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"Liver-on-a-Chip" cultures of primary hepatocytes and Kupffer cells for hepatitis B virus infection

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TITLE:

"Liver-on-a-Chip" Cultures of Primary Hepatocytes and Kupffer Cells for Hepatitis B Virus Infection

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KEYWORDS:

Hepatitis B virus, microfluidic device, tissue culture, organ-on-a-chip, bioengineering, hepatocytes, Kupffer cells

SUMMARY:

The goal of this protocol is to provide a step-by-step guide to perform 3-D "liver-on-a-chip" infection experiments with the hepatitis B virus.

ABSTRACT:

Despite the exceptional infectivity of the hepatitis B virus (HBV) *in vivo*, where only three viral genomes can result in a chronicity of experimentally infected chimpanzees, most *in vitro* models require several hundreds to thousands of viral genomes per cell in order to initiate a transient infection. Additionally, static 2-D cultures of primary human hepatocytes (PHH) allow only short-term studies due to their rapid dedifferentiation. Here, we describe 3-D liver-on-a-chip cultures of PHH, either in monocultures or in cocultures with other nonparenchymal liver-resident cells. These offer a significant improvement to studying long-term HBV infections with physiological host cell responses. In addition to facilitating drug efficacy studies, toxicological analysis, and investigations into pathogenesis, these microfluidic culture systems enable the evaluation of curative therapies for HBV infection aimed at eliminating covalently closed, circular (ccc)DNA. This presented method describes the set-up of PHH monocultures and PHH/Kupffer cell co-cultures, their infection with purified HBV, and the analysis of host responses. This method is particularly applicable to the evaluation of long-term effects of HBV infection, treatment combinations, and pathogenesis.

INTRODUCTION:

The study of HBV has been complicated by the poor susceptibility of culture systems, requiring several hundreds to thousands of HBV genome copies per cell to initiate the infection[1].

Furthermore, primary human hepatocytes are generally exceptionally fragile and rapidly dedifferentiate during conventional cultures[2]. This is mainly due to the fact that the flat and hard plastic surfaces do not mimic the natural extracellular environments found within the liver and the general lack of oxygenation of the cultures in the absence of microfluidic circulation. Conventional static hepatocyte cultures on collagen-coated plates rapidly dedifferentiate and lose their susceptibility to HBV infection[3]. Here, we describe the set-up and infection of PHH grown in 3-D liver-on-a-chip cultures, which are vastly advantageous over conventional 2-D static PHH cultures on collagen-coated plates due to their extended metabolic and functional competence, facilitating long-term cultures of at least 40 days[4]. In this system, PHH are seeded on collagen-coated scaffolds, which are continually perfused with growth medium to supply oxygen and nutrients to the cells. Even though alternative culture systems for PHH based on complex cocultures of murine fibroblasts or 3-D growth in spheroids have been validated and are susceptible to HBV infection using multiplicities of infection of 500 genome equivalents (GE) of HBV per cell, 3-D liver-on-a-chip cultures remain the sole *in vitro* model system susceptible to 0.05 GE of HBV per cell[4]. This is additionally underpinned by the necessity of using high concentrations of dimethyl sulfoxide (DMSO) and polyethylene glycol (PEG) to establish HBV infection in these cultures, which is dispensable for the infection of -3D liver-on-a-chip culture systems[4]. Among the major hallmarks of HBV infection is the cccDNA pool, which acts as the transcriptional template for all *de novo*-produced virions[5,6]. Even though cccDNA can be detected in conventional hepatocyte cultures[7,8], it remains unclear as to whether the regulation of cccDNA and any therapeutic approaches aimed at its elimination are recapitulated in partially or completely dedifferentiated hepatocytes. We have shown that cccDNA is functionally formed in 3-D liver-on-a-chip cultures, responds to physiological stimuli, and can be targeted by interfering with the accessibility of the transcriptional machinery to the cccDNA genome[4].

Host responses to HBV infection in 3-D liver-on-a-chip mimic those observed in HBV-infected patients, enabling the identification of biomarkers for infection, as well as therapeutic success. Among the unique features of liver-on-a-chip cultures is the ability to evaluate long-term host responses between PHH and other nonparenchymal cells within the liver, including Kupffer cells[4], stellate cells[9], and liver sinusoidal endothelial cells[10,11]. This offers the unique opportunity to evaluate cell/cell interactions in a complex 3-D microenvironment.

Additionally, the extended culture period of these platforms facilitates the evaluation of sequential drug treatments and their impact on HBV persistence, which are not possible using conventional hepatocyte culture systems.

This protocol describes how 3-D liver-on-a-chip cultures are generated, either for monocultures of PHH or for cocultures of PHH with Kupffer cells. Furthermore, we describe the production of purified HBV for low-multiplicity-of-infection studies, as well as the subsequent analysis of host and viral responses.

PROTOCOL:

1. Assembly and Equilibration of Plates

1.1. Ensure that both the compressor and the vacuum pump associated with the LiverChip platform are turned on. Perform the assembly and equilibration of the plates in a class II cabinet.

1.2. Aseptically assemble the microfluidic plates by placing a sterile membrane between the plate base and adding the well-containing top plate (**Figure 1a**).

1.3. Ensure that the sterile membrane smoothly rests on the two pins of the base plate, since uneven membrane placement compromises the microfluidic circulation.

1.4. Add a sterile plate lid and tighten the screws at the base of the plate using an automated precision torque to 33 lb using a spiral tightening sequence. Ensure that all screws are tightened to 35 lb using a manual torque. During this step, ensure the screws are tightened symmetrically (**Figure 1a**).

1.5. Prewarm hepatocyte seeding medium containing Williams E medium, primary hepatocyte thawing, and plating supplements, 5% fetal bovine serum (FBS), and 1 µM dexamethasone to 37 °C before priming.

1.6. Prime the completely assembled plate by placing it within the washing dock and adding 400 µL of hepatocyte seeding medium to the reservoir side of each well. Ensure the plate snaps into the washing dock completely.

1.7. Initiate flow in the upward direction for 3.5 min at 1 µL/s. The successful function of the microfluidic circulation can be ascertained through the red indicators at the side of the plate (**Figure 1a**).

1.8. Once the medium is pumped to the cell growth side of the plate, ensuring the correct assembly of the flow channel, add an additional 1.2 mL of hepatocyte seeding medium.

1.9. Carefully transfer the plate into the docking station within a humidified incubator at 37 °C and 5% CO_2 and initiate flow in the upward direction at a flow rate of 1 µL/s for 16 h (**Figure 1c**).

1.10. Transfer the plate to the washing dock and eliminate bubbles in the well by gently pipetting up and down.

1.11. Add one sterile, round filter paper, followed by a cell attachment scaffold and a retaining ring, to each well using sterile forceps. Press down each well with a sterile plunger to lock the retaining rings and scaffolds into place (**Figure 1b**).

1.12. Aspirate all medium and add 400 µL of prewarmed hepatocyte seeding medium gently over the scaffold and initiate flow in the downward direction for 3.5 min at 1 µL/s.

1.13. Aspirate all medium pumped out of the reservoir side of the plate. This step is necessary to replace the medium contained in the flow channel.

1.14. Add 1.4 mL of hepatocyte seeding medium to each well before returning the plate to the dock to complete the total volume per well. The volume per well is now 1.6 mL (1.4 mL in the well and 0.2 mL in the flow channel).

2. Thawing and Seeding of Hepatocytes for Monocultures

2.1. Prewarm hepatocyte thawing medium and hepatocyte seeding medium to 37 °C prior to thawing one vial of PHH according to the suppliers' instructions. Use a centrifuge at room temperature during this step to avoid abrupt temperature changes. Perform the thawing and seeding of the hepatocytes in a class II cabinet.

2.2. Resuspend the cells in 1 mL of hepatocyte seeding medium and count the cells using trypan blue. Ensure that the viability of the cells is above 90%.

2.3. Keep the cells on ice until they are added to the wells.

2.4. Transfer the equilibrated and fully assembled plate to the washing dock and aspirate all medium from the wells.

2.5. Add 600,000 hepatocytes to each well in a 500-µL volume of hepatocyte seeding medium.

2.6. Initiate flow in the downward direction at a flow rate of 1 µL/s and bring the total volume in the well to 1.6 mL by adding 900 µL of hepatocyte seeding medium.

2.7. Transfer the plate to the docking station within a humidified incubator at 37 °C and with 5% CO_2.

2.8. Initiate flow in the downward direction at a flow rate of 1 µL/s for 8 h, followed by a flow reversal to the upward direction at a flow rate of 1 µL/s for 8 h.

