecular Probes' ATP Determination Kit provides all of the reagents needed to perform this luminescence assay. In this kit, D-luciferin and firefly luciferase are packaged separately rather than premixed. The separate packaging allows researchers to optimize the proportions of D-luciferin and firefly luciferase for their particular system. This flexibility can be useful because the detection range of ATP will vary depending on instrument and sample requirements. A standard protocol is included that should be suitable for many routine applications; this protocol should also provide a starting point for customizing the assay. In addition to firefly luciferase and D-luciferin, this kit supplies a 20X concentrate of reaction buffer, dithiothreitol (DTT) for use in the reaction and an ATP solution for preparing standard curves.

Materials

Kit Contents

- **D-Luciferin** (Component A, MW 302, blue cap), 5 vials, each containing 3 mg of lyophilized powder
- **Luciferase, firefly recombinant** (Component B, red cap) 40 µL of a 5 mg/mL solution in 25 mM Tris-acetate, pH 7.8, 0.2 M ammonium sulfate, 15% (v/v) glycerol and 30% (v/v) ethylene glycol
- **Dithiothreitol (DTT)** (Component C, MW 154, black cap) 25 mg
- **Adenosine 5′-triphosphate (ATP)** (Component D, green cap), 400 µL of a 5 mM solution in TE buffer
- **20X Reaction Buffer** (Component E) 10 mL of 500 mM Tricine buffer, pH 7.8, 100 mM $MgSO_4$, 2 mM EDTA and 2 mM sodium azide

The ATP Determination Kit provides sufficient reagents to perform 200 ATP assays using 500 µL sample volumes or 1000 ATP assays using 100 µL sample volumes. The contents of this kit are sufficient to make at least 100 mL of a standard reaction solution (see step 2.1) containing 0.5 mM D-luciferin, 1.25 µg/mL firefly luciferase, 25 mM Tricine buffer, pH 7.8, 5 mM $MgSO_4$, 100 µM EDTA and 1 mM DTT.

Storage And Handling

Upon receipt, this kit should be stored at ≤–20°C, protected from light. For long term storage and infrequent use, store the

firefly luciferase frozen at –80°C. Avoid repeated freezing (at –80°C) and thawing. When properly stored, these reagents are stable for six months to one year.

Experimental Protocol

The following protocol has been developed as a generalized procedure suitable for many experimental applications. Optimal reaction conditions for specific experimental circumstances may be determined empirically by adjusting the amounts of D-luciferin and firefly luciferase added to the reaction. In the protocol below, a 10 mL "standard reaction solution" is prepared. This volume of standard reaction solution is sufficient for about twenty 500 µL reactions or about one hundred 100 µL reactions, depending upon luminometer requirements. For fewer or more assays, modify the protocol accordingly.

Special Precautions

- Because of the high sensitivity of the luciferin–luciferase reaction, avoid contamination with ATP from exogenous biological sources, such as bacteria or fingerprints.
- Protect the D-luciferin and firefly luciferase reagents from light as much as possible.
- Mix solutions containing firefly luciferase *gently*, for example, by inversion; vortex mixing may denature the enzyme.
- Arsenate compounds may inhibit the reaction.
- The optimum temperature for the reaction is 28°C. At higher temperatures, the reaction is slower.

Reagent Preparation

1.1 Make 1.0 mL of 1X Reaction Buffer by adding 50 µL of 20X Reaction Buffer (Component E) to 950 µL of deionized water (dH_2O). This volume will be sufficient to make 1 mL of 10 mM D-luciferin stock solution.

1.2 Make 1 mL of a 10 mM D-luciferin stock solution by adding 1 mL of 1X Reaction Buffer (prepared in step 1.1) to one vial of D-luciferin (Component A, blue cap). Protect from light until use. The D-luciferin stock solution is reasonably stable for several weeks if stored at ≤–20°C, protected from light.

