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July 5th, 2019

Dr. Ronald Myers
Senior Science Editor
JoVE, Peer Reviewed Scientific Video Journal
1 Alewife Center, Suite 200, Cambridge, MA 02140

Re: Revisions required for your JoVE submission JoVE60367

Response to Editorial Comments

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 proofread our manuscript to avoid spelling or grammar issues.

2. Please provide the emails and affiliations for each author in the written manuscript.
   Our revised manuscript includes the emails and affiliations for each author.

Sanghee Lee[1,2], Danielle N. Burner[1,2], Theresa R. Mendoza[1,2], Michelle T. Muldong[1,2], Catalina Arreola[1,2], Christina N. Wu[2,3], Nicholas A. Cacalano[4], Anna A. Kulidjian[2,5], Christopher J. Kane[1,2], Christina A.M. Jamieson[1,2,6]

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3. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to…"
Revised as following (Line 529).

Here, we present a protocol to establish, image and process cultured 3D organoid with detailed tips for successful completion of the desired procedures.

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols ™, registered symbols ®, and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently reference in the Table of Materials and Reagents.
Revised accordingly as following in a revised manuscript.
line 139 and 162, ~~QuadroMACS™~~
line 143, ~~Accumax~~ Cell Dissociation Solution ~~(Stem Cell Technologies)~~
line 267, ~~Keyence~~ microscope
line 277 and 355, ~~BZ-X800~~

Revision above was also reflected to the Table for Materials and Reagents.

5. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.
Revised as following (Line 106); This study was carried out in strict accordance with the recommendations in the Guide for the University of California San Diego (UCSD) Institutional Review Board (IRB). IRB#090401 Approval was received from the UCSD institutional review board (IRB) to collect surgical specimens from patients for research purposes. An informed consent was obtained from each patient and a surgical specimen from fractured bone having prostate cancer metastasis was harvested and processed. All animal protocols were performed under a University of California San Diego (UCSD) animal welfare and Institutional Animal Care and Use Committee (IACUC) approved protocol #S10298. Harvest and processed patient tumor cells were intra-femorally injected in 6 to 8 week old male *Rag2*$^{-/-}$;$\gamma_c^{-/-}$ mice as previously described. Tumor volume was determined using an in vivo bioluminescence imaging system and caliper measurement. Upon tumor growth up to 2.0 cm (the maximal allowable size approved by IACUC), the tumor was harvested for 3D organoids establishment.

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6. Please convert centrifuge speeds to centrifugal force (x g) intead of revolutions per minutes (rpm).
Revised accordingly (line 135, line 188, line 322, line 328, line 333, line 336, line 315).

7. The protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.
Our original version of protocol contained only 2-3 actions per step unless an additional note were written to share tips specifically applicable to each step.
In our revised version, some of the previously written notes were moved to Discussion to fulfil both request #7 and #14 shown as following.

Removed from line 134 and the edited sentences added to line 444;
After a one-time process of filtering the tumor tissue suspension, the left over tissue should be processed again by taking the filtered through supernatant from the bottom of the 50 ml conical tube back and pushed back through the 70 μm cell strainer with the tumor tissue for a repeated suspension.

Removed from line 195 and added to line 449;
In general, the establishment of 3D organoids from patient primary tumor tissue is less successful than the establishment of 3D organoids from PDX tumor tissue. Therefore, it is recommended to have a higher seeding density (up to four times higher) of cells for the establishment of 3D organoids from patient primary tumor tissues than from PDX tumors.

Removed from line 207 and edited sentences added to line 485;
For all three different methods, this protocol recommends using the lower volume of media and the higher volume of Matrigel for the resuspension process because the domes will last longer in culture. A dome is formed with a 1:4 ratio of media and Matrigel. Of note, this method increases the formation of bubbles while pipetting the cell suspension up and down since the added volume of media does not circumvent the viscosity of Matrigel. Therefore, it is recommended to set up 1.5 ml tubes such that there is one cell pellet per each dome instead of having one cell pellet for several domes in one 1.5 ml tube. In doing so, the formation of bubbles in one tube for one dome will not serially affect the other domes.

