

# Journal of Visualized Experiments

## Serum Free Production of Three-Dimensional Human Hepatospheres from Pluripotent Stem Cells --Manuscript Draft--

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5th March 2019

Dear Dr Weldon,

I would be grateful if you would consider our manuscript, "Serum free production of 3D human liver tissue from pluripotent stem cells" for publication in JoVE.

Liver disease is an escalating global health issue. Although liver transplantation is an effective mode of therapy, patient mortality increases due to the shortage of donor organs. Therefore, the establishment of pluripotent stem cell based systems, which serve a renewable source of human tissue, have generated much excitement in the field.

Despite significant progress, *in vitro* derived hepatocyte-like cells generally display a mix of adult and foetal features (Szkolnicka et al., Stem Cells. 34(6): 1421–1426, 2016). This limits the application of the technology to study disease 'in a dish' or develop pioneering therapies for the clinic.

To improve cell phenotype, recent research as focussed on liver tissue formation by cell self-aggregation. The embedding of somatic cells or stem cells in cancer-derived matrix has opened new avenues to model human liver biology *in vitro*. However, current 3D systems are difficult to scale, suffer from batch to batch variation, and consequently are not yet suitable for cell based modelling at scale or clinical translation (Takebe *et al.*, Nature 499, 481–484, 2013; Huch M *et al.*, Cell. 160(1-2):299-312, 2015; Camp *et al.*, Nature 546, 533–538, 2017; Takebe *et al.*, Cell Reports. 2017; 21(10):2661-2670).

To deal with these outstanding issues, we have employed pluripotent stem cells to generate renewable sources of human liver tissue using a chemically defined process. Specifically, the work presented in our manuscript demonstrates that stem cell derived liver tissue can be produced reliably and with stable phenotype from both hESC and iPSC lines.

We believe our paper will be of interest to the readers of JoVE and are grateful to you for your consideration. We look forward to hearing from you in due course.

With best wishes

A handwritten signature in blue ink, which appears to read "David C Hay".

Professor David C Hay,

Chair of Tissue Engineering.

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**KEYWORDS:**

pluripotent stem cells, three-dimensional culture, hepatospheres, drug metabolism and protein secretion, stable cell phenotype, defined culture system

**SUMMARY:**

This protocol describes an approach to produce hepatospheres from human pluripotent stem cells using a defined culture system and cell self-assembly. This protocol is reproducible in a number of cell lines, cost effective and allows the production of stable human hepatospheres for biomedical application.

**ABSTRACT:**

The development of renewable sources of hepatospheres represents an attractive approach to tackle the shortage of primary cell types for cell-based modelling, and donor organs for

transplantation. Human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) represent a promising source of renewable human hepatospheres. We have developed a serum free and defined method of cellular differentiation to generate three-dimensional (3D) human liver spheres formed from human pluripotent stem cells. A potential limitation of the technology is the production of dense spheres with dead material inside. Importantly, the use of the microwell technology at defined cell densities allows the user to control the size of the 3D spheres, preventing the generation of apoptotic and/or necrotic cores. Notably, the spheres generated by our approach display liver function and stable phenotype, representing a valuable resource for basic and applied scientific research. We believe that our approach could be used as a platform technology to develop further tissues to model and treat human disease and in the future may permit the generation of human tissue with complex tissue architecture.

## **INTRODUCTION:**

The ability of human pluripotent stem cells (hPSCs) to self-renew, whilst retaining pluripotency, provides an opportunity to produce human cell types and tissues on demand. hPSCs have been efficiently differentiated into hepatocyte-like cells (HLCs) using two-dimensional (2D) adherent culture systems<sup>1-10</sup>. These systems have been used to successfully model monogenic disease, virus lifecycle, drug induced liver injury (DILI), fetal exposure to toxins and non-alcoholic fatty liver disease (NAFLD)<sup>11-15</sup>. However, these models do possess some drawbacks, which limit their routine use. Those include fetal marker expression, unstable phenotype and poor tissue architecture<sup>16-19</sup>, which could also result in poor translation to organ function in vivo.

To overcome these limitations, three-dimensional (3D) differentiation platforms have been developed to mimic in vivo tissue architecture. On the whole, those approaches rely on the use of animal derived products and matrices to drive tissue genesis<sup>20-22</sup>, limiting scale-up and widespread application.

Here, we detail procedures to generate large quantities of 3D hepatospheres from hPSCs using defined materials and cell self-assembly. Notably, the tissue generated by our procedure remains functional for more than one year in cell culture and is capable of supporting liver function in vivo<sup>23</sup>.

In summary, our defined differentiation approach allows the generation of stable human hepatospheres from both human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs). We believe the described procedure represents a significant breakthrough in the generation of 3D hepatospheres for basic and applied scientific research.

## **PROTOCOL:**

### **1. Preparation of agarose microplate molds**

NOTE: Media used for these experiments must be sterile and at room temperature (RT) for cell culture.

### 1.1. Prepare 2% agarose molds.

1.1.1. Dissolve 2 g of low melting temperature agarose into 100 mL of sterilized distilled water. Carefully heat in a microwave with interval shaking to dissolve completely.

1.1.2. Add 520  $\mu$ L of melted agarose to a 256-well format mold and leave to solidify.

1.1.3. Transfer each agarose microplate into a single well of a 12-well plate.

1.1.4. Add 1.5 mL of 1x Dulbecco's phosphate-buffered saline (DPBS) with  $\text{Ca}^{2+}$ / $\text{Mg}^{2+}$  to each well and remove the air bubbles from the microwells by gently pipetting up and down several times using a P1000 pipette tip. This is performed to ensure uniform cell seeding.

NOTE: Agarose microplates can be stored for up to 6 months at 4 °C in 1x DPBS.

## 2. Seeding human pluripotent stem cells into agarose microwell plates

### 2.1. Preparation of cell suspension

2.1.1. Aspirate the medium from an undifferentiated culture of hPSCs as previously described<sup>8</sup>.

NOTE: hPSCs are cultured on LN-521 in mTeSR1 with the medium changed every 24 h, and passaged regularly once the cells reach 75% to 85% of confluency as previously described<sup>8</sup>.

2.1.2. Rinse the cells with 5 mL of RT 1x DPBS without  $\text{Ca}^{2+}$ / $\text{Mg}^{2+}$  and remove the buffer.

2.1.3. Add 5 mL of 1x cell dissociation reagent (**Table of Materials**) to the cells and allow cell dissociation by incubating cells at 37 °C for 6–8 min.

NOTE: To stop the reaction, examine cell detachment under the microscope. The cells should be partially detached from the plate. Extend the incubation for an extra 1–2 min if longer time is required.