1.3 Prepare a 100 mM DTT stock solution by adding 1.62 mL of dH_2O to the bottle containing 25 mg of DTT (Component C, black cap). Aliquot into ten 160 µL volumes and store frozen at ≤–20°C. Stock solutions of DTT stored properly are stable for six months to one year. Thawed aliquots should be kept on ice or at 4°C until ready for use.

1.4 Prepare low-concentration ATP standard solutions by diluting the 5 mM ATP solution (Component D, green cap) in dH_2O. The concentrations and volumes to make depend upon the sensitivity and design of the luminometer to be used. Typically, ATP concentrations ranging from 1 nM to 1 µM are appropriate. These dilute solutions are stable for several weeks when stored at ≤–20°C.

Standard Reaction Solution

2.1 We suggest combining the components of the reaction as follows to make 10 mL of a standard reaction solution. Adjust the volumes according to particular requirements.

- 8.9 mL dH_2O
- 0.5 mL 20X Reaction Buffer (Component E)
- 0.1 mL 0.1 M DTT (from step 1.3)
- 0.5 mL of 10 mM D-luciferin (from step 1.2, store the remaining 0.5 mL at ≤–20°C for up to several weeks)
- 2.5 µL of firefly luciferase 5 mg/mL stock solution

2.2 Gently invert the tube to mix, **do not vortex;** the firefly luciferase enzyme is easily denatured. Keep the reaction solution protected from light until use. Although the solution may be stored at 2–6°C protected from light for several days, assay sensitivity will diminish with time (see *Notes*).

Standard Curve

3.1 Place an appropriate volume of the standard reaction solution (prepared in steps 2.1 and 2.2) in the luminometer and measure the background luminescence.

3.2 Start the reaction by adding the desired amount of dilute ATP standard solution (prepared in step 1.4) and read the luminescence. The volume of the dilute ATP standard solution that is added to the standard assay solution (prepared in step 2.1) should be no more than 10% of the total assay volume. For example, a 100 µL total assay volume should contain 10 µL or less of the ATP standard solution.

3.3 Subtract the background luminescence.

3.4 Generate a standard curve for a series of ATP concentrations. Be sure to always add a constant sample volume of the ATP-containing solution.

Sample Analysis

4.1 Follow the directions given in *Standard Curve*, substituting ATP-containing samples for the ATP standard solutions. Please note that the total volume of the experimental sample assays should be equal to that of the ATP standard assays, with the amount of sample added amounting to no more than 10% of the total assay volume.

4.2 Calculate the amount of ATP in the experimental samples from the standard curve.

Notes

Standards must be run with each experiment. Fluorescence background levels of the working solutions increase over time, decreasing the sensitivity of the assay.

References

1. J Appl Biochem 3, 473 (1981); **2.** Biotechniques 31, 420 (2001); **3.** J Biol Chem 276, 3090 (2001); **4.** Anal Biochem 175, 14 (1988); **5.** Bio/Technology 6, 634 (1988); **6.** J Clin Microbiol 20, 644 (1984); **7.** J Clin Microbiol 18, 521 (1983); **8.** Meth Enzymol 57, 3 (1978); **9.** J Biolumin Chemilumin 6, 193 (1991); **10.** J Natl Cancer Inst 77, 1039 (1986).

Product List *Current prices may be obtained from our website or from our Customer Service Department.*

Cat #	Product Name	Unit Size
A22066	ATP Determination Kit *special packaging* *200-1000 assays*	1 kit

Contact Information

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Molecular Probes, Inc.
29851 Willow Creek Road, Eugene, OR 97402
Phone: (541) 465-8300 • Fax: (541) 335-0504

Customer Service: 6:00 am to 4:30 pm (Pacific Time)
Phone: (541) 335-0338 • Fax: (541) 335-0305 • probesorder@invitrogen.com

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Phone: (541) 335-0353 • Toll-Free (800) 438-2209
Fax: (541) 335-0238 • probestech@invitrogen.com

Invitrogen European Headquarters
Invitrogen, Ltd.
3 Fountain Drive
Inchinnan Business Park
Paisley PA4 9RF, UK
Phone: +44 (0) 141 814 6100 • Fax: +44 (0) 141 814 6260
Email: euroinfo@invitrogen.com
Technical Services: eurotech@invitrogen.com

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Several Molecular Probes products and product applications are covered by U.S. and foreign patents and patents pending. All names containing the designation ® are registered with the U.S. Patent and Trademark Office.