Liquid status of commercially available Matrigel is often variable. Therefore, new batches of Matrigel need to be tested for the doming

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process. If the Matrigel is more viscous than usual, then up to additional 10 μl of adDMEM complete media (details written in protocol 1) can be added to dilute the mixture of cell pellet and matrigel.

Removed from line 205 and edited sentences added to line 437,
To minimize bubble formation during the pipetting process, it is recommended to pipette up a lower volume than the actual volume of cells, media and Matrigel mixture. For example, to dome in 24 well plate, 40 μl of Matrigel and up to 10 μl of adDMEM complete media is mixed with the cell pellet. In this case, the pipette can be set to handle a volume of 40 μl instead of 50 μl while pipetting up and down in order to minimize bubble formation. If the duration of culture condition is previously optimized and known to be short (up to a month), Matrigel can be diluted more to circumvent a viscosity issue of the Matrigel. For this second method, cell pellet resuspension for several wells can be prepared in one tube to minimize cell loss and variability.

8. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.
   Revised accordingly.

9. The preparation of the material steps can be removed or please refer to the Table of Materials.
   The preparation of the material steps was removed from line 127, 260 and line 319.

10. What is the age/gender/strain of the mouse used?
    Revised as following in line 113;
    Harvested and processed patient tumor cells were intra-femorally injected in 6 to 8 week old male *Rag2*$^{-/-}$;$\gamma_c^{-/-}$ mice as previously described.

11. 1.2.d: The units of the cell strainer are wrong.
    Revised accordingly in line 132, line 133 and line 447.

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12. 1.2.4: Wash with what volume of media.
Revised accordingly.

In line 166, following statements were added;
As shown in Table 3, we aim to provide a protocol applicable to different culture conditions. Volume of media for washing step should be matched with volume of media suggested in Table 3. For example, for a 24 well plate culture condition, the volume for the wash should be 500 µl.

13. Please specify the volumes of all washes and resuspensions.
Revised accordingly.

14. Please discuss limitations and critical steps on the protocol in the discussion.
Revised accordingly as following;

After a one-time process of filtering the tumor tissue suspension, the left over tissue should be processed again by taking the filtered through supernatant from the bottom of the 50 ml conical tube back and pushed back through the 70 µm cell strainer with the tumor tissue for a repeated suspension.

In general, the establishment of 3D organoids from patient primary tumor tissue is less successful than the establishment of 3D organoids from PDX tumor tissue. Therefore, it is recommended to have a higher seeding density (up to four times higher) of cells for the establishment of 3D organoids from patient primary tumor tissues than from PDX tumors.

For all three different methods, this protocol recommends using the lower volume of media and the higher volume of Matrigel for the resuspension process because the domes will last longer in culture. A dome is formed with a 1:4 ratio of media and Matrigel. Of note, this method increases the formation of bubbles while higher chance pipetting the cell suspension up and down since the added volume of media does not circumvent the viscosity of Matrigel.
Therefore, it is recommended to set up 1.5 ml
tubes such that there is one cell pellet per each dome instead of having one cell pellet for several domes in one 1.5 ml tube. In doing so, the formation of bubbles in one tube for one
dome will not serially affect the other domes. If the duration of the culture condition has been previously optimized and is known to be short (up to a month), Matrigel can be diluted more to circumvent a viscosity issue of Matrigel. Cell pellet resuspension for several wells can be prepared in one tube, utilizing a higher volume of media to desired volume of Matrigel to minimize cell loss and variability.

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15. Please do not abbreviate journal titles in the References.
Revised accordingly.

16. Please provide scale bars for all microscope images.
Revised accordingly.

17. Figure 3: Please use the SI abbreviation for minutes (min).
Revised accordingly.

18. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or xlsx file.
Revised accordingly.

