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25/01/2021,

RESPONSE TO EDITORIAL AND REVIEWER COMMENTS

Dear Editor,

Thank you very much for considering our manuscript for publication and for your feedback. We thank the reviewers for their constructive and supportive comments and have edited the manuscript to address their concerns. We hereby provide a substantially revised manuscript, now entitled **"Staining and high-resolution imaging of 3D organoid and spheroid models"**, in which we have integrated all of the reviewers' comments.

We hope that with these changes and additions, JoVE will deem our manuscript suitable for publication.

With kind regards,

Dr. Laura Broutier

Editorial comments:

Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.	✓
Please revise the following lines to avoid previously published work: 35-36, 91-93, 103-104, 135-136, 139-140, 149-150, 171-172, 182-183, 189-190, 196-199, 207-208, 245-2456, 282-283, 291-292, 308-313, 343-345, 358-360, 430-433.	✓
JoVE cannot publish manuscripts containing commercial language. 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: e.g., Matrigel, OPERA Phenix, PerkinElmer, Harmony 4.9, Q Path, Cytoblock, Cytoblock Cassettes, Cytoblock Reagent #1, Cytoblock Reagent #2, Thermo Scientific, Panoramic SCAN II, Axio- Imager, Halo, Indica labs, etc. We must maintain our scientific integrity and prevent the subsequent video from becoming a commercial advertisement.	✓
Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.	✓
Line 111/126/132/141: Please italicize “g” which indicates the centrifugal speed.	✓
Line 352-366/ 367-377: Please ensure that each Figure Legend includes a title and a short description of the data presented in the Figure and relevant symbols. The Discussion of the Figures should be placed in the Representative Results. Details of the methodology should not be in the Figure Legends, but rather the Protocol.	✓
Please remove the lines 419-422	✓
Please do not use the &-sign or the word “and” when listing authors. Authors should be listed as last name author 1, initials author 1, last name author 2, initials author 2, etc. End the list of authors with a period. Example: Bedford, C. D., Harris, R. N., Howd, R. A., Goff, D. A., Koolpe, G. A. Quaternary salts of 2-[(hydroxyimino)methyl]limidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).	✓
Please remove the commercial names (Harmony, Halo, Panoramic Scan II, Zeiss AxioImager).	✓
Figure 2B: Please remove the commercial name “Cytoblock”.	✓
Figure 3: Please provide the scale bars for all the images. Please define the scale bars either in the image or the Figure Legend.	✓
Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.	✓
Files uploaded to the “Supplemental Files (as requested by JoVE)” section of your Editorial Manager account are only for JoVE’s internal use and will NOT be published with your article. If you would like your files to be available for download with your article, then please move them to the “Supplemental Code Files” section of your Editorial Manager account. However, if these files are only to help the scriptwriter visualize the computational steps, then they can remain in the current section.	✓
14. Supplementary File 1: Please consider removing the commercial name Harmony 4.9	✓
15. Supplementary File 2: Please consider removing the commercial name Halo.	✓

Reviewers' comments:

Reviewer #1: Manuscript Summary: This protocol substantially covers 3D tissue fixation and embedding that can be standardized across many 3D model systems. The authors have included an exceptional level of detail that can be replicated by other researchers. Overall, I find the protocol and manuscript of good quality and of high value to the scientific community. As an organoid researcher myself, the methods within research publications are often too abbreviated to replicate. Thus, this new protocol will likely be highly utilized and cited.

We thank the Reviewer #1 for providing such constructive feedback on our manuscript. We have attempted to address all concerns raised to submit an improved version of our manuscript that we renamed: **Staining and high-resolution imaging of 3D organoid and spheroid models**. Below a point-by-point response to Reviewer #1 questions is provided and the manuscript has been adjusted accordingly. All modifications made to the manuscript are highlighted in red.

Major Concerns:

1) The protocol is general to 3D cultures, which is a strength, yet the introduction is unfocused and goes into too much depth about several types of 3D cultures. This should be revised to be general and provide examples without going into too much detail.