2.9. Transfer the plate to the washing dock and aspirate all medium from the wells.

2.10. Add 400 µL of hepatocyte maintenance medium (Williams E medium supplemented with hepatocyte maintenance supplements and 100 nM dexamethasone) to each well and initiate flow in the downward direction at a flow rate of 1 µL/s for 3.5 min.

2.11. Aspirate all medium from the reservoir and add 1.4 mL of hepatocyte maintenance medium (**Figure 1d**).

2.12. Replace the medium with hepatocyte maintenance medium every 48 h. To ensure all medium in the well is replaced, perform a washing step before the addition of fresh hepatocyte

maintenance medium.

2.13. For the washing step, transfer the plate to the washing dock, aspirate all medium from the wells, and add 400 µL of maintenance medium.

2.14. Initiate flow in the downward direction at 1 µL/s for 3.5 min. Aspirate all medium appearing on the reservoir side of the wells.

2.15. Add 1.4 mL of hepatocyte maintenance medium and, within a humidified incubator at 37 °C and with 5% CO_2, transfer the plate into the docking station and initiate flow in the upward direction at a flow rate of 1 µL/s for 48 h (**Figure 1f**).

Note: For hepatocyte monocultures used as controls for cocultures, in order to ensure controlled conditions, a second type of maintenance medium is used, which is specific for use in the cocultures with primary human Kupffer cells. 48 h after replacing the hepatocyte seeding medium with hepatocyte maintenance medium (day 3 post-seeding), the regular hepatocyte maintenance medium will be replaced with coculture maintenance medium II, especially in monocultures of PHH when comparing to cocultures of both PHH and Kupffer cells, as the medium components differ slightly.

3. Thawing and Seeding of Kupffer Cells and Hepatocytes for Cocultures

3.1. In order to ensure an accurate comparison of results, always compare PHH/Kupffer cell cocultures to PHH monocultures

3.2. For cocultures of PHH and Kupffer cells, thaw one vial of Kupffer cells in advanced Dulbecco's modified Eagle's medium (AdDMEM) without dexamethasone but supplemented with primary hepatocyte thawing and plating supplements (coculture seeding medium) according to the suppliers' instructions. Perform the thawing and seeding of the Kupffer cells and hepatocytes in a class II cabinet.

3.3. Resuspend the cells in 1 mL of coculture seeding medium and count the cells using trypan blue. Ensure that the viability of the cells is above 90%.

3.4. Keep the cells on ice prior to adding them to the wells to avoid cell adhesion.

3.5. Follow the instructions in steps 2.1 - 2.3 for the thawing of primary human hepatocytes.

3.6. Transfer the equilibrated and fully assembled plate to the washing dock and aspirate all medium from the wells.

3.7. Add 60,000 Kupffer cells and/or 600,000 hepatocytes to each well in a total volume of 250 µL each of coculture seeding medium.

3.8. Initiate flow in the downward direction at a flow rate of 1 µL/s and add 900 µL of coculture seeding medium to each well.

3.9. Transfer the plate into the docking station within a humidified incubator at 37 °C and with 5% CO_2.

3.10. Initiate flow in the downward direction at a flow rate of 1 µL/s for 8 h, followed by flow reversal to the upward direction at a flow rate of 1 µL/s for 8 h.

3.11. Transfer the plate to the washing dock and aspirate all medium from the wells.

3.12. Add 400 µL of coculture maintenance medium I (AdDMEM without dexamethasone but supplemented with hepatocyte maintenance supplements) to each well and initiate flow in the downward direction at a flow rate of 1 µL/s for 3.5 min.

3.13. Aspirate all medium from the reservoir side and add 1.4 mL of coculture maintenance medium I to each well.

3.14. Transfer the plate into the docking station within a humidified incubator at 37 °C and with 5% CO_2 and initiate flow in the upward direction at a flow rate of 1 µL/s for 48 h.

3.15. Transfer the plate to the washing dock and aspirate all medium from the wells. Add 400 µL of coculture maintenance medium II (Williams E medium without dexamethasone but supplemented with 100 nM hydrocortisone and hepatocyte maintenance supplements) and initiate flow in the downward direction at 1 µL/s for 3.5 min.

3.16. Aspirate all medium appearing on the reservoir side of the wells.

3.17. Add 1.4 mL of coculture maintenance medium II and transfer the plate into the docking station within a humidified incubator at 37 °C and with 5% CO_2.

3.18. Initiate flow in the upward direction at a flow rate of 1 µL/s for 48 h (**Figure 1e**).

3.19. Replace the medium every 48 h with coculture maintenance medium II. To ensure all medium in the well is replaced, perform a washing step before the addition of fresh medium.

3.20. To wash, transfer the plate to the washing dock, aspirate all medium from the wells, and add 400 µL of coculture maintenance medium II.

3.21. Initiate flow in the downward direction at 1 µL/s for 3.5 min. Aspirate all medium appearing on the reservoir side of the wells.

3.22. Add 1.4 mL of coculture maintenance medium II and transfer the plate into the docking station within a humidified incubator at 37 °C and with 5% CO_2, and initiate flow in the upward

direction at a flow rate of 1 µL/s for 48 h (**Figure 1f**).

4. Production of an Infectious Hepatitis B Virus for Infection Studies

4.1. Perform this section of the protocol in a containment level III lab. Do the seeding, medium changes, medium collection, and virus concentration in a class II cabinet.

4.2. Culture HBV-producing cells (*e.g.*, HepDE19, HepAD38) in collagen-coated T1000 5-layer flasks in 120 mL of complete DMEM/F12 (10% FBS, penicillin/steptomycin, nonessential amino acids, 500 µg/mL G418, and 1 µg/mL tetracycline) until they reach 90% confluency.

4.3. Change the medium to induction medium (complete DMEM without tetracycline) to induce the HBV production.

4.4. Collect the complete medium volume every 48 h for 12 d post-withdrawal of tetracycline and store it at 4 °C.

4.5. Filter the collected medium through a 0.45-µm bottle top filter.

4.6. Add sterile PEG 8000 in phosphate-buffered saline (PBS) to the collected medium to a final concentration of 4% w/w, mix by inverting 8x - 10x, and incubate at 4 °C for 16 h. Centrifuge at 10,000 x *g* for 1 h at 4 °C to collect the PEG-precipitated virus and resuspend the pellet in PBS containing 10% FBS.

4.7. Combine the PEG-precipitated virus from all harvesting time points and layer it on top of a 20% sucrose cushion. Centrifuge at 140,000 x *g* for 16 h at 4 °C using an SW28 rotor.

4.8. Aspirate the supernatant and resuspend the pellet in PBS supplemented with 10% FBS, and aliquot and store it at -80 °C.

4.9. Determine the HBV DNA copy number present in the supernatant by HBV DNA qPCR (step 6).

5. Infection of 3-D Cultures with HBV

5.1. Perform infections in a class II cabinet within a containment level III lab.

5.2. 3 d after seeding the monocultures or cocultures, thaw the required number of HBV-containing aliquots at room temperature and dilute the required virus dose in 1.8 mL of hepatocyte maintenance medium or coculture maintenance medium II per well, respectively.

5.3. This 1.8-mL diluted virus is sufficient for the washing step (400 µL) and the replacement of medium in the well (1.4 mL). However, the required multiplicity of infection needs to be adjusted to account for the final culture volume of 1.6 mL.

5.4. Transfer the plate to the washing dock and aspirate all medium from the wells. Add 400 µL of HBV-containing medium and initiate flow in the downward direction at 1 µL/s for 3.5 min. Aspirate all medium appearing on the reservoir side of the wells.

5.5. Add 1.4 mL of HBV-containing maintenance medium/coculture maintenance medium II per well and transfer the plate into the docking station within a humidified incubator at 37 °C and with 5% CO_2. Initiate flow in the downward direction at a flow rate of 1 µL/s for 8 h, followed by a reversal to the upward direction at a flow rate of 1 µL/s.

5.6. 24 h following the addition of HBV, transfer the plate to the washing dock and aspirate all medium from the wells.

5.7. Wash each well in the plate 3x with the corresponding medium, according to the type of culture as outlined in steps 2.12 - 2.14, to eliminate leftover virus from the well. In contrast to steps 2.12 - 2.14, add 1.6 mL of medium in every well to account for the extra volume to be sampled to exclude inoculum carryover (**Figure 1g**).

5.8. Following these washing steps, collect 200 µL of medium from each well to confirm the complete removal of the HBV inoculum.

5.9. Transfer the plate to the docking station within a humidified incubator at 37 °C and with 5% CO_2, and initiate flow in the upward direction at a flow rate of 1 µL/s for 48 h.

5.10. 48 h later, collect the complete well volume for downstream analysis, followed by three washes with hepatocyte maintenance medium as outlined in steps 2.12 - 2.14. Replace the medium and wash each well 3x every 48 h until experimental termination.