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None of figures in our manuscript has previously been published. Tables were obtained from previous publication as written in our original and revised manuscript and shared with readers for convenience. 'Copyright Permission Letter' is submitted for Tables. For table legends for Table 1, 2 and 3, a following statement was added in a revised manuscript.

Response to Reviewer #1' Comments

1. One question is raised by the fact that it is not clear why this protocol should be applied only to bone metastatic prostate tumors since it looks like just a good example of trying to culture organoids from limited amount of starting material. I would suggest revising the title to increase the applicability of the methods to different types of metastatic sites (specifying the amount of material used as a starting). Otherwise why the protocol is applicable only to bone? Authors should explain that in details.
Our manuscript is submitted to JoVE Methods Collection, which aims to introduce several manuscripts to describe the establishment of 3D orgsion in our revised manuscript line 497;
Culture media for 3D organoids introduced in our manuscript

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is specifically for prostate-derived cells. However, other details described in our protocol are applicable to different types of tumor tissues and metastatic sites.

2. Author should clarify why FBS is needed in their culture since it will allow the growth of non-tumor cells (example fibroblasts). How the author eliminates non-tumor cells from the culture?
   Our group recently determined that serum supplementation can help support 3D organoid growth. For 3D organoid cultures from tumor specimens, we do not aim to selectively culture a pure population of tumor cells but rather to support tumor growth with the patient-derived microenvironment cells as well. However, as previously published by J Drost et al, culture media used for our protocol is most effective for human prostate-derived epithelial tumor cells. In parallel, our goal for the establishment of 3D organoids from bone metastatic prostate cancer is to provide culture conditions for heterogeneous tumor cells to be able to retain their properties as closely as possible to their *in vivo* condition. *In vivo*, tumor cells require interaction with non-tumor cells of the microenvironment.

   The statement regarding FBS supplementation was revised (line 451) to the following:
   In other words, the culture conditions described here which were adapted from Drost et al and modified to have FBS serum supplementation have been shown to be optimal for the cultures of patient prostate cancer bone metastasis-derived cells so that they can survive, grow and also retain their heterogeneity (manuscript in prepration).

3. How the authors check that they have in culture is really tumor from a bone metastasis? And no other cell types? Any test on the tumors to include in the protocols?
   As written above in response to Reviewer #1's comment 2, there can be other cell types. During culture, we do not aim to selectively culture pure population of tumor cells for organoids growth. However, after termination of experiment, we can perform IFC to characterize heterogeneous population of cells using different cell markers.

4. Page 2 line 101 it looks like the protocol is just for organoids derived from PDX since a Mouse Cell Depletion Kit is mentioned. Please specify that this protocol is for tumor coming from mouse at the beginning of the paragraph.
   Revised accordingly in line 123,
   for tumor derived from a xenograft mouse model

5. Is the protocol explained from line 189 the one to be used for Imaging? Please specify if the two paragraphs are connected.
   Each protocol to be used for each figure was labelled with 'Figure #' in our original and revised manuscript. Protocols which will be video filmed were yellow highlighted in response to a request by JoVE

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editorial team.

6. Literature references for prostate organoids establishment and applications are missing as Vela et al, 2014 and Puca et al., 2018.
   Revised accordingly.

7. Page 4 line 186 please remove the word "either"
   Revised accordingly.

8. Authors should clarify what they are referring to when they talk about "beads"
   Following sentence was added to our revised manuscript (line 259); NOTE: This protocol is named as floating beads since the mixture of Matrigel, media and organoids looks like a bead.

Response to Reviewer #2' Comments

1. When plating the organoids Line 157:
   "(NOTE: For this process, it is recommended to set up 1.5 ml tubes such that there is one cell pellet per each dome instead of having one cell pellet for several domes in one 1.5 ml tube. In this way, cell loss is minimized and cell density between domes is uniform when plating.)"
   This rather seems to induce more variability akin to making a separate master mix for each qPCR well. The viscosity of the Matrigel is the main factor in creating seeding variability. However, by diluting the Matrigel to 70% with medium this can be circumvented.
   a. **We prefer to use the lower volume of media and higher Matrigel for resuspension process because the domes last longer in culture. A dome formed with ratio less than 1:4 media:Matrigel shows slower de-polymerization and degradation in culture for long term cultures.**

   b. We recommend preparing the mix for each dome individually rather than from a common stock. This is to avoid bubbles that would arise from successive resuspension if a master mix of Matrigel + cells were to be used. Unlike qPCR, an exponential amplification reaction, this is a more accurate way to prepare the cells + Matrigel which is highly viscous.