We have revised the Introduction to be more general and provide examples.

2) The figures are a great addition to the manuscript, but the legends should include the cell type and days in culture. This also may provide more focus to the introduction in which they may refer to the examples shown.

We have adjusted the legend and cited the examples in the Introduction.

3) I'm not sure that the inclusion of the Axio imager with Halo software are permitted in JoVE. Also, although acquisition mode settings are included for one instrument, this section is not particularly powerful in reproducibility, as many laboratories utilize different confocal microscopes. Thus, this section may need to be removed, in which case, the title could be revised to something like "Staining and preparation of 3D cell models for high-resolution imaging".

We agree with the Reviewer #1 than we should avoid commercial language. Since imaging is a critical part of the procedure and details on acquisition mode settings and analyses are necessary and helpful for users, we decided to provide broader acquisition mode settings to help users regardless of their tools. Moreover, we kept details of our own acquisition and analysis settings in figure legends and supplementary figures but emphasized in the text that they can be used as helpful examples but should be adapted to the microscope and analysis software used.

4) A video for harvesting 3D cell cultures is only somewhat helpful, but the field would greatly benefit from videos of procedures 2 (whole mount staining) and 3 (paraffin embedding).

We thank the reviewer for this interesting feedback and have changed the highlighted sections accordingly in the manuscript.

5) The following is used interchangeably throughout the text, and is a source of confusion: spheroids, 3D cell cultures, 3D-cells, tumor spheroids, organoids. The authors should clarify the terminology throughout the protocol.

We apologize for the confusion. We have now clarified our terminology in the manuscript. We have carefully defined what we consider in this manuscript as *in vitro* 3D cell culture models, spheroids and organoids. Moreover, to help with manuscript clarity towards the procedure itself we only use the term “3D structures” to refer to *in vitro* 3D cell culture models.

6) The discussion can be more thorough by integrating more than one comparison with previously published protocols to this one for each section of the procedure.

As suggested, we have revised our Discussion to include comparisons of our protocol with other previously published protocols.

Minor Concerns:

7) Areas of odd wording throughout the manuscript

We have carefully edited our manuscript.

8) Add cell type and days in culture to the figure legends

We apologize for this oversight. We have adjusted the legends.

9) Figure 1:
   - Overall clear schematics. The organization could be improved to have clearer zones. Consider splitting this figure into "a" and "b" sections for the separate procedures described above.
   - Change "sectionning" to "sectioning"
   - Consider including "&" to read as "2D Sectioning & Staining"
   - Refer to the different zones in your figure to guide the reader. Example, line 354: "...and then either prepared for immunofluorescence or embedded in paraffin." to "...and then either prepared for 3D whole mount staining (top) or 2D sectioning and staining (bottom)."

We thank the Reviewer#1 for the suggestions. We have adjusted Figure 1 and the corresponding Legend to address all comments.

10) Figure 2:
   - It would be useful to guide the reader through the text by referring to figure parts. Also the legend (or the text) should describe "good embedding" versus "failed embedding." Here the authors should indicate arrowheads point to 3D cultures. This are much larger than the actual organoids and not useful. A higher magnification to see the organoids would help.

We have adjusted Figure 2 and the corresponding Legend to address all comments.

11) Figure 3:
   - In reference to figure, define "Weak positive" in the legend or in the text.

As suggested, we defined "Weak positive" in the legend of the Figure 3.

Reviewer #2: Overall, the aim to provide simple, robust, and reproducible protocols for quantifying cells in 3D environments is very worthwhile, and I applaud the authors for choosing to address this challenge. While I have no doubt that the protocol as described here works well for the cell models used by the authors, I wonder whether they would scale well for larger organoids or different types of organoids. As it stands, the description of the procedure lacks key details and does not adequately address limitations. As such, I think the broad claims of the title and text need to be significantly narrowed down in order to avoid misleading future readers in trying a protocol that has not been vetted for their desired conditions. Also, the authors fail to distinguish the relative advantages and disadvantages of their protocol to a plethora of already published 3D staining, clearing, and quantification protocols. More details in the individual comments below.