2.1.4. Stop the reaction by removing the cell dissociation reagent and add 5 mL of fresh mTeSR1 medium supplemented with 10  $\mu$ M Rho-associated kinase (ROCK) inhibitor Y27632 to the cells. Dissociate cells by pipetting up and down several times by using a P1000 pipette tip.

2.1.5. Count the viable cells using a hemocytometer and trypan blue staining exclusion.

Following this, prepare the cell suspension at the required concentration.

2.1.6. Calculate the total number of cells needed. For 3D hepatosphere differentiation, seed  $3.84 \times 10^5$  cells per agarose microplate to generate spheroids with 100–150  $\mu\text{m}$  in diameter.

2.1.7. Transfer the desired number of cells into a sterile 15 mL or 50 mL centrifuge tube and centrifuge the tube at  $200 \times g$  for 5 min at RT to pellet the cells.

2.1.8. Aspirate and discard the supernatant and resuspend cells in mTeSR1 medium plus 10  $\mu\text{M}$  ROCK inhibitor Y27632. Dilute the cell pellet in the appropriate volume of medium to a final concentration of  $2.1 \times 10^6$  cells/mL.

## 2.2. Seeding the cells to the prepared agarose microplates

NOTE: If using fridge-stored agarose microplates, place them in the cell incubator at 37 °C for at least an hour prior to use and aspirate the 1x DPBS from the well before cell seeding.

2.2.1. Add 190  $\mu\text{L}$  of the cell suspension into the agarose microwell.

2.2.2. After seeding, return the plates to the cell incubator at 37 °C and 5%  $\text{CO}_2$  for 2 h to allow the cells to settle.

2.2.3. After 2 h, add 1 mL of fresh and warm mTeSR1 medium supplemented with 10  $\mu\text{M}$  ROCK inhibitor Y27632 to each well.

2.2.4. Return the plates to the cell incubator at 37 °C and 5%  $\text{CO}_2$  for 24 h and examine the formation of spheres the next day to allow the cells to attach.

## 3. Differentiating hPSCs to 3D hepatospheres on agarose microwells

### 3.1. Preparation of poly 2-hydroxyethyl methacrylate (poly-HEMA) coated wells

3.1.1. Dissolve 2 g of poly-HEMA in 100 mL of 95% ethanol. Stir the solution overnight using a hot plate at 55 °C. Add 250  $\mu\text{L}$  of poly-HEMA solution per well of a 24-well plate and dry overnight at 60 °C using an oven.

### 3.2. Preparation of the differentiation medium

3.2.1. Prepare a 1000x stock solution of human activin A by dissolving human activin A lyophilized protein in sterile 0.2% bovine serum albumin (BSA)/DPBS to a final concentration of 100  $\mu\text{g/mL}$ . Store at -20 °C in small aliquots. Use at 1:1000.

3.2.2. Prepare a 1000x stock solution of Wnt3a by dissolving mouse Wnt3a lyophilized protein in sterile 0.2% BSA/DPBS to a final concentration of 10 µg/mL. Store at -20 °C in small aliquots. Use at 1:200.

3.2.3. Prepare a 1000x stock solution of human hepatocyte growth factor (HGF) by dissolving human HGF lyophilized protein in sterile 0.2% BSA/DPBS to a final concentration of 10 µg/mL. Store at -20 °C in small aliquots. Use at 1:1000.

3.2.4. Prepare a 1000x stock solution of oncostatin M (OSM) by dissolving OSM in sterile 0.2% BSA/DPBS to a final concentration of 20 µg/mL. Store at -20 °C in small aliquots. Use at 1:1000.

3.2.5. Prepare a 1000x stock solution of epithelial growth factor (EGF) by dissolving the lyophilized protein in sterile 0.2% BSA/DPBS to a final concentration of 10 µg/mL. Store at -20 °C in small aliquots. Use at 1:1000.

3.2.6. Prepare a 1000x stock solution of basic fibroblast growth factor (bFGF) by dissolving the lyophilized protein in sterile 0.2% BSA/DPBS to a final concentration of 10 µg/mL. Store at -20 °C in small aliquots. Use at 1:1000.

3.2.7. Prepare a 1000x stock solution of vascular endothelial growth factor (VEGF) by dissolving the lyophilized protein in 0.2% BSA/DPBS to a final concentration of 10 µg/mL. Store at -20 °C in small aliquots. Use at 1:1000.

3.2.8. Make endoderm differentiation medium consisting of Roswell Park Memorial Institute 1640 (RPMI 1640) basal medium supplemented with 2% B27 supplement (50x, without insulin), and 1% penicillin/streptomycin (final concentrations: 100 IU/mL and 100 µg/mL, respectively). Unless indicated, at each medium change, supplement the required volume with Wnt3a and activin A at a final concentration of 50 ng/mL and 100 ng/mL, respectively.

NOTE: Store at 4 °C and use within two weeks.

3.2.9. Make hepatoblast differentiation medium consisting of knockout Dulbecco's modified Eagle medium (KO-DMEM) with 20% knockout serum replacement (KOSR) and supplemented with 0.5% of an alternative supplement to L-glutamine, 1% non-essential amino acids (NEAA), 0.1 mM beta-mercaptoethanol, 1% dimethyl sulfoxide (DMSO), and 1% penicillin/streptomycin (final concentrations at 100 IU/mL and 100 µg/mL, respectively). Filter under vacuum.

NOTE: Store at 4 °C and use within two weeks.

3.2.10. Make hepatocyte maturation medium consisting of hepatocyte medium supplemented

with 1% of an alternative supplement to L-glutamine, 10  $\mu$ M hydrocortisone 21-hemisuccinate sodium salt (HCC), 1% penicillin/streptomycin (final concentrations at 100 IU/mL and 100  $\mu$ g/mL, respectively). For each medium change, supplement the required volume with OSM and HGF (final concentrations at 20 ng/mL and 10 ng/mL, respectively).

NOTE: Store the stock at 4 °C and use within two weeks.

3.2.11. Make hepatocyte maintenance medium consisting of William's E medium supplemented with 10% knockout serum replacement, consisting of 1% of an alternative supplement to L-glutamine, 1% penicillin/streptomycin (final concentrations at 100 IU/mL and 100  $\mu$ g/mL, respectively). For each medium change, supplement the required volume with HGF, EGF, bFGF and VEGF (final concentration of each growth factor at 10 ng/mL).

NOTE: Store the stock at 4 °C and use within two weeks.

3.3. Check hepatosphere formation 24 h post seeding and initiate hepatocyte differentiation. Carefully remove the mTeSR1 medium and replace it with 1 mL of fresh endoderm differentiation medium supplemented with 100 ng/mL activin A and 50 ng/mL Wnt3a.

3.4. Change supplemented endoderm priming medium every 24 h for 3 days for hESCs. When working with hiPSCs, extend this stage for a further 2 days supplementing the media with activin A (100ng/mL) alone.