6. Quantification of Extracellular HBV DNA

6.1. Isolate total DNA from the culture supernatants according to the manufacturer's instructions with the addition of 1 µg of carrier RNA in a containment level III lab to ensure the virus inactivation in the samples prior to moving them to a different area.

6.2. Prepare a master mix containing quantitative PCR master mix, 600 nM forward primer, 600 nM reverse primer, and 300 nM of probe.

6.3. Add 7 µL of the master mix into each well of a 384-well plate.

6.4. Add 5 µL of DNA samples in duplicate, a no-template control, and duplicates of serially diluted HBV genome-containing plasmid-based standard (*e.g.*, pCMV-HBV) ranging from 10^9 copies per reaction to 10^2 copies per reaction to each well of the qPCR plate.

6.5. Place an adhesive cover over the plate and ensure that each well is sealed correctly.

6.6. Centrifuge the plate for 1 min at 300 x *g*.

6.7. Start the qPCR run according to the manufacturer's instructions. The cycle conditions for real-time PCR are 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

6.8. Quantify the number of HBV DNA copies within the unknown samples according to the standard curve.

7. Quantification of Intracellular HBV Pregenomic (pg)RNA

7.1. Isolate total RNA from the scaffolds according to the manufacturer's instructions. In order to ensure complete cell lysis, vortex each scaffold 3x for 30 s followed by centrifugation at 300 x *g* for 1 min between each vortexing. Perform the cell lysis in the containment level III lab to ensure the virus inactivation in the samples prior to moving them to a different area.

7.2. Transcribe cDNA from the isolated RNA according to the manufacturer's instructions.

7.3. The cycle conditions for the retrotranscription are 25 °C for 10 min, 37 °C for 120 min, and 85 °C for 5 min.

7.4. Keep the cDNA samples at 4 °C for short-term or at -20 °C for long-term storage.

7.5. Prepare master mixes for pgRNA and RPS11 containing quantitative PCR master mix and forward and reverse primers for pgRNA and RPS11 (used as housekeeping gene) at a final concentration of 0.2 µM.

7.6. Add 7.5 µL of the master mix and 2.5 µL of cDNA per well on a 384-well plate. Measure RPS11 and pgRNA of each sample in duplicate and include a no-template control well for both genes.

7.7. Place an adhesive cover over the plate and ensure that each well is sealed completely.

7.8. Centrifuge the plate for 1 min at 300 x *g*.

7.9. Insert the plate into the qPCR cycler and start the qPCR run using the standard quantitative PCR protocol according to the manufacturer's instructions. The cycle conditions for real-time PCR are 50 °C for 2 min, 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

7.10. Calculate the expression of pgRNA relative to RPS11.

8. Immunofluorescence Staining of Viral Antigen

8.1. Remove the retaining ring from the well and remove the scaffold with forceps in a class II cabinet in the containment level III lab.

8.2. Fix the cell-containing scaffolds with 4% paraformaldehyde in 1 mL of PBS for 30 min at room temperature in the containment level III lab. The following steps can be performed in a different area.

8.3. Wash the scaffolds 3x with 1 mL of PBS.

8.4. Permeabilize the cells using 0.1% Triton-X 100 in 1 mL of PBS for 1 h at room temperature.

8.5. Wash the scaffolds 3x with 1 mL of PBS.

8.6. Block non-specific binding by incubation of the scaffolds with 1% BSA in 1 mL of PBS for 16 h at 4 °C.

8.7. Wash the scaffolds 3x with 1 mL of PBS.

8.8. Perform primary antibody staining using rabbit anti-hepatitis B virus core antigen at a dilution of 1:200 in 1% BSA in 1 mL of PBS for 16 h at 4 °C.

8.9. Wash the scaffolds 1x with 0.1% Tween in 1 mL of PBS (PBS-Tween) and 3x with 1 mL of PBS.

8.10. Perform secondary antibody staining using goat anti-rabbit IgG (H+L) cross-adsorbed Alexa Fluor 594-conjugated secondary antibody at a dilution of 1:2,000 in 1% BSA in 1 mL of PBS for 16 h at 4 °C.

8.11. Wash the scaffolds 1x with 1 mL of 0.1% PBS-Tween and 3x with 1 mL of PBS.

8.12. Counterstain the scaffolds using DAPI in 1 mL of PBS at a concentration of 2 µg/mL for 10 min at room temperature.

8.13. Wash the scaffolds 1x with 1 mL of 0.1% PBS-Tween and 3x with 1 mL of PBS.

8.14. Transfer the scaffolds to a microscope slide and mount it for imaging.

8.15. Image the scaffolds using a fluorescence microscope.

9. Human Albumin ELISA

9.1. Perform this section of the protocol in a class II cabinet allocated in the containment level III lab if working with infectious material.

9.2. To evaluate the viability and metabolic functionality of PHH, evaluate human albumin production by ELISA.

9.3. Coat 96-well plates with 50 µL per well of goat anti-human antibody diluted 1:800 in coating buffer (100 mM bicarbonate/carbonate, pH 9.6). Cover the plates and incubate for 2 h at 37 °C or overnight at 4 °C.

9.4. Aspirate the coating antibody from the plate and wash it 4x with 200 µL of 0.05% PBS-Tween.

9.5. Add 200 µL of blocking buffer (1% BSA in PBS), cover the plates, and incubate them for 1 h at 37 °C or store them at 4 °C for 3 months. For long-term storage, add 0.05% sodium azide to the blocking buffer.

9.6. Aspirate the blocking buffer and wash 1x with 200 µL of 0.05% PBS-Tween.

9.7. Add 50 µL of previously diluted samples per well (1:100). The sample diluent contains 1% BSA in 0.05% PBS-Tween. Incubate for 1 h at 37 °C or overnight at 4 °C.

Note: Incubate the standards at the same time as the samples. A concentration range of 500 - 0.488 ng/mL (1:2 serial dilutions) is recommended. Perform all serial dilutions of human albumin in sample diluent.

9.8. Aspirate the samples from the plate and wash it 4x with 200 µL of 0.05% PBS-Tween.

9.9. Add 50 µL of HRP-conjugated goat anti-human albumin antibody previously diluted 1:10,000 in sample diluent. Incubate for 2 h at 37 °C or overnight at 4 °C.

9.10. Aspirate the antibody from the plate and wash it 6x with 200 µL of 0.05% PBS-Tween.

9.11. Add 100 µL of TMB reagent and, as soon as the highest standards are fully developed, add 100 µL 1 M H_2SO_4 to stop the colorimetric reaction.

9.12. Read the absorbance at 450 nm on a 96-well plate reader for analysis.

10. Interleukin (IL)6 and Tumor Necrosis Factors (TNF)α Production in 3-D Cocultures

10.1. Perform this section of the protocol in a class II cabinet allocated in the containment level III lab if working with infectious material.

10.2. Quantify the IL6 and TNFα production to evaluate the functionality and viability of the primary Kupffer cells. To induce the production of these cytokines by Kupffer cells, treat cocultures with 1 µg/mL lipopolysaccharide (LPS) 9 d post-seeding in the coculture maintenance medium II for 48 h.

10.3. At day 11 post-seeding, harvest the medium from each well and store it at -80 °C.

10.4. Measure the IL6 and TNFα concentration in the culture medium by an appropriate assay

and according to the manufacturer's instructions.

REPRESENTATIVE RESULTS:

We describe a simple and versatile platform for the long-term culture of primary human Kupffer cells and/or hepatocytes and their infection with HBV. Primary human cells are seeded on collagen-coated polystyrene scaffolds within a microfluidic plate assembly, which continuously perfuses the cells with growth medium (**Figure 1a**).

PHH, which usually are only stable for a limited amount of time in conventional culture systems, can be functionally maintained for extended periods of time. Human albumin, which is secreted by functional hepatocytes and is considered the best marker for the evaluation of hepatic metabolism, is stably and highly expressed by 3-D cultures until day 40 post-seeding (**Figure 2**). For cocultures, Kupffer cell functionality and viability can be evaluated by the secretion of specific cytokines (*e.g.*, IL6 and TNFα). To measure cytokine production, the use of capture-based detection means upon LPS-stimulation of cocultures is recommended (**Figure 3**).

Cells form hepatic microtissues, usually within 3 days of seeding of PHH, demonstrating functional bile canaliculi and complete cell polarization (**Figure 2**). In addition to retaining their physiological cellular metabolism, these cultures become exceptionally susceptible to HBV infection. HBV DNA and other viral markers, in contrast to other culture systems, become readily detectable from day 2 post-infection (**Figure 4**). In addition to secreted markers of viral infection, hepatocyte-containing scaffolds can be retrieved from the cultures and used for the immunofluorescence detection of viral antigens (*e.g.*, HBsAg, HBcAg) (**Figure 4**). Where conventional hepatocyte cultures require inoculation with at least 500 HBV GE per cell and the addition of 2% DMSO and 4% PEG, as few as 0.05 HBV GE are able to initiate infection in 3-D cultures without the requirement of DMSO or PEG (**Figure 4**).