We thank the Reviewer #2 for providing constructive feedback on our manuscript. We have attempted to address all concerns raised to submit an improved version of our manuscript that we renamed: **Staining and high-resolution imaging of 3D organoid and spheroid models**. Below a point-by-point response to Reviewer #2 questions is provided and the manuscript has been adjusted accordingly. All modifications made to the manuscript are highlighted in red.

Major Concerns:

1) The authors claim a "Staining and high-resolution imaging of fixed 3D cell models". The field of 3D cell models is quite vast, spanning many different tissue and cell-types and aggregates with small (tens of microns) to very large diameters (several millimeters). Many organoid protocols result in tissues that are larger than one millimeter, and the broad claims in this manuscript appear not to have been vetted with samples larger than a few hundred micrometers. There is a sentence in the discussion mentioning this, but the title and introduction may mislead readers looking for a protocol to fix, stain, and clear organoid models that commonly result in structures in the millimeter range. The authors should either considerably narrow their claims and amend their title and text, or they need to provide data showing the applicability to various 3D spheroid and organoid models spanning at least tissues derived from the three germ layers to account for the heterogeneity of tissue behaviors of different origin during fixing, staining, and clearing.

We apologize for the lack of clarity. Our procedure presents two different and complementary types of staining and imaging acquisition to deal with a large variety of sizes (from 50-100 µm to several millimeters) and types of *in vitro* 3D cell culture models. The choice of one (3D whole-mount analysis) or the other (2D sectioning analysis) will depend on the model used and aim of the study. 3D whole-mount analysis by confocal enables the visualization of cells with a depth of field of 200 µm, irrespective of the overall size of the 3D structure, whereas the 2D sectioning allows the analysis of larger samples reaching several millimeters, but providing only 2D dimensional results. We have now clarified the procedure and the Figure 1 to help users choose the best experimental set-up according to their needs.

We agree with Reviewer #2 and considering the large variety of 3D *in vitro* cell culture models we have limited our procedure to organoids and spheroids, and carefully defined in the introduction what these structures represent. We have amended our title accordingly. Moreover, following Reviewer #2 suggestions, we have included in the revised manuscript references and new staining in Figure 3 to show the validity of our procedure for cells arising from the three germ layers to account for the heterogenous tissues. Please find further details in the following answers.

2) The authors only test their claims using one, maybe two different 3D cell culture models. They also fail to identify what their model system is (or are they two different ones in figure 3?), making it additionally difficult for readers to judge whether this protocol might be of use for their own samples. The authors need to clearly identify what their samples are and how they were generated.

We apologize for this oversight. In the first version of this manuscript, we used one of our models, a primary human rhabdomyosarcoma culture grown in 3D structures in low attachment suspension culture conditions for 15 days. We have adjusted the legend of Figure 3 accordingly. We have now added more models (see below), and have clarified this point.

3) The authors do present their example data without any discernible controls. How can a reader judge whether the technique results in abnormally high background stainings? How do we know whether the technique alters morphology of the markers? The study should include control samples imaged without primary antibodies and treated and acquired with identical settings. These should be presented side-by-side with the fully stained samples. Similarly, to judge fidelity of the segmentation, it would be advantageous to count a few samples by hand and compare results with the automated segmentation. The way things are presented here, it is impossible to judge the quality of the technique employed due to a lack of conditions to compare it to.

For all acquired images, we established settings using a negative control to control the lack of fluorescence when the primary antibody is absent (see Figure 3a) and to validate the specificity of our staining. In the procedure and in the legend of Figure 3, we have now clarified this step, which we agree with Reviewer #2, is a prerequisite for any analysis. Nevertheless, we would prefer not to include all these control images in our Figure 3 to maintain a level of clarity.
Moreover, to provide data supporting our automated segmentation methods, we have now performed manual counting and obtained highly consistent results, though this procedure was 10 times longer than the automated segmentation. We have modified Figure 3 accordingly.