3.5. Following definitive endoderm induction, switch to hepatoblast differentiation medium for hepatoblast specification for 5 days replacing the medium every 2 days and perform the last change on the last day of the hepatoblast specification.

3.6. Transfer hepatospheres into the poly-HEMA coated wells.

3.6.1. Wash the cells once with hepatocyte maturation medium without supplements after removing KSR/DMSO medium and add 1 mL of hepatocyte maturation medium supplemented with 10 ng/mL HGF and 20 ng/mL OSM.

3.6.2. Using a P1000 pipette, lift up the hepatospheres from the agarose microplate by pipetting up and down the solution several times.

3.6.3. Transfer the medium containing the hepatospheres to a poly-HEMA coated well.

3.6.4. Wash the agarose microplate using 1 mL of hepatocyte maturation medium supplemented with 10 ng/mL HGF and 20 ng/mL OSM and transfer the medium to the poly-HEMA coated well.

3.6.5. Repeat step 3.6.4 as many times as possible in order to transfer all the hepatospheres from the agarose microplate.

NOTE: It is critical to carefully pipette up and down the solution containing the hepatospheres to avoid damaging them.

3.7. Carefully aspirate excess medium without removing hepatospheres by using a P100 pipette until ~ 1 mL of medium containing the hepatospheres remains in the poly-HEMA coated well.

3.8. Change the medium every 48 h for 12 days.

3.9. At day 20 when working with hESCs or day 22 if working with hiPSCs, switch the medium to hepatocyte maintenance medium.

3.9.1. Remove hepatocyte maturation medium and wash cells once with hepatocyte maintenance medium without the supplements. Add 1 mL of hepatocyte maintenance medium supplemented with 10 ng/mL of HGF, EGF, FGF and VEGF.

3.10. Change the medium for fresh hepatocyte maintenance medium every 48 h.

#### **4. Functional analysis of long-term cultured 3D hepatospheres**

4.1. Switch to hepatocyte maturation medium supplemented with 10  $\mu$ M HCC, L-glutamine, 1% penicillin/streptomycin (final concentrations at 100 IU/mL and 100  $\mu$ g/mL, respectively) and 10 ng/mL HGF 48 h prior to performing functional analysis.

4.2. Analyze hepatocyte metabolic function using cytochrome (CYP) P450 assays.

4.2.1. Replace medium with 1 mL of fresh hepatocyte maturation medium supplemented with 50  $\mu$ M luciferin-6'-pentafluoro-benzyl ether (luciferin-PFBE) substrate to detect CYP3A basal activity or 100  $\mu$ M luciferin-methyl ether (luciferin-ME) substrate to detect CYP1A2 basal activity (number of replicates = 3). Use tissue culture media as a negative control.

NOTE: In order to avoid cross-reactivity, it is recommended not to use the same wells containing 3D hepatospheres to test different CYP P450 activities but to perform them in individual wells.

4.2.2. Incubate cells for 24 h at 37 °C.

4.2.3. Collect the supernatants using a P100 pipette tip and carry out the assay as per

manufacturer's instructions.

4.2.4. Measure the relative levels of basal activity and normalize to per mg protein as determined by the bicinchoninic acid assay (BCA).

4.3. Test serum protein production using enzyme-linked immunosorbent assay (ELISA).

4.3.1. Replace medium with 1 mL of fresh hepatocyte maturation medium supplemented with 10 ng/mL HGF and 20 ng/mL OSM (number of replicates = 3). Use tissue culture media as a negative control.

4.3.2. Incubate cells for 24 h at 37 °C.

4.3.3. Collect the supernatant using a P100 pipette tip and measure the relative levels of serum protein production as per manufacturer's instructions.

4.3.4. Normalize to per mg protein as determined by the BCA Assay.

## **5. Immunocytochemistry**

5.1. Preparation of paraffin sections containing hepatospheres.

5.1.1. Wash the hepatospheres three times with 1x DPBS.

5.1.2. Fix the hepatospheres with ice-cold methanol for 30 min.

5.1.3. Wash three times with 1x DPBS.

5.1.4. Embed the hepatospheres in 300 µL of a tempered solution of 2% agarose dissolved in H<sub>2</sub>O using an empty well of a 24-well plate as a mold and leave it to solidify for 30 min.

5.1.5. Embed the agarose containing hepatospheres in paraffin.

5.1.6. Section the paraffin block containing fixed hepatospheres in 4 µm thick sections using a microtome.

5.2. De-waxing and rehydration of the sections.

NOTE: Use fresh solutions whenever it is possible. Do not use solutions that have been on the staining trough for longer than a week.

329 5.2.1. Place sections in a slide rack.  
330  
331 5.2.2. Immerse slide rack containing sections in a staining trough containing 300 mL of xylene  
332 for 5 min.  
333  
334 5.2.3. Repeat step 5.2.2.  
335  
336 5.2.4. Immerse in absolute ethanol for 20 s.  
337  
338 5.2.5. Immerse in 95% ethanol for 20 s.  
339  
340 5.2.6. Immerse in 90% ethanol for 20 s.  
341  
342 5.2.7. Immerse in 80% ethanol for 20 s.  
343  
344 5.2.8. Immerse in 70% ethanol for 20 s.  
345  
346 5.2.9. Rehydrate the sections with water for 5 min.  
347  
348 NOTE: Maintain the slides in the same slide rack and move it from one staining trough to the  
349 next one. Volume used (generally ~300 mL) depends on the size of the staining trough.  
350  
351 5.3. Antigen retrieval of paraffin sections containing hepatospheres.  
352  
353 5.3.1. Heat de-waxed and rehydrated sections in 1x Tris-EDTA (TE) pH 9.0 buffer solution for 15  
354 min in a microwave at 800 W.  
355  
356 5.3.2. Cool down samples by immersing them in tap water for 5 min.  
357  
358 5.4. Immunostaining.  
359  
360 5.4.1. Incubate slides with blocking solution made of PBS with 0.1% polysorbate 20 (PBS/T)/10%  
361 BSA for 1 h at RT.  
362  
363 5.4.2. Replace blocking solution with the appropriate primary antibody diluted in PBS/T/1% BSA  
364 and incubate at 4 °C with gentle agitation overnight.  
365  
366 5.4.3. Wash cells with PBS/T for 5 min and repeat three times.  
367  
368 5.4.4. Incubate with the appropriate secondary antibody diluted in PBS/T/1% BSA and incubate  
369 in the dark at RT for 1 h with gentle agitation.

NOTE: The optimized primary and secondary antibodies are listed in **Table of Materials**.

5.4.5. Wash cells with PBS/T for 5 min and repeat three times.

5.4.6. Add 4',6-diamidino-2-phenylindole (DAPI) to the cells according to manufacturer's instructions and place a glass coverslip gently to reduce air bubbles. Keep fixed cells at 4 °C in the dark. Observe staining under a microscope with appropriate filter and fluorescent lamp.