FIGURE AND TABLE LEGENDS:

Figure 1: Set-up of 3-D liver-on-a-chip cultures. (**a**) This is a schematic layout for the assembly of the culture plate in order to ensure the establishment of microfluidic circulation. (**b**) This panel shows a close-up view of the culture wells, including the filter paper, scaffold, and retaining ring. (**c**) This panel shows the process of plate equilibration prior to seeding the cultures. The next two panels show the process of seeding for (**d**) hepatocyte monocultures and (**e**) hepatocyte/Kupffer cell cocultures. (**f**) This panel shows the washing steps involved in medium changes. (**g**) This panel shows the HBV infection set-up, including the removal of inoculum. S.M. = seeding medium, M.M. = maintenance medium.

Figure 2: Hepatic microtissue formation and hepatocyte viability. (**a**) This panel shows longitudinal brightfield images of 3-D hepatocyte monocultures demonstrating microtissue formation following seeding. (**b**) This panel shows immunofluorescence imaging of cultures for nuclei (blue) and human albumin (green). (**c**) This panel shows longitudinal total albumin, as well as per cell adjusted albumin production, during 40 days of hepatocyte monocultures, as determined by ELISA. The data shown are mean ± SD. This figure is adapted from Ortega-Prieto

et al.[4].

Figure 3: Kupffer cell functionality in 3-D cocultures. These panels show the secretion of (**a**) IL6 and (**b**) TNFα in hepatocyte monocultures and hepatocyte/Kupffer cell cocultures 11 days post-seeding in response to exogenously added LPS at day 9 post seeding, as determined using Human Magnetic Luminex assay. This figure is adapted from Ortega-Prieto *et al.*[4].

Figure 4: HBV infection in liver-on-a-chip cultures. (**a**) This panel shows the immunofluorescence microscopy detection of HBcAg (red), HBsAg (green), and nuclei (blue) 10 days following the infection of the cultures with HBV. (**b**) This panel shows the susceptibility of the cultures to HBV infection using different multiplicities of infection, as determined by a quantification of HBV DNA in the culture supernatants. (**c**) This panel shows the quantification of the longitudinal accumulation of HBV pgRNA relative to the housekeeping gene RPS11. The data shown are mean ± SD. This figure is adapted from Ortega-Prieto *et al.*[4].

DISCUSSION:

The challenges in maintaining long-term cultures of PHH have driven the development of several culture models with increased functionality and longevity, each exhibiting differential advantages and disadvantages. It is now widely acknowledged that static 2-D cultures of PHH are mimicking certain aspects of hepatocyte biology for very limited amounts of time. Thus, micropatterned cocultures[12,13], spheroid cultures[14,15], and 3-D liver-on-a-chip cultures[16,17] are rapidly replacing these more basic systems. Especially when studying infectious diseases, which have coevolved with their host to utilize specific microenvironments, the requirement for providing physiological environments is underpinned by the often challenging nature of culturing human-tropic infectious diseases, including hepatitis C virus, HBV, and malaria.

The most critical step in performing 3-D liver-on-a-chip cultures is the quality of the initially sourced primary cell types. These cells should be tested for their adherence capacity and only plateable PHH lots should be used in order to ensure successful tissue formation and culture generation. Even though freshly isolated PHH can be used, their cryopreservation is usually complicated and requires special rate-controlled freezers.

In contrast to conventional static 2-D cultures, the host genetic background is negligible in regard to susceptibility to HBV infection, and all thus-far tested hepatocyte donors are able to establish HBV infection[4].

Even though patient-derived HBV establishes infections of 3-D cultures, it is imperative to utilize PEG-precipitated and sucrose cushion-purified HBV whenever using inducible HBV producer cell lines for the generation of viral inocula. Cell culture supernatants directly applied to 3-D liver-on-a-chip cultures, either through the presence of inhibitory factors or due to an incompatibility of present growth factors with hepatocytes, do not readily result in infection. Additionally, when selecting patient-derived viral inocula, only serum should be used, since plasma inevitably coagulates and clogs the microfluidic circulation of the culture platform.

Irrespective of the viral inoculum used, assuring cellular viability and differentiation, as well as ensuring complete removal of the initial HBV inoculum, is key to successful long-term infection studies. The most convenient way to do this is sampling cultures following the removal of the viral inoculum, as well as measuring human serum albumin levels throughout the culture period. Of note, similarly to all other described platforms, HBV infection, once established, does not readily spread to uninfected cells. The underlying mechanism for this remains elusive since HBV infection *in vivo* readily infects the majority of the hepatocytes within the liver.

In regard to cocultures of PHH and Kupffer cells, it is advisable to perform lot tests of Kupffer cells to evaluate IL6 and TNFα secretion in response to LPS stimulation, since not all commercially available Kupffer cell donors have an equal responsiveness.

Importantly, for all drug treatments or initial infection of cultures with HBV, the total volume of the well (1.4 mL), as well as of the microfluidic channel (0.2 mL), must be taken into account for the calculation of drug or inoculum concentrations. In order to assure accurate dosing, one washing step with medium containing HBV or drugs is performed in order to prime the microfluidic channel.

The platform used utilizes 600,000 PHH per well, which ensures multilayered hepatocytes within the scaffolds. Even though the cell number can be varied, the chosen cell concentration ensures optimal results. The plate format holds a total of 12 scaffolds, which can be upgraded to 36 scaffolds. However, due to microfluidic requirements, scaling up to higher well numbers is not possible to date.

Using these approaches, cultures can be maintained with optimal cell performance for at least 40 days, which, thus far, offers unprecedented opportunities to evaluate novel drug candidates, as well as study the complex interplay between different hepatic cell populations during HBV infection.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

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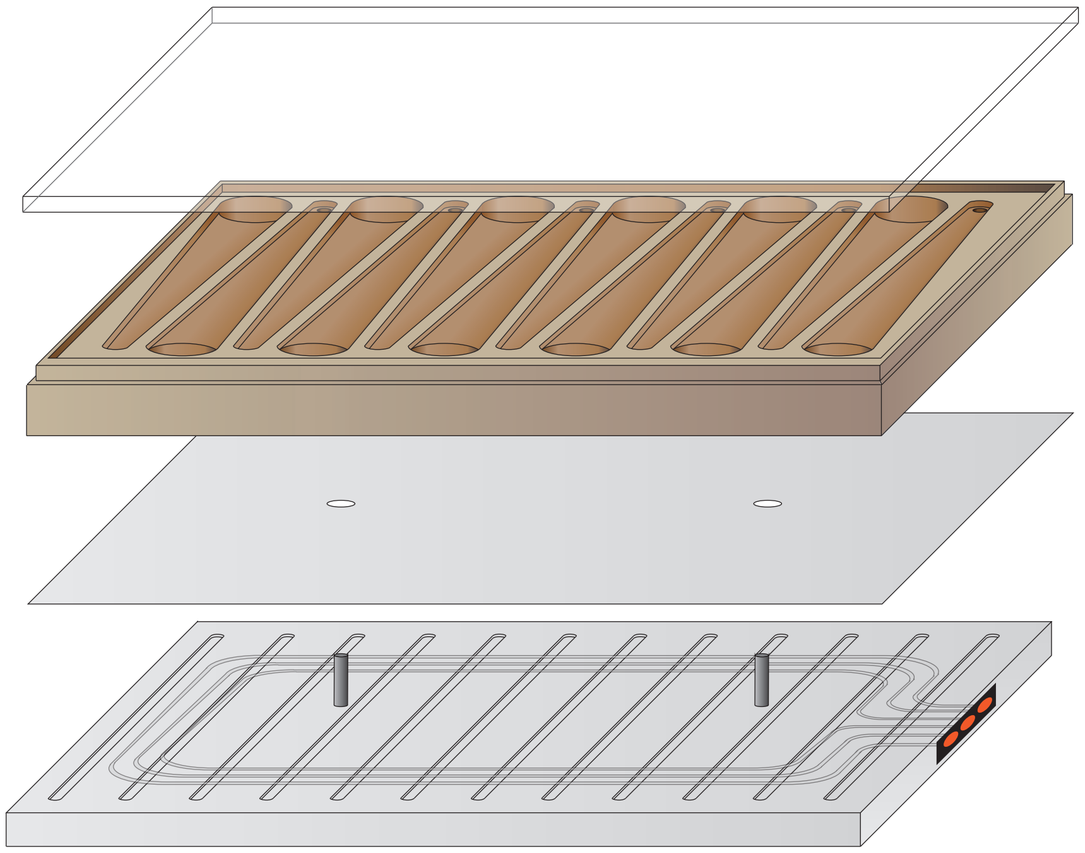
a