4) The authors use a single nuclear stain (Ki67) to test and present their protocol. This should be considerably widened to include cytoplasmic and membrane-based stains to ascertain whether their techniques also function for these classes of antigens.

We thank the Reviewer #2 for this comment, which has significantly improved our manuscript. In the first version of this manuscript, we decided to present an example using a nuclear marker. To our knowledge, if nuclear staining works, and considering that the penetration of the antibodies is the limiting step here, membrane-based and cytoplasmic staining should follow. However, this protocol was already performed using a variety of markers (cytoplasmic, membrane-based and nuclear) in my previously published work (Broutier et al. Nature medicine, 2017). Please find below a panel with examples of different staining obtained using the described procedure on primary liver cancer organoids.

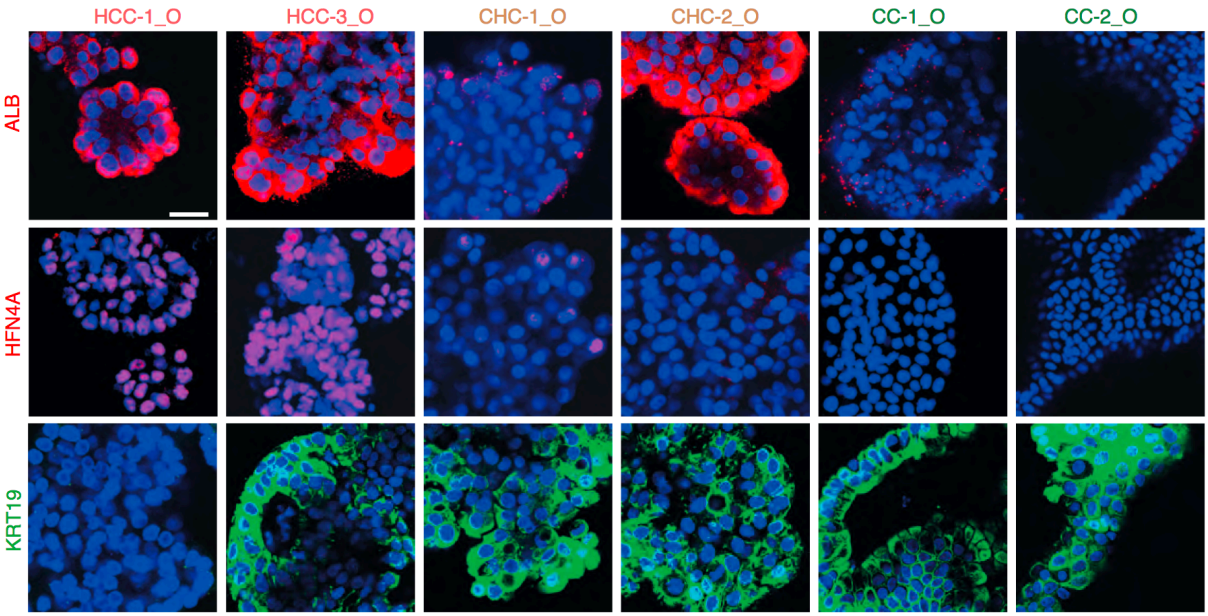

Figure A: IF analysis for the hepatocyte markers ALB and HFN4A (red) and ductal/CC marker KRT19 (green) on tumoroids expanded in culture for at least 3 months. Nuclei were counterstained with Hoechst33342 (blue). Scale bar, 30µm.

We are now mentioning this reference in the introduction to help readers to evaluate the quality of our procedure. Moreover, following Reviewer #2 suggestions, we have now included in Figure 3 cytoplasmic and nuclear staining on 3 different models from different origins (human rhabdomyosarcoma, human glioma, murine neural crest cells). Together with our cited previous work, we consequently applied our procedure to cells arising from the three germ layers to account for the effect on heterogenous tissues of fixing, staining, and clearing.