NOTE: Store plates at 4 °C in the dark until imaging.

#### **REPRESENTATIVE RESULTS:**

Three-dimensional aggregates from the embryonic stem cell line (H9) or the induced pluripotent stem cell line (P106) were differentiated towards the hepatocyte lineage using our defined procedure (**Figure 1**). 3D spheres of pluripotent stem cells were first primed towards definitive endoderm prior to hepatoblast specification. Following this, hepatoblasts were matured into 3D hepatospheres which could be maintained in culture for up to one year<sup>23</sup>.

To study the structure of 3D spheres, 30-day-old hESC- or iPSC-derived spheres were fixed, sectioned and stained to detect the presence of proteins expressed in hepatocytes and mesenchymal cells. Hepatocyte nuclear factor 4 alpha (HNF4α) and the mesenchymal marker vimentin were employed, revealing the presence of an outer layer composed of hepatocyte like cells surrounding a core of mesenchymal cells (**Figure 2**). We followed these experiments to analyze the expression of proteins expressed in hepatocytes: albumin, CYP3A and E-cadherin. Staining revealed that the expression of these markers was restricted to the outer layer of the spheres (**Figure 3**).

Functional analyses of the hepatospheres were performed in day 30 cultures. CYP1A2 and CYP3A are important enzymes within functional hepatocytes. Their activity was assessed using established assays. H9-derived hepatospheres exhibited CYP3A activity at  $220375 \pm 74514$  RLU/mL/mg protein and CYP1A2 activity at  $732440 \pm 33330$  RLU/mL/mg protein (**Figure 4A**). CYP3A activity in P106-derived hepatospheres displayed at  $132117 \pm 43391$  RLU/mL/mg protein and CYP1A2 activity at  $409907 \pm 121723$  RLU/mL/mg protein (**Figure 4B**). When compared with two batches of human primary hepatocytes, 3D liver spheres displayed respectable levels of CYP activity<sup>10</sup>.

Analysis of the synthesis and secretion of albumin and alpha-fetoprotein (AFP) revealed that H9-derived hepatospheres secreted  $683.9 \pm 84$  and  $159 \pm 20$  ng/mL/24 h/mg protein of albumin and alpha-fetoprotein, respectively (**Figure 5A**). Whereas P106-derived hepatospheres secreted  $497 \pm 41$  and  $756 \pm 24$  ng/mL/24 h/mg of albumin and alpha-fetoprotein (**Figure 5B**).

## FIGURE LEGENDS:

**Figure 1: Stepwise differentiation procedure to generate 3D hepatospheres from hESCs.** Blue brackets represent days of differentiation for hiPSCs.

**Figure 2: Structural reorganization of 3D hepatospheres.** Representative images of the expression of hepatocyte nuclear factor 4 alpha (HNF4 $\alpha$  - green) and vimentin (red) in (A) H9-derived 3D hepatospheres and (B) P106-derived 3D hepatospheres and their corresponding immunoglobulin G (IgG) controls. Scale bars represent 60  $\mu$ m.

**Figure 3: Evaluation of hepatic marker expression in 3D hepatospheres.** Representative images of the expression of hepatocyte markers — albumin, CYP3A, E-cadherin and their corresponding IgG controls in (A) H9- and (B) P106-derived 3D hepatospheres. Scale bars represent 60  $\mu$ m.

**Figure 4: Cytochrome P450 function in 3D hepatospheres.** Measurement of the cytochrome P450 1A2 and 3A activity in (A) H9-derived 3D hepatospheres and (B) P106-derived 3D hepatospheres. The data represents the mean of three biological replicates, and the error bars represent the standard deviation (SD). Activity is quoted as relative light units (RLU) per mL per mg of protein.

**Figure 5: Analysis of hepatosphere protein secretion.** The secretion of albumin and alpha-fetoprotein (AFP) was analyzed in (A) H9-derived 3D hepatospheres and (B) P106-derived 3D hepatospheres. The data is representative of three biological replicates, and the error bars represent the SD. Secreted protein is quoted as nanograms of protein per mL per 24 h per mg of protein.

## DISCUSSION:

The development of defined and xeno-free systems to produce human hepatospheres in 3D is required for both in vitro and in vivo endeavors. At present most of the current hepatocyte differentiation approaches from human pluripotent stem cells are performed in two dimensional adherent cultures. These environments lack many of the environmental cues involved in tissue genesis and homeostasis which include; heterotypic cell interactions, matrix production and remodeling, which can lead to failure in translation to the human situation in vivo<sup>18,19</sup>.

As a result, research has focused on alternative approaches to generate hepatospheres from pluripotent stem cells. A number of 3D studies have advanced the field, but those are reliant on animal products<sup>20,22,24</sup> to provide support and/or require the use of human tissue<sup>21,22</sup> which complicates technology scale-up and compromises experimental reproducibility and application.

The procedure described in our article (**Figure 1**) is defined, efficient, highly reproducible and cost-effective, allowing the production of functional liver spheres, which remain functional over a year in vitro and provide critical liver support in vivo<sup>14</sup>. Importantly, this platform allows the user to control the size of the 3D liver spheres, limiting the formation of dense necrotic centers and loss of phenotype.

The transfer of the 3D hepatospheres to the poly-HEMA coated plates represents a critical step in this protocol. It is important to pipette gently at this stage in the procedure in order to avoid cell damage. In addition, media changes must be performed carefully to avoid shear stress and distortion of sphere structure.

In these studies, 3D hepatospheres displayed an organized structure (**Figure 2** and **Figure 3**), CYP1A2 and CYP3A metabolic activities (**Figure 4**) and secreted liver proteins, including albumin and alpha-fetoprotein (**Figure 5**). This procedure has been successfully performed in four pluripotent stem cells lines with comparable outcomes. Looking ahead, this technology could be employed as a platform to develop further endodermal and mesenchymal tissues with complex architectures.

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#### **DISCLOSURES:**

David C. Hay is a co-founder and shareholder of Stemnovate Ltd and HigherSteaks Ltd. The rest of the authors certify that they have no conflicts of interest in the subject matter or materials discussed in this article.