Appendix VII: Stereolithography materials, detailed description of composition

Photopolymer R05

Supplier: Envisiontec

Mixture acrylic acid esters and photoinitiator

Chemical composition:

Components	Approximate % by weight	C.A.S. No. & EINECS No.	UK/EU Classification according to Directive 67/548/EEC
1. Acrylated oligomer	20-60%	Proprietary	Xi; R36/38, R43
2. Acrylated monomer	5-30%	Proprietary	Xi: Irritant
3. Acrylated polyol	5-30%	5888-33-5	None
4. 1,6-Hexanediol acrylate	1-10%	13048-33-4	Xi; R36/38, R43
5. Photoinitiator	0.1-5%	Proprietary	F: Highly flammable Xn: Harmful N: Dangerous for the environment

PIC100

Supplier: Envisiontec

Chemical composition:

Chemical composition	CAS-No.	Percentage	Symbol(s)	R-phrase(s)
Methacrylate monomer	Trade secret	60-90 %	Xi	36
Acrylate monomer	Trade secret	0-35 %	Xi	36/38/43
Photoinitiator	119313-12-1	0.1 - 5%	Xn	48/22

Appendix VIII: Courses and other activities

Courses

Course name	Moment of participation	Course provider	Trainer	Credits	Course Fee
3D Printing and Biofabrication	July 2014	Utrecht University, Faculty of Medicine	multiple trainers	1.5 ECTS	€250,-
Modern Methods in Data Analysis	January 2015	Utrecht University, Faculty of Veterinary Medicine	multiple trainers	4.5 ECTS	-
Writing a Scientific Paper	March-July 2015	Utrecht University, Faculty of Veterinary Medicine	Annemarie van der Zeeuw	3 ECTS	€300,-
Presenting in English	July 2015	Utrecht University, Faculty of Veterinary Medicine	Margo de Wolf	1 ECTS	Free of charge for PhD candidates registered with the Graduate School of Life Sciences and employed by the faculties of Science, Veterinary Medicine or UMC Utrecht

Course description:

3D Printing and Biofabrication
A 1-week full time course with plenty of different speakers from all over the world. Very intriguing presentations, for instance the replacement of the upper part of a skull with a custom designed 3D printed part. The course ended with presentations given by groups of students.

Modern Methods in Data Analysis
A 3-week course about various statistical methods. Per method, the theory as well as the application were discussed. Exercises were performed using the computer programs R and SPSS. At the end of the course an exam was performed, which was successfully completed.

Writing a Scientific Paper
The course consists of 6 3-hour sessions, accompanied by some homework per session and a writing assignment. The writing process as well as the content of the several sections of a Scientific Paper were discussed. In addition, the most important pitfalls of writing a scientific paper were discussed and some explanation was given about popular science. For the writing assignment a popular scientific article was written.

Presenting in English
The course consists of 3 3-hour sessions in a small group of only 4 people. For every session, one or two presentations were prepared by the student. Because of the small group size, very personal feedback could be given.

Other activities

14-01-2015	Presentation in Dutch (1 hour) at 'Wetenschapscafé' (in a retirement home). Public: Retired people, interested in scientific topics. Some suffering from dementia or other mental/physical disadvantages.
15-07-2015 & 16-07-2015	2 presentations in English (30 minutes per presentation) at Utrecht Summer School: 3D Printing and Biofabrication. Public: Some students, but mainly PhD candidates and postdocs, from all over the world.