5) The removal of ECM suggested by the authors amounts to a relatively "blind" mechanical abrasion. These risks damaging the outer structures of organoids, some of which can extend small cellular processes into the ECM. How can the process guarantee not removing the outer layer of morphologies of embedded 3D cellular structures? See below, our combined response to point 5 and 6.
6) Authors caution the readers that "Remaining 3D matrix can hamper proper antibody penetration of the organoid structure or lead to high background staining". This is a big caveat. Removal of ECM may drastically alter morphology and the understanding of the cellular niche in the outer segments of organoids. If this protocol requires removal of ECM to function, then it only provides an incomplete picture of the very cellular niches that 3D cell culture aims to understand. Authors should amend their claims.

As Reviewer #2 noted, the 3D matrix can hamper proper antibody penetration of the organoid structure or lead to high background staining. We thus suggest whenever possible to remove ECM before staining. In many cases, this removal, based on very gentle washes, results in the preservation of the 3D structures of the organoids, as nicely exemplified by *Dekkers et al.* (Nature protocols, 2019). Owing to the wide variety of 3D *in vitro* cell culture models, we agree that ECM removal may alter the morphology of the outer segments of organoids (notably in case of small cellular protrusions) and partially hamper the analysis. To clarify these points, we have now (i) limited our procedure to organoids and spheroids, and carefully defined these structures in the introduction (ii) added a note in the appropriate section to encourage the user to adapt the “Harvesting of 3D cell culture models embedded in a matrix” step according to their systems and needs. Moreover, we have given tips and included a reference to help in the process of keeping the ECM and pursuing with our procedure.

7) The evidence for the ability to quantify in 3D in figure 3 is based on confocal stacks spanning 75 x 0.7 um = 52.5 um. These are very small samples and the side views indicate that the stacks do not even remotely include the rather small spheroids. The authors should quantify whole microtissues to support their claims or describe potential limitations in detail. Ideally, they showcase proper segmentation at the bottom, center, and top of the spheroids using a single image segmentation algorithm.

We have re-acquired a more accurate sample to sustain our claim. We have re-acquired a more accurate sample to sustain our claim. The choice of the step in between each focal plan was carefully adjusted to the specifications of each objective use and its depth of focus. For larger structures, we acquired up to 200µm to facilitate the analysis of larger 3D structures.

8) "Notably, since cystic organoids tend to collapse during fixation, we advise not to let them grow over 400 µm in size to avoid this issue." Most organoids above a certain size will develop necrotic centers due to diffusion-limited nutrient and oxygen supply. As cells die, the center hollows, turning them essentially into cystic organoids. The incompatibility of this protocol with cystic structures prohibits the use of this protocol for a vast group of organoid structures.

We apologize for the lack of clarity. By cystic organoids, we meant monolayered organoids (see answer to comment 18 from Reviewer #2). Hence, we respectfully disagree with Reviewer #2. Indeed, necrotic 3D structures are usually empty in their middle but very rarely (if never) mono-layered. Thus, to our knowledge, they do not collapse during fixation, nor during the clearing step. Moreover, whenever possible we void studying 3D structures suffering massive necrotic events due to diffusion-limited nutrients and oxygen supply, since this will affect the physiology of the studied system. Depending on the question addressed, setting-up smaller 3D structures, to limit necrosis, could drastically improve the culture system and subsequent high-resolution imaging.

Minor Concerns:

9) "Reagent changes and transfer of 3D structures can cause sample loss throughout the following procedure" -- a very useful cautionary note. It would be helpful to be more specific in describing different avenues of sample loss as to sensitize the reader to particular caution during these steps.

We have now included the following **NOTES** throughout the manuscript to emphasize the different avenues of sample loss and sensitize the reader to taking extra care during these steps.