Sterile plate lid

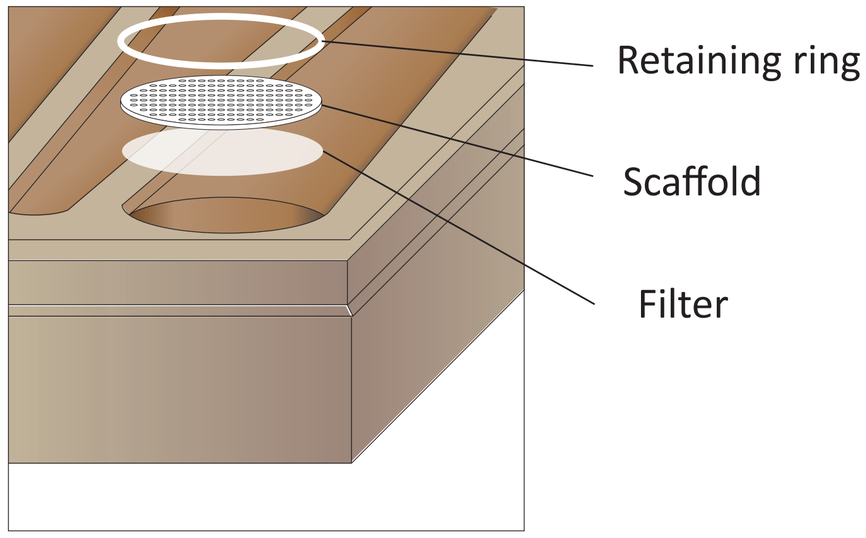
Sterile well-containing top plate

Sterile membrane

Base plate



b



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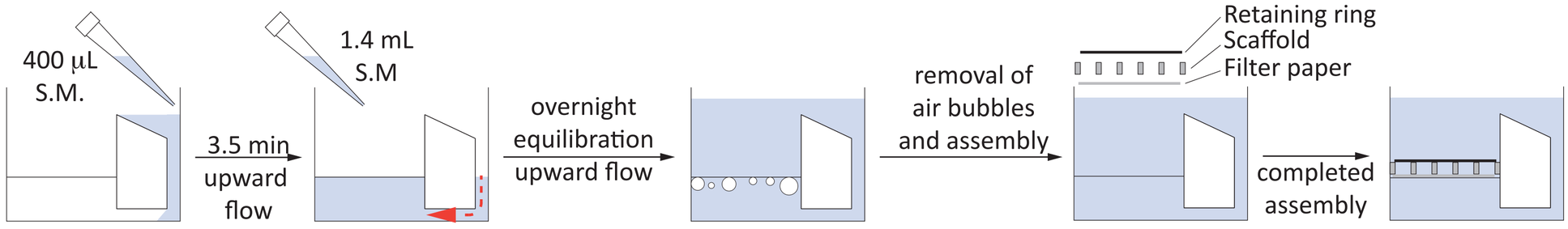
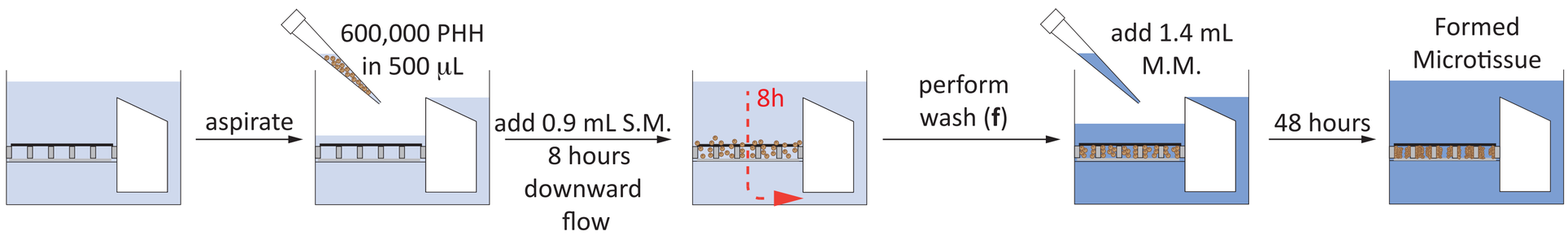
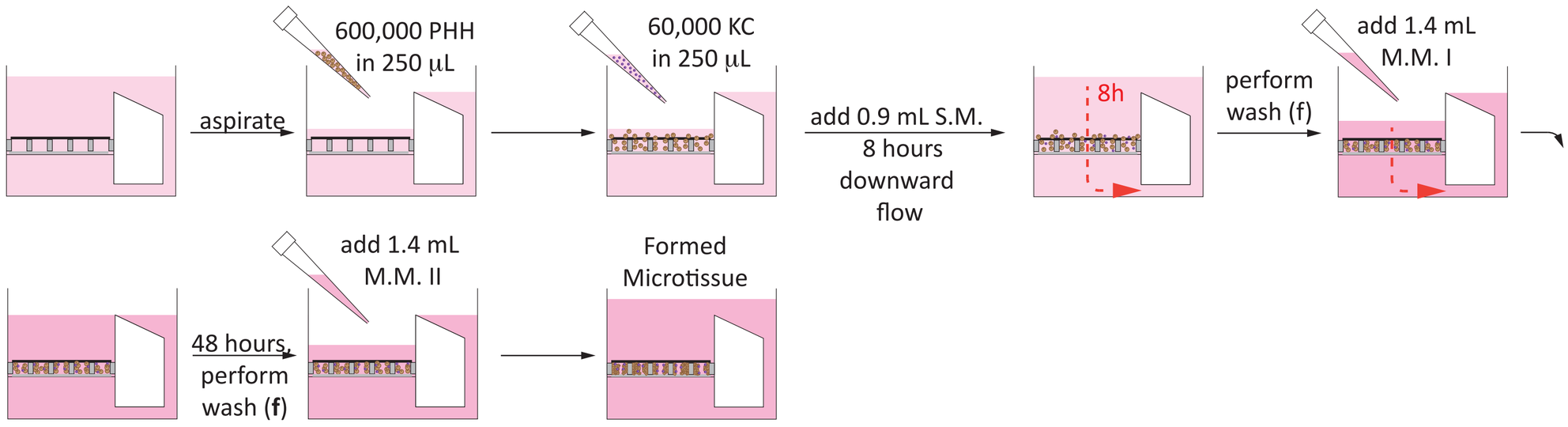


