Imperial College London
Hepatitis virus group
Department of Medicine
Medical School, Room 309
Norfolk Place
London, W2 1PG
United Kingdom

Jaydev Upponi
JOVE
United Kingdom
50 Westminster Bridge Road
London, SE1 7QY
United Kingdom

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.

2. Please print and sign the attached Article And Video License Agreement – UK. Please then scan and upload the signed ALA with the manuscript files to your Editorial Manager account.

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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.