- ***NOTE:*** *Be careful not to aspirate the 3D structures, which will be only loosely attached to the tube wall.*
- ***NOTE:*** *If necessary, cut the top of the tip to avoid breaking the 3D structures.*
- ***NOTE:*** *Tip precoating will prevent the cells from sticking to the tip and minimize any loss of 3D structures.*

10) We recommend using 3D cell culture models with a size ranging from 100 to 500 µm. -This is very small for many organoid models. Neural organoids can grow to 4 mm and larger. Is the protocol capable of dealing with this type of sample? What if any adjustments need to be made? Can you show sample data from larger organoids or at least provide an upper bound of suitable size for your protocol?

We apologize for the lack of clarity. Our procedure presents two different and complementary types of staining and imaging acquisition to deal with a large variety of sizes and types of *in vitro* 3D cell culture models. The choice of one (3D whole-mount analysis) or the other (2D sectioning analysis) will depend on the model used and issue addressed. 3D whole-mount analysis by confocal enables the visualization of cells at a depth of field of up to 200 µm, irrespective of the overall size of the 3D structures, whereas the 2D sectioning method allows the analysis of samples of any size, but results remain 2D dimensional. We have now clarified the procedure and the Figure 1 to help users choose the best experimental set-up according to their needs.

11) Spinning as part of the protocol is surprising, as most 3D cell cultures tend to settle quickly by gravity. Why are potentially compromising centrifugation steps part of the protocol? Could they be omitted to maximize quality of the resulting samples?

This is indeed an optional step for some 3D structures that sink rather slowly (10-30 minutes) and partially. Readers should be able to choose carefully considering the advantages/disadvantages of this optional step according to the aim of their study. We have rephrased the manuscript to emphasize this point.

12) "Prepare the permeabilization-blocking (PB) solution. PB solution is PBS-1X supplemented with 0.1%-1% Triton X-100" -- this is a very wide range. It would be helpful to add a table with specific use scenarios as to maximize utility to the reader.

We have carefully rephrased this sentence to provide the range of Triton X-100 concentration we routinely use depending on the cellular localization.

13) Does a 1h blocking time allow penetration to deeper tissue layers in large samples? How would the authors suggest the reader to optimize this step for their own samples?

To our knowledge, 1h is sufficient to allow adequate penetration into the 3D structures analyzed, for a field of depth of 200 µm. Users working on larger samples and having to do 3D-whole mount staining should optimize blocking time (increase), clearing step (the specimen should be totally cleared) and image acquisition (e.g. light sheet microscopy) for their own samples. However, to our knowledge resolution obtained for such deep structures remains suboptimal, compared to confocal microscopy, for obtaining sub-cellular resolution. This has now been stated in the revised version.

14) Why is the PB solution diluted for the antibody step?

We carefully optimized this protocol. The use of PB 1:10 solution provides better results than pure PB solution or classically used PBS1X and PBS1X-BSA solutions -probably thanks to the presence of a lower concentration of Triton X-100, helping the spread of primary antibodies without damaging them, and BSA and donkey serum helping with lowering non-specific binding. Moreover, we decided to use a PB 1:10 as this was more cost-effective.

15) Washing after primary ab is 5x3 min. Is this enough to remove the primary antibodies from the tissues? If it takes 3-4 days to penetrate into the tissue, why are 5x3 min sufficient for proper removal? Would the protocol not risk the formation of larger primary/secondary antibody complexes in the tissue? Alternatively, could the incubation time with primary ab be reduced?

To our knowledge, this is sufficient to remove the non-bound primary antibodies. Indeed, we never observed the presence of larger primary/secondary antibody complexes in the 200µm sections analyzed. This is also sufficient to prevent background noise.

16) 1:250 is a very high secondary concentration. Most 3D protocols use 1:1000 or 1:2000. Why use them at 10x higher concentration than this?

We carefully optimized this protocol and in our setting this concentration of secondary antibodies gave the most robust and qualitative results for a large variety of antibodies.

17) Hoechst diluted 1:1000 from which stock concentration?

We apologize for this oversight. We have now provided Hoechst stock concentration in the manuscript.

18) Please define the term "mono-layered organoid". It seems like an oxymoron.