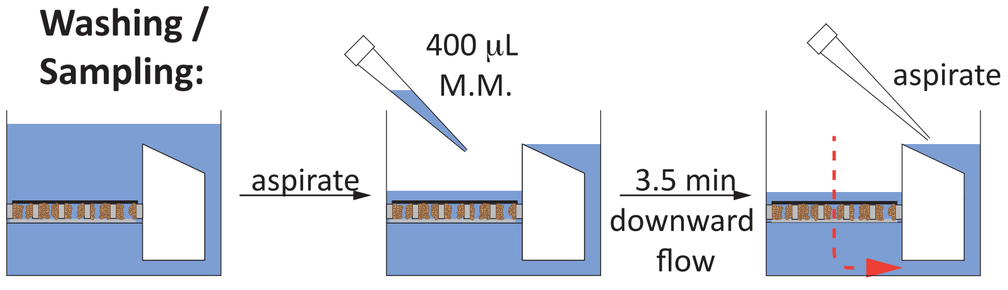
d **Seeding (PHH monoculture):**



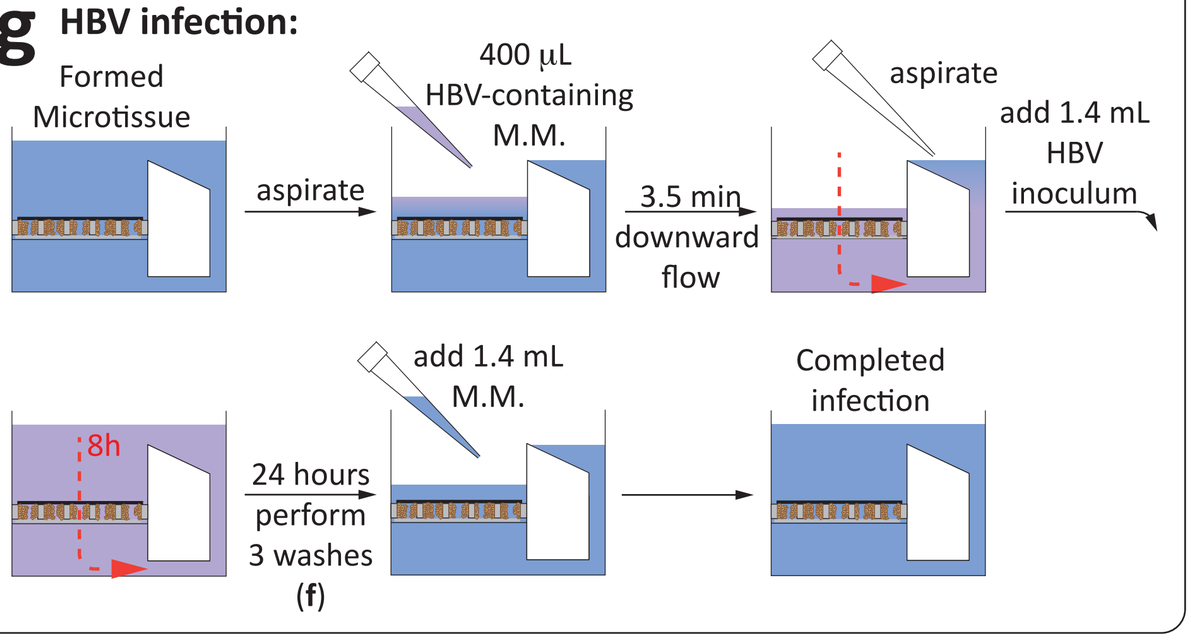
e **Seeding (PHH/KC coculture):**



f **Washing / Sampling:**



g **HBV infection:**



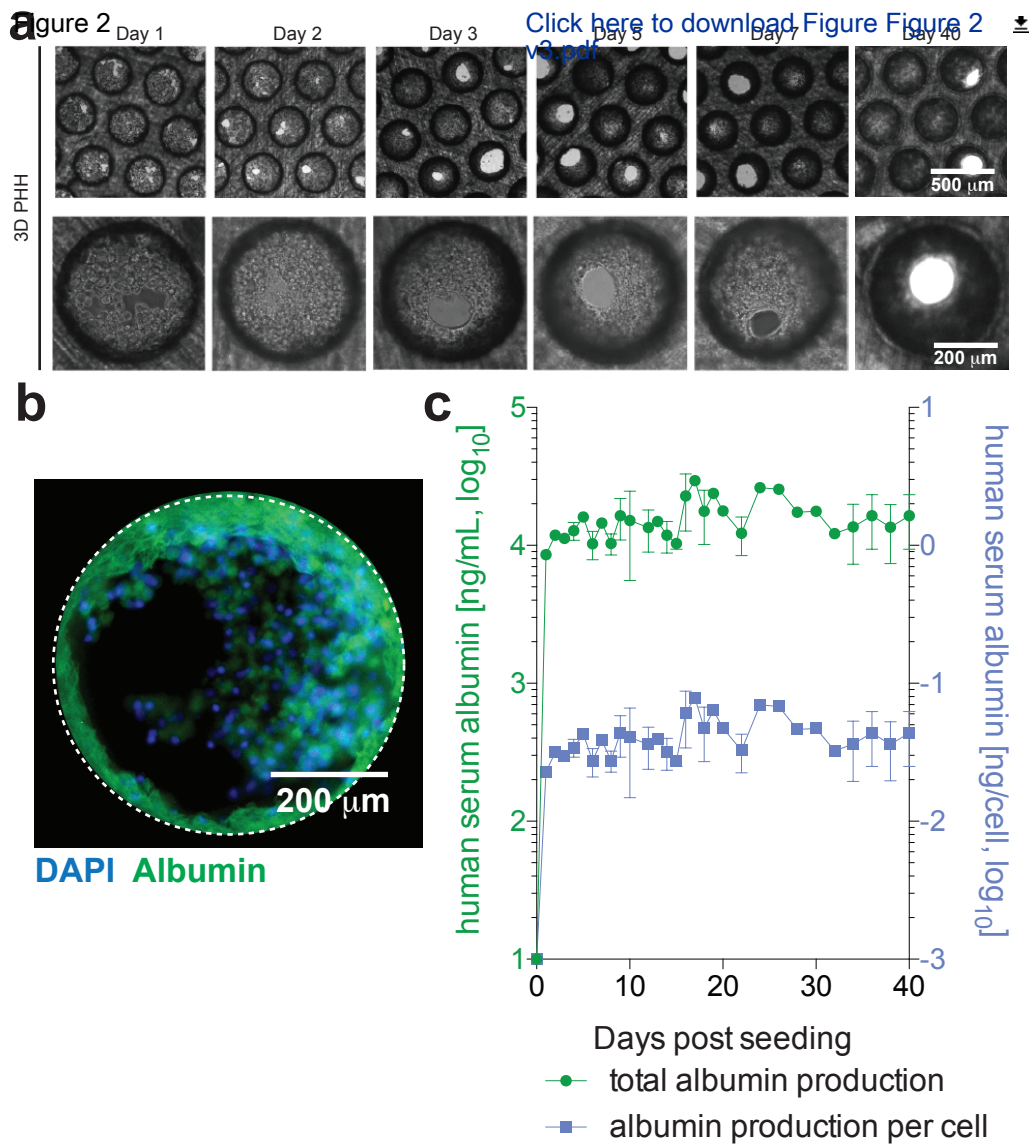


Figure 3

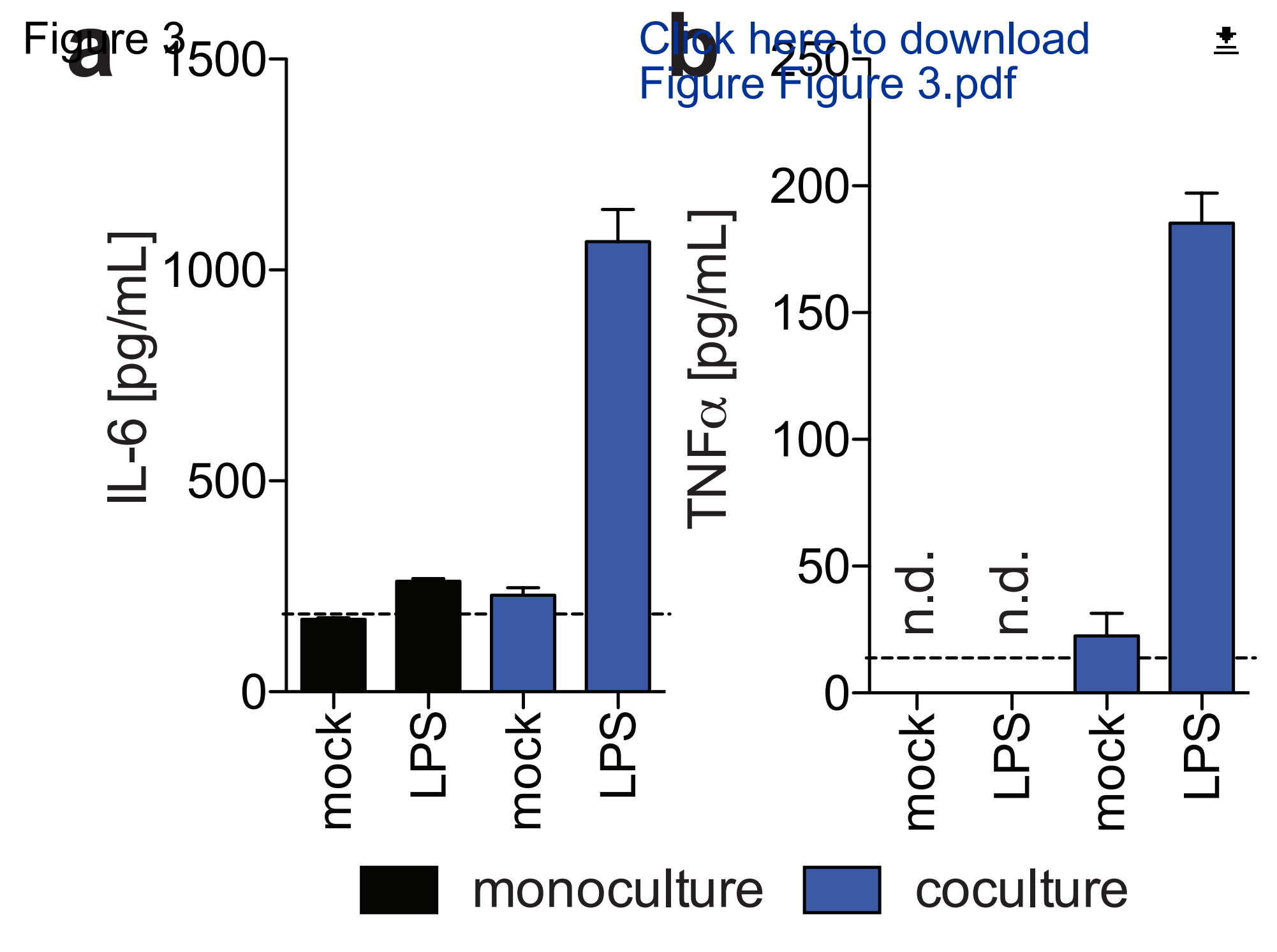


Figure 4

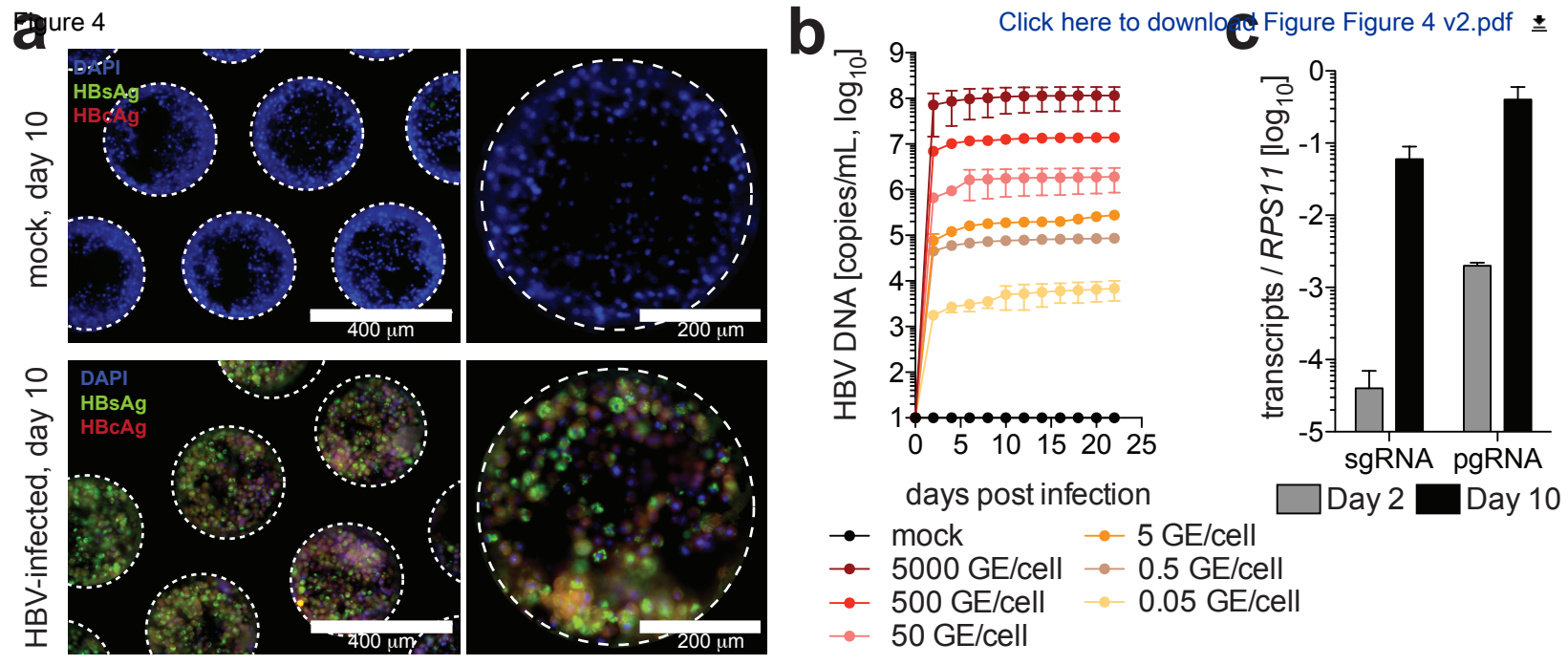


Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Reagents			
William's E Medium, no phenol red	GIBCO	A12176-01	
Hepatocyte Thaw Medium	GIBCO	CM7500	
Primary Hepatocyte Thawing and Plating Supplements	GIBCO	CM3000	
Primary Hepatocyte Maintenance Supplements	GIBCO	CM4000	
DMEM/F-12	GIBCO	11320-033	
Advanced DMEM	GIBCO	12491023	
DPBS, no calcium, no magnesium	GIBCO	14190-144	
MEM Non-Essential Amino Acids (NEAA) 100X	GIBCO	11140050	
Penicillin-Streptomycin (10,000 U/mL)	GIBCO	15140-122	
Fetal Bovine Serum, USA origin, Heat Inactivated, sterile-filtered, suitable for cell culture	SIGMA	12106C	
Hydrocortisone	SIGMA	H0888	
Trypan blue	Merck	T8154	
Collagen from calf skin	Merck	C9791	
G418	SIGMA	G418-RO	
Tetracycline	SIGMA	T3258	
Polyethylene glycol 8000	SIGMA	P2139	
Sucrose	SIGMA	SO389	
Sodium carbonate anhydrous	SIGMA	451614-25G	
Sodium bicarbonate	SIGMA	S5761	
Sodium azide	SIGMA	S2002-5G	
Sulfuric acid, 99.999%	SIGMA	339741	
4% Paraformaldehyde	SIGMA	252549	
Triton-X 100	SIGMA	X100	
Tween 20	SIGMA	P1379	
DAPI	SIGMA	D9564	
Albumin (human)	SIGMA	A9731	
Fisher BioReagent Bovine Serum Albumin, Fraction V, Heat Shock Treated	Fisher Scientific	BP9701-100	
ProLong Gold Antifade Mountant	Invitrogen	P36930	
TaqMan Universal Master Mix II, no UNG	Applied Biosystems	4440040	
SYBR Select Master Mix	Applied Biosystems	4472903	
Lipopolysaccharide from *Escherichia coli K12*	InvivoGen	tlrl-eklps	
Kits/Consumables			
Sterile membrane	CN Bio innovations	LC-SC	
LiverChip Perfusion cell culture plate	CN Bio innovations	LC12	
LiverChip culture plate lid	CN Bio innovations	LC-SC	
Sterile round filter paper	CN Bio innovations	LC-SC	
Cell attachment scaffold	CN Bio innovations	LC-SC	
Retaining ring	CN Bio innovations	LC-SC	
Sterile plunger	CN Bio innovations	LC-ST	
Dneasy blood & tissue kit	Qiagen	69506	
Rneasy mini kit	Qiagen	74106	
Human Magnetic Luminex assay	R&D Systems		
1-Step Ultra TMB-ELISA Substrate Solution	ThermoFisher Scientific	34028	
High Capacity cDNA Reverse Transcription Kit	ThermoFisher Scientific	4368814	
QIAamp Viral RNA Mini Accessory Set	Qiagen	1048147	Containing RNA carrier
Millicell HY 5-layer cell culture flask, T-1000, sterile	Millipore (Merck)	PFHYS1008	
MicroAmp Optical 384-Well Reaction Plate with Barcode	Life technologies	4309849	
MicroAmp Optical Adhesive Film	Life technologies	4311971	