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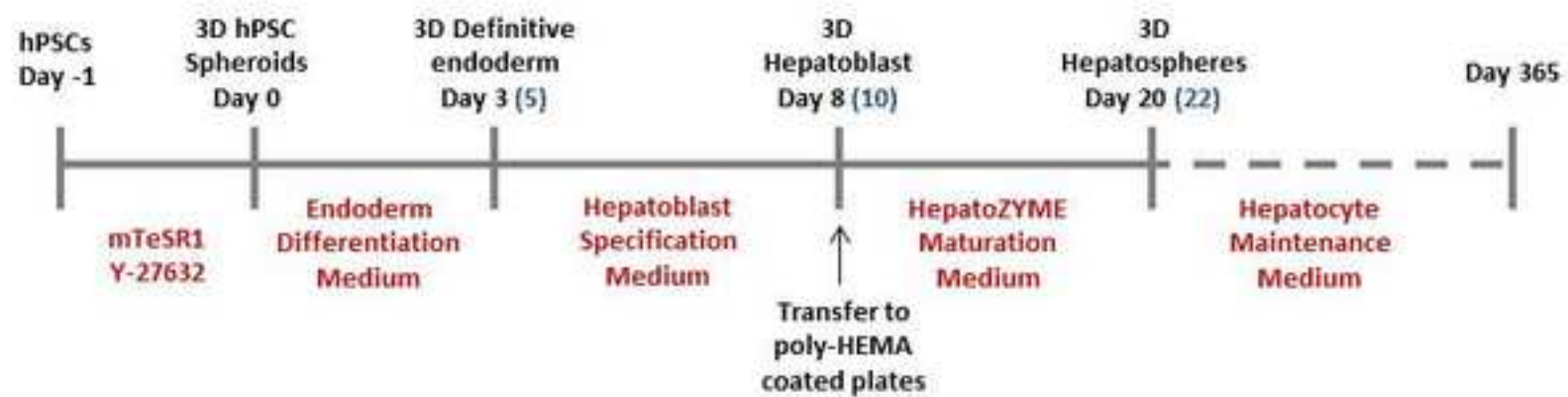
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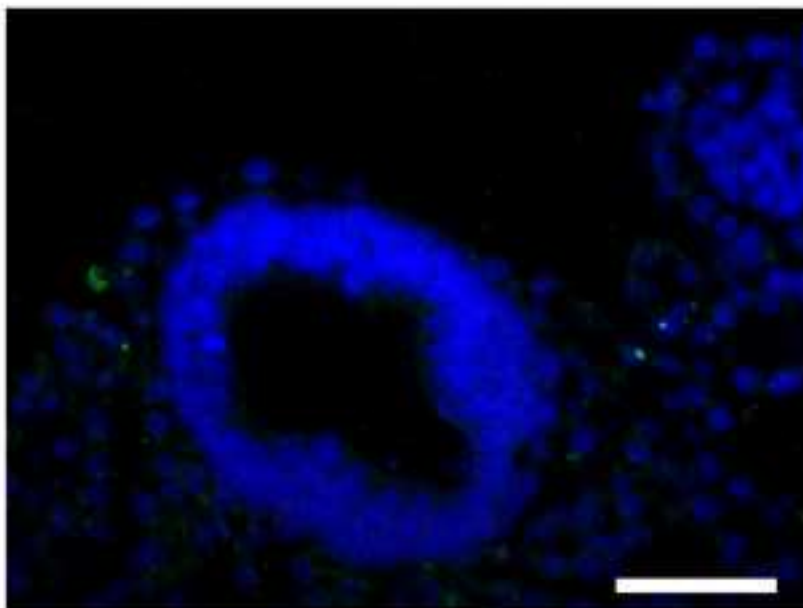
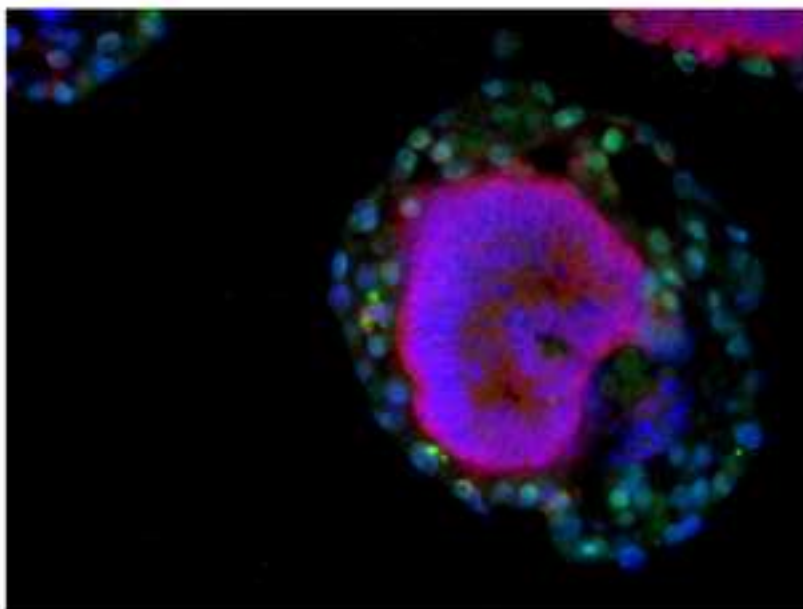
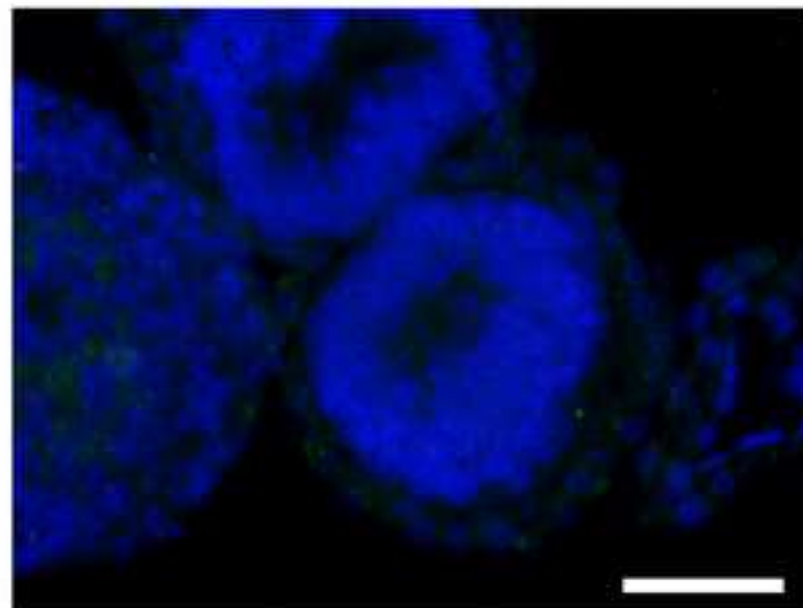
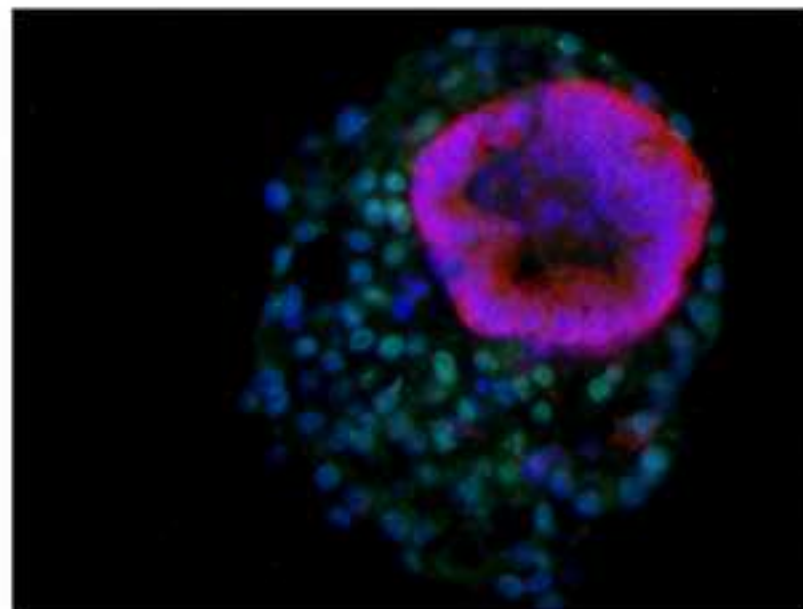
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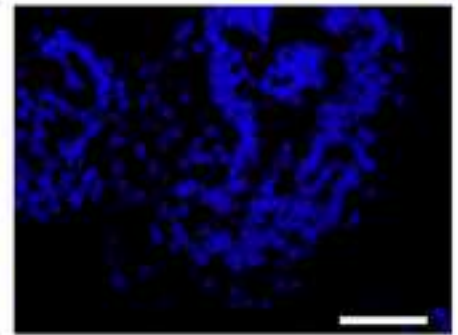