   Original statement was removed from line 157 and then revised and added to Discussion in response to Editorial request.
   For all three different methods, this protocol recommends using the lower volume of media and the higher volume of Matrigel for the resuspension process because the domes will last longer in culture. A dome is formed with a 1:4 ratio of media and Matrigel. Of note, this method increases the formation of bubbles while pipetting the cell suspension up and down since the added volume of media does not circumvent the viscosity of Matrigel.
   Therefore, it is recommended to set up 1.5 ml tubes such that there is one cell pellet per each dome instead of having one cell pellet for several domes in one 1.5 ml tube. In doing so, the formation of bubbles in one tube for one dome will not serially affect

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the other domes. If the duration of the culture condition has been previously optimized and is known to be short (up to a month), Matrigel can be diluted more to circumvent a viscosity issue of Matrigel. Cell pellet resuspension for several wells can be prepared in one tube, utilizing a higher volume of media to desired volume of Matrigel to minimize cell loss and variability.

To minimize bubble formation during the pipetting process, it is recommended to set up the pipette up a lower volume than the actual volume of the cells, media and Matrigel mixture For example, for a dome within 24 well plates, 40 µl of Matrigel and up to 10 µl of adDMEM complete media are mixed with cell pellet. In this case, the pipette can be set to handle a volume of 40 µl instead of 50 µl while pipetting up and down in order to minimize bubble formation. If the duration of culture of culture condition is previously optimized and known to be short (up to a month), Matrigel can be diluted more to circumvent a viscosity issue the Matrigel. For this second method, cell pellet resuspension for several wells can be prepared in one tube to minimize cell loss and variability.

2. In the image analysis is it possible to see the difference between dying cells that blow up with big vacuoles and cysts?
We have not tried to determine dying cells in the image. We have optimized a condition for IFC staining with specific markers.

3. This is the biggest point of critique.
They show different ways to plate Matrigel discs and domes, attached or unattached, which is fine. However, the discussion of when you would use each of these techniques and the pros and cons of each is largely absent. A couple of examples are given in the discussion but this needs to be much more complete as this comprises a large proportion of the protocol presented. For instance, there are a couple different ways to plate floating domes, why use one or the other? As one is clearly much easier than the other.

Following sentences were added to line 477;
In addition, both types of floating domes can be easily transferred from one well to the another, making them suitable for co-culture with adherent cells after setting up 3D organoids cultures and the adherent cells in the separate wells. Attached dome and floating dome released from being attached have shown to retain organoids for up to 10 weeks. Interestingly, a floating dome from floating beads method has shown to retain organoids for up to 4 months. Floating beads method introduced in our protocol involves more steps and skills but can be considered for a long-term culture. In parallel, despite their use to establish 3D organoids from different tumors and metastasis, the role of doming method and Matrigel shape on organoids formation have not been deeply determined. In that sense, floating beads method can be considered

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for use when the two other methods have been unsuccessful.

4. Line 7: its should be their
   We thank for your suggestion. In our revised version of manuscript, we revised a title so that this is not applicable to a current version of title.

5. Line 173: typo (Issue instead of Tissue)
   Revised accordingly
6. Line 265: Some comments from other authors during manuscript preparation phase remain.
   Revised accordingly

We truly appreciate the careful consideration to give us an opportunity to publish or manuscript JoVE60307 in JoVE Methods Collection. If you have any questions and further request, please let us know.

Sincerely,

Christina A.M. Jamieson, Ph.D