Clear Flat-Bottom Immuno Nonsterile 96-Well Plates	ThermoFisher Scientific	442404	
Sealing Tape for 96-Well Plates	ThermoFisher Scientific	15036	
Nalgene Rapid-Flow Sterile Disposable Bottle Top Filters with PES Membrane	ThermoFisher Scientific	295-3345	
Fisherbrand Microscopic Slides with Ground Edges, Twin Frost	Fisher Scientific	FB58628	
Tube, Thinwall, Ultra-Clear, 38.5 mL, 25 x 89 mm	Beckman Coulter	344058	
Primary cells / Cell lines			
Human Plateable Hepatocytes, Transporter Qualified	Thermo Fisher Scientific	HMCPTS	
Cryopreserved Human Kupffer Cells	Thermo Fisher Scientific	HUKCCS	
HepDE19 cell line	Haitao Guo (Indiana University, IN, USA)		
Primers/Probes/Standards			
HBV DNA forward primer	Invitrogen		5'-GTGTCTGCGGCGTTTTATCA-3'
HBV DNA reverse primer	Invitrogen		5'-GACAAACGGGCAACATACCTT-3'
HBV DNA probe	Invitrogen		5'FAM-CCTCTKCATCCTGCTGCTATGCCTCATC-3'TAMRA
pgRNA forward primer	Invitrogen		5'-GAGTGTGGATTCGCACTCC-3'
pgRNA reverse primer	Invitrogen		5'-GAGGCGAGGGAGTTCTTCT-3'
RPS11 forward primer	Invitrogen		5'-GCCGAGACTATCTGCACTAC-3'
RPS11 reverse primer	Invitrogen		5'-ATGTCCAGCCTCAGAACTTC-3'
pCMV-HBV	Professor Christoph Seeger (Fox Chase Cancer Centre, PA, USA)		
Antibodies			
Anti-Hepatitis B virus core antigen IgG fraction (polyclonal)	DAKO	discontinued	Lot 10102505
Human Albumin Antibody, A80-129A	Bethyl Laboratories. inc	A80-129A	
Human Albumin cross-adsorbed Antibody, A80-229P	Bethyl Laboratories. inc	A80-229P	
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 594	ThermoFisher Scientific	# A-11072	Lot 1431810
Equipment			
LiverChip Vacuum pump	CN Bio innovations	LC-PN	
LiverChip Pneumatic Hookup	CN Bio innovations	LC-PN	
LiverChip platform	CN Bio innovations	LC-PN	
LiverChip plate washing dock	CN Bio innovations		
Autoclavable metal forceps	VWR	232-0106	
Vortex Genie 2	Scientific industries	SKU: SI-0236	
Optima XPN-80 Ultracentrifuge	Beckman Coulter	A95765	
Heraeus Multifuge X3R Centrifuge	Thermo Scientific	75004500	
SAM-12 Medical Suction High Vacuum High Flow	MGE worldwide	SAM12/01010101	
NUAIRE 5800 SERIES incubator	NUAIRE	NU-5841	
Automated precision torgue	CN Bio innovations		
Manual torque	CN Bio innovations		
LiverChip compressor	CN Bio innovations		
Luminex LX-200 Instrument with xPONENT 3.1	Luminex		
Millipore Hand-Held Magnetic Separator Block	ThermoFisher Scientific	Millipore™ 40-285	
FluoStar Optima Plate Reader	BMG Labtech		
KOLVER Precision electric screwdrivers	VTECH ltd	FAB10RE/FR	
KOLVER Power supply	VTECH ltd	EDU1FR	
BAMBI VT575D	Air Equipment	Discontinued	
Integra Vacuboy	INTEGRA		
ViiA 7 Real-Time PCR System with 384-Well Block	ThermoFisher Scientific	4453536	