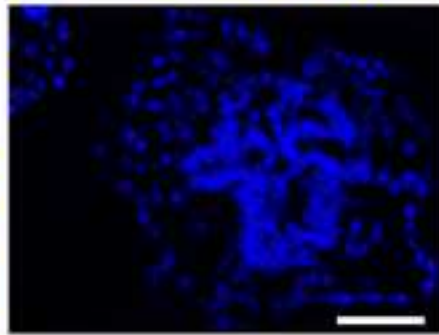
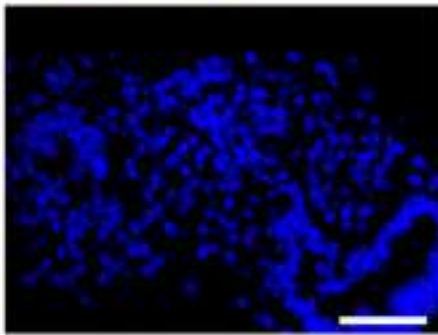
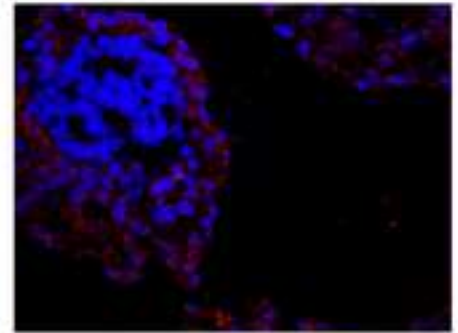
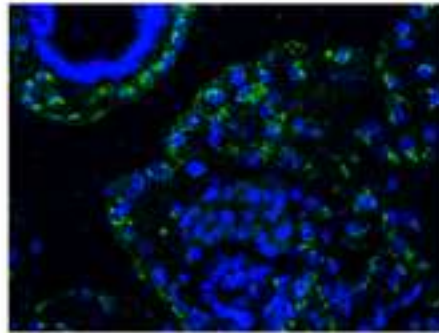
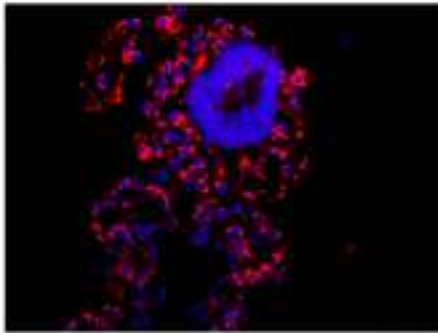
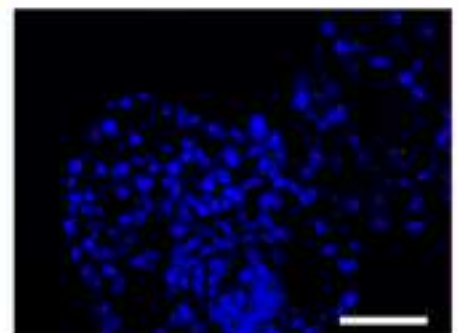
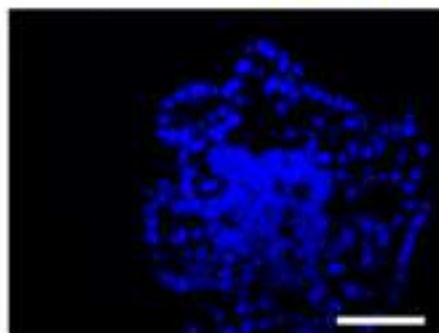
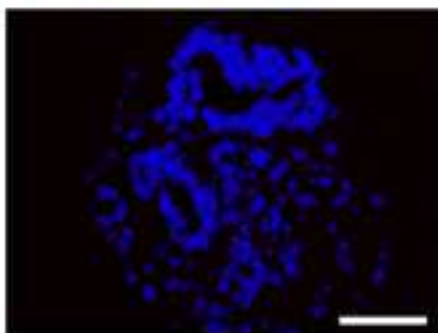
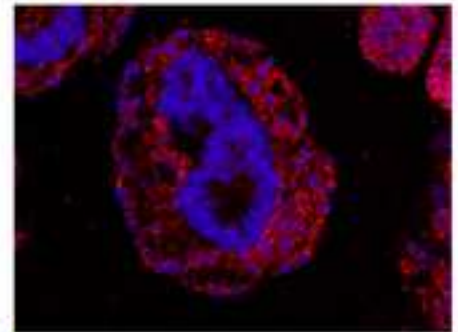