Please find below a panel from my work published in Nature medicine in 2017 with an example of non-layered organoid (left) versus more compact multi-layered cancer organoids.

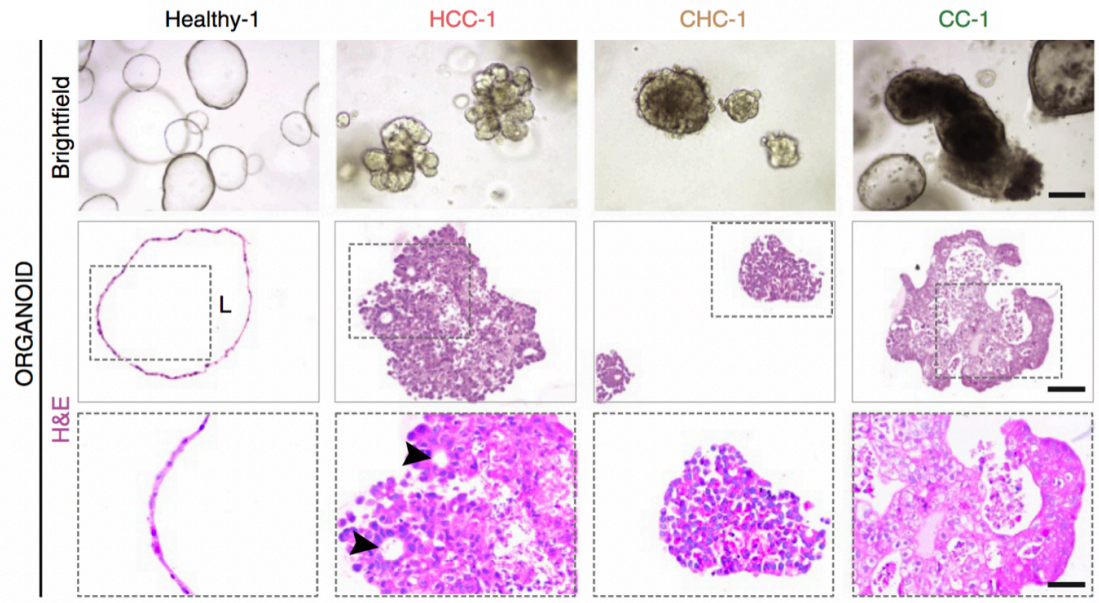

Figure B: Representative brightfield microscopy images (top row) and H&E histological analysis of primary liver cancer organoids (middle and bottom row). Note that, whereas healthy liver-derived organoids (left) grew as a single-layered epithelium of ductal-like cells surrounding a central lumen (L, lumen), tumor-derived organoids (right) formed compact structures. HCC-1 tumoroids, similarly to their parental tissue, exhibit pseudoglandular rosettes (arrowheads), a hallmark of HCC. CC-1 tumoroids present a glandular lumen, similar to the patient's tumor (top row). Scale bars, middle row, 100 µm; top and bottom rows, 50 µm.

19) Reversal of clearing can be very useful. Please provide a brief summary of steps for proper reversal. Incidentally, are the samples damaged by drastic changes in osmolarity? In any case, the authors should include data/images if they include this claim.

We do not have ready-to-publish data to support this claim and did not have time to generate them in the short time of this rebuttal. Consequently, the claim has been removed but mentioned in the discussion with an appropriate reference.

20) "Place the biopsy pad containing the 3D structures into the mold and gently fold it until all of the organoids drop to the bottom of the mold" - I do not understand what is being folded how in this step. Please describe in more detail.

We have carefully rephrased this sentence in our manuscript.

21) Cytoblock kit steps: do these instructions differ from the manufacturer's manual? If so, how?

We did follow the instructions from the manufacturer’s manual. This has now been stated in the revised version.

22) Quote: "Polymerized gel containing 3D structures is carefully handle using forceps for block completion." Please fix sentence.

We thank the reviewer for noticing this mistake. We have carefully rephrased this sentence.