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Institution:	Imperial College London
Article Title:	"Liver-on-a-Chip" cultures of primary hepatocytes and Kupffer cells for hepatitis B virus infection
Signature:	 Date: 22/04/2018

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London, 10 June 2018

RE: Resubmission of manuscript "Liver-on-a-Chip" cultures of primary hepatocytes and Kupffer cells for hepatitis B virus infection

Dear Jaydev,

Please find enclosed our revised manuscript entitled ""Liver-on-a-Chip" cultures of primary hepatocytes and Kupffer cells for hepatitis B virus infection" including a point-by-point response to the editorial as well as reviewers' comments.

Should you have any further questions please do not hesitate to contact me.

Sincerely,

Marcus Dorner, Ph.D.
Non-Clinical Senior Lecturer / Associate Professor in Immunology
Head of hepatitis virus group

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We have proofread the manuscript and have corrected all errors.

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4. Figure 1: Please include a space between all numbers and their corresponding units: 0.9 mL, 400 µL, 48 h; etc.

We have adapted all units throughout the manuscript

5. Figure 2/4: Please include a space between the number and the units of the scale bar.

We have adapted the formatting of numbers and units throughout the manuscript

6. Figures 3/4: Please define error bars in the figure legend.

All error bars were defined in the corresponding figure legend

7. Table of Equipment and Materials: Please remove trademark (™) and registered (®) symbols. Please provide lot numbers and RRIDs of antibodies, if available.

All trademarks and registered symbols were removed from the table of equipment and materials

8. Abstract (150-300 word): Please include a statement about the purpose of the method. A more detailed overview of the method and a summary of its advantages, limitations, and applications is appropriate.

We have included a section on the purpose as well as an overview of the method

9. Please revise the Introduction to include all of the following:
a) A clear statement of the overall goal of this method
b) The rationale behind the development and/or use of this technique
c) The advantages over alternative techniques with applicable references to previous studies
d) A description of the context of the technique in the wider body of literature
e) Information to help readers to determine whether the method is appropriate for their application
10. Please define all abbreviations before use.
11. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.
12. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Qiagen, Via7, Applied Biosystems, EVOS FL Auto microscope, Nunc, etc.
13. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.
14. 1.4/2.5/3.7-3.9: Please break up into sub-steps.
15. PCR: Please ensure that for all PCR, conditions and primers are listed.
16. 8.3: What volume of PBS is used to wash? Please specify throughout.
17. Please include single-line spaces between all paragraphs, headings, steps, etc.
18. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
19. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

20. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s).
21. References: Please do not abbreviate journal titles. Please include volume and issue numbers for all references.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
Manuscript describes use of microfluidic device to culture primary human hepatocytes,including normal, HBV infected and in-situ infection of cultured normal PHH. Authors also describe analysis of extracellular DNA and intracellular HBV RNA. Protocols are reasonably well written and are of interest to the scientific community. In my opinion this manuscript should be published after addressing with several concerns outlined below.

Major questions:
1. Can these experiments be performed in transwells using organotypic 3D culture? it is worth mentioning it either in Introduction or Results. Do authors recommend control experiments outside microfluidic device?

We thank the reviewer for bringing this up. We now have included a section in the introduction, describing the utility of alternative models and where the strengths of the microfluidic cultures lie. We also included a section on appropriate controls.

2. Please include description of the microfluidic chip and its support system in the introduction. Expand section 1 to provide more details details regarding "LiverChip platform" (this can also be done in introduction).

We now have included a description of the LiverChip platform in the introduction

3. How would a user obtain samples for analyses described in Sections 6 and 7? Is there a concern of withdrawing too much fluid from the chip? Can this sampling be done multiple times during a single culture period to "evaluate long-term host responses" (line 65)?

We have added a description of which samples are used for sections 6 and 7. Since all liquid of the cultures is replaced at the washing steps, there is no risk of withdrawing too much culture supernatant.

Minor Concerns:
1. Figure 1 needs to be modified to improve readability and understanding. Fig 1C steps 1 and 2: Red arrow shows logical and expected direction of fluid flow based on fluid levels in adjacent compartments. However, the same arrow in Fig 1D steps 4 and 5 indicates the flow that is counter intuitive. In step 4 one would expect no flow and in step 6 it would be in the opposite direction of the arrow. Same in Fig 1F step 3 and 1G steps 3 and 4. Please adjust the drawings and associated text (including figure captions) that explains / corrects apparent visual flow abnormalities.

The flow directions in the figures are correct. Figure 1C is describing the setup of the plates and the removal of the air bubbles is essential. Thus, the flow is adjusted to push air bubbles towards the culture area of the well. Figure 1D subsequently describes the seeding of the cells, where it is essential not to introduce air bubbles below the filter paper as well as to ensure all cells are pushed towards the scaffold.

2. Please spell out HBV on line 49 and cccDNA on line 56.

We have corrected this

Reviewer #2:
Manuscript Summary:
This manuscript describes a method for 3D culture of primary human liver cells suitable for in vitro hepatitis B viral infection studies.

Minor Concerns:
1. It would be beneficial if the authors included an expanded discussion of the scale of their platform, including some context on how the cell numbers and volumes were optimized, for example. Further, what potential steps (or limitations) would be relevant if the scale was increased or decreased.

We have now added this in the discussion

2. Overall, additional details regarding the viral dosing capabilities and procedures should be given. For example, is it expected or required to typically adjust the infection protocol in co-cultures versus monocultures? The abbreviation definition for genome equivalents (GE) should be provided in the text and in the figure legend for figure 4.

We have added the clarification of genome equivalents and furthermore added recommendations as to the used controls for co-culture experiments.

3. The use of DMSO or PEG for viral inoculation procedures is clearly non-physiologic. However, are there other specific advantages to use a system that does not require these additives?

Even though the omission of DMSO or PEG is a great advantage over other culture methods, the most important aspect is that this system enables HBV infection at physiological inoculum levels. We have made this point more clear in the introduction.

4. It may be relevant to include any specific safety procedures in place for working with HBV- within the context of this experimental platform.

We have added requirements for working under the appropriate biosafety levels wherever applicable throughout the protocol section.

5. Is loss of cells, or a reduction in cell viability, a potential failure mode for this platform at longer time points? Are there potential troubleshooting steps that could be undertaken if viable cell numbers are not maintained for the longer duration experiments?

In our experience, we never observed declining cell viability in long-term cultures. We have added the requirement for the evaluation of albumin levels throughout the cultures, since this is the best means of ensuring hepatocyte viability and functionality.