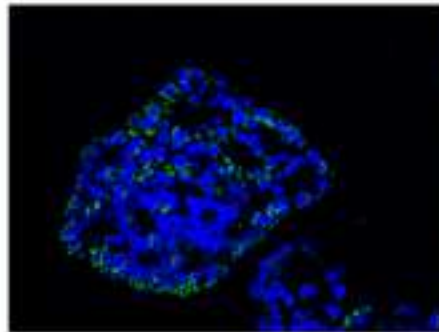
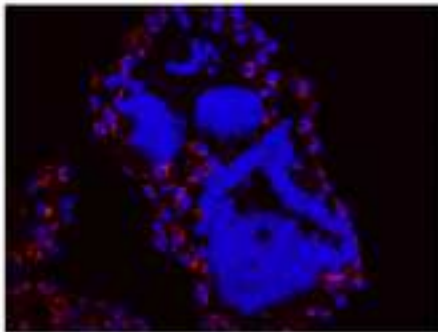
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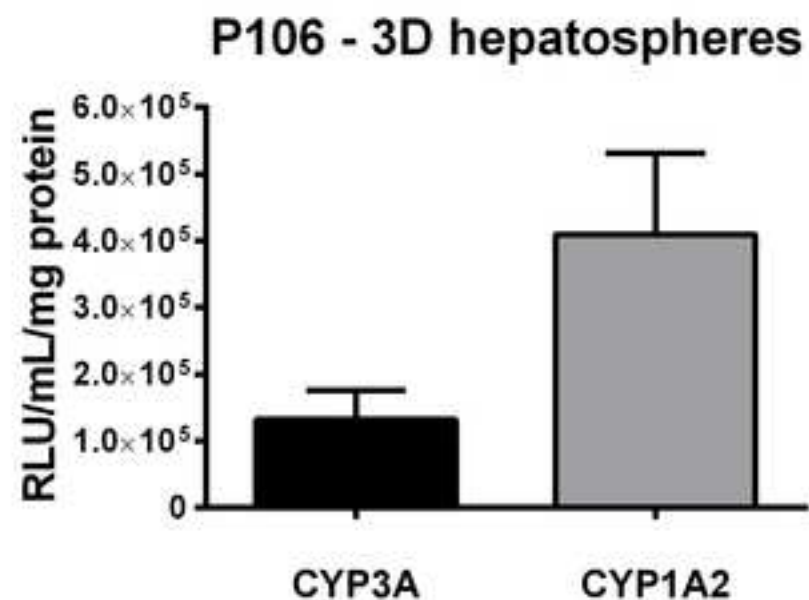
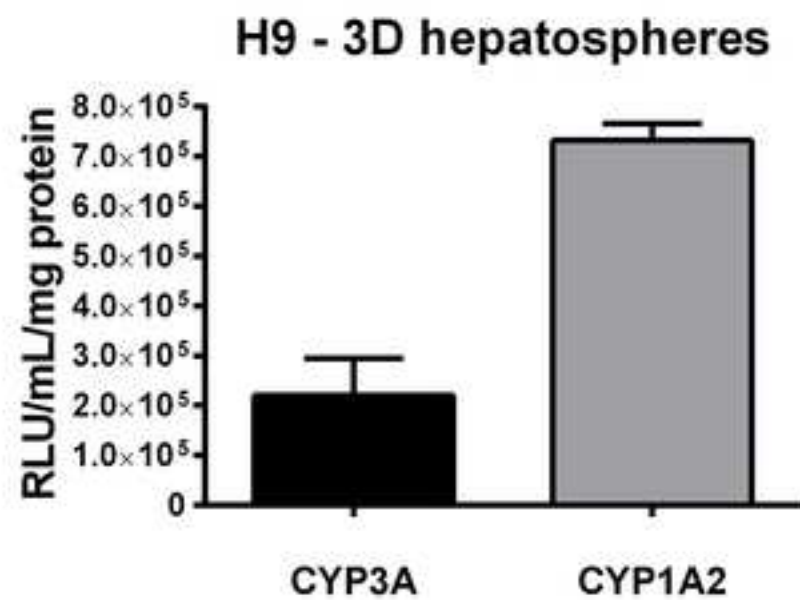
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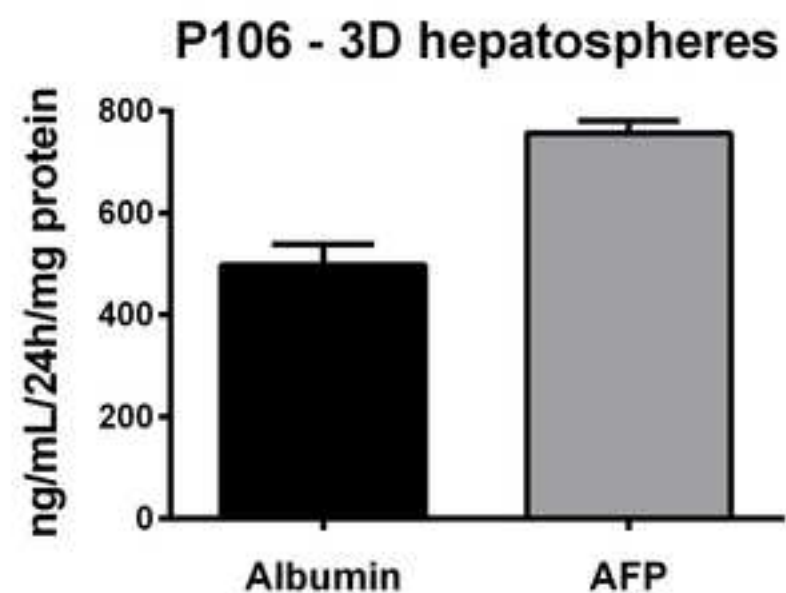
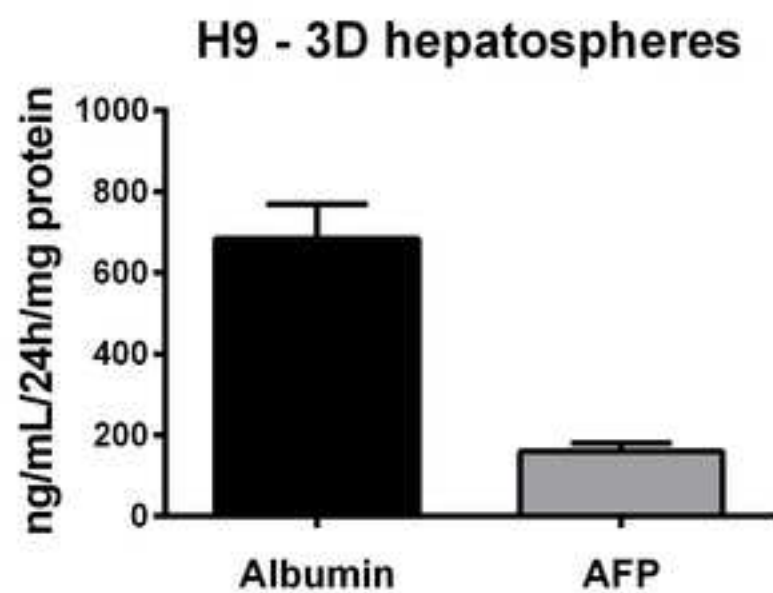
Figure 1



**A****B**

**A****Albumin****CYP3A4****E-Cadherin****B****Albumin****CYP3A4****E-Cadherin**





**Cell Culture and functional assays**

<b>Name of Material</b>	<b>Company</b>	<b>Catalog Number</b>
Agarose	Fisher Bioreagents	10766834
B27 supplement	Life Technologies	12587-010
beta-mercaptoethanol	Life Technologies	31350
Bovine Serum Albumin	Sigma-Aldrich	A2058
DAPI	Invitrogen	D1306
DMSO	Sigma-Aldrich	D5879
DPBS, no calcium, no magnesium	ThermoFisher	14190250
DPBS with Calcium and Magnesium	ThermoFisher	14040133
Gentle cell dissociation reagent	STEMCELL Technologies	7174
GlutaMax	Life Technologies	35050
HepatoZYME-SFM	Life Technologies	17705021
Human Activin A	Peptotech	120-14E
Human Alpha Fetoprotein ELISA	Alpha Diagnostics	500
Human Basic Fibroblast Growth Factor	Peptotech	100-18B
Human Epithelial Growth Factor	Peptotech	236-EG
Human Hepatocyte Growth Factor	Peptotech	100-39
Human Oncostatin M	Peptotech	300-10
Human Recombinant Laminin 521	BioLamina	LN521-02
Human Serum Albumin ELISA	Alpha Diagnostics	1190
Human Vascular Growth Factor	Bio-technie	293-VE
Hydrocortisone 21-hemisuccinate sodium salt	Sigma-Aldrich	H4881
Knockout DMEM	Life Technologies	10829
Knockout Serum Replacement	Life Technologies	10828
Micro-mold spheroids	Sigma-Aldrich	Z764000
mTeSR1 medium	STEMCELL Technologies	5850
Non-essential amino acids	Life Technologies	11140
P450-Glo CYP1A2 Assay and Screening System	Promega	V8771
P450-Glo CYP3A4 Assay and Screening System	Promega	V8801

Penicillin-Streptomycin (10,000 U/mL)  
poly-HEMA (Poly 2-hydroxyethyl methacrylate)  
Recombinant mouse Wnt3a  
RPMI 1640  
Rho-associated kinase (ROCK)inhibitor Y27632  
Trypan Blue solution  
William's E-medium

Life Technologies  
Sigma-Aldrich  
Bio-technie  
Life Technologies  
Sigma-Aldrich  
Sigma-Aldrich  
Life Technologies

15140122  
P3932  
1324-WN-500/CF  
21875  
Y0503-1MG  
T8154  
A1217601

## Equipment

### Name of Material

Microwave  
Microtome  
Oven

### Company

Bosch  
Leika  
Thermoscientific

### Catalog Number

RM2125RT

## Antibodies

### Primary antibodies

#### Name of Material

Albumin  
CYP3A4  
E-cadherin  
HNF-4 $\alpha$   
IgG  
Vimentin

#### Company

Sigma-Aldrich  
University of Dundee  
Abcam  
Santa Cruz  
DAKO  
DAKO

#### Catalog Number

A6684  
University of Dundee  
ab76055  
sc-8987  
X0943  
M0725

### Secondary antibodies

Donkey anti-sheep, Alexa Fluor 488  
Goat anti-mouse, Alexa Fluor 568

Life technologies  
Life technologies

A-11015  
A-11004

Goat anti-rabbit, Alexa Fluor 488  
Rabbit anti-mouse, Alexa Fluor 488

Life technologies  
Life technologies

A-11008  
A-11059

## Immunostaining and fixatives

Name of Material	Company	Catalog Number
0.5M EDTA	Life Technologies	15575020
Boric Acid	vWR International	2549
Cover Glass	vWR International	631-0135
Ethanol	Fisher Scientific	15637850
Methanol	Sigma-Aldrich	34860
Slide rack	Agar Scientific	AGH112
Staining trough	DWK Life Scientific	631-0856
Tris (Base)	vWR International	4109
Tween	Sigma-Aldrich	P9416
Xylene	Sigma-Aldrich	534056



**Dilution employed**

- 1:100 (mouse)
- 1:200 (sheep)
- 1:200 (mouse)
- 1:100 (rabbit)
- 1:400
- 1:100 (sheep)

- 1:400
- 1:400

1:400

1:400



Agarose	Fisher Bioreagents	10766834
B27 supplement	Life Technologies	12587-010
beta-mercaptoethanol	Life Technologies	31350
Bovine Serum Albumin	Sigma-Aldrich	A2058
DAPI	Invitrogen	D1306
DMSO	Sigma-Aldrich	D5879
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DPBS with Calcium and Magnesium	ThermoFisher	14040133
Gentle cell dissociation reagent	STEMCELL Technology	7174
GlutaMax	Life Technologies	35050
HepatoZYME-SFM	Life Technologies	17705021
Human Activin A	Peprtech	120-14E
Human Alpha Fetoprotein ELISA	Alpha Diagnostics	500
Human Basic Fibroblast Growth Factor	Peprtech	100-18B
Human Epithelial Growth Factor	Peprtech	236-EG
Human Hepatocyte Growth Factor	Peprtech	100-39
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Human Vascular Growth Factor	Bio-technie	293-VE
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Recombinant mouse Wnt3a	Bio-technie	1324-WN-500/CF
RPMI 1640	Life Technologies	21875
Rho-associated kinase (ROCK) inhibitor	Sigma-Aldrich	Y0503-1MG
Trypan Blue solution	Sigma-Aldrich	T8154
William's E-medium	Life Technologies	A1217601

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Serum free production of 3D human hepatospheres from pluripotent stem cells

Author(s):

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**Editorial comments:**

We are grateful to the editorial team for their time and comments. We have addressed those comments below:

Changes to be made by the author(s):

*1. Please note that the editor has formatted the manuscript to match the journal's style. Please retain the same. The updated manuscript is attached and please use this version to incorporate the changes that are requested.*

We have modified the manuscript without altering the format.

*2. Please revise lines 105-109, 112-114, 186-188, 200-202, 219-221, and 364-365 to avoid previously published text.*

We have tried to use original language wherever possible. However, we are limited in the ways in which we can describe certain steps. We hope that the current changes are acceptable

*3. As we can only film 2.75 pages of the protocol, please review and shorten the highlighted portion to 2.75 pages. For example, some of the shorter protocol steps can be combined; however, please ensure that there are no more than 2-4 actions per step. Please note that the highlighted content should be continuous and contain 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. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.*

We have highlighted the sections that we wish to be filmed

*4. Abstract: Please expand to include an overview of the method and a summary of its advantages, limitations, and applications.*

We have expanded the abstract to include the detail requested

*5. Discussion: As we are a methods journal, please also discuss critical steps within the protocol and any limitations of the technique.*

We have extended the discussion to take account of this request.

*6. Please address specific comments marked in the attached manuscript.*

We have addressed the specific comments and modified them accordingly to the editor's comments whenever it was convenient.