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Dear Dr. Myers,

Thank you for the review of our manuscript entitled: "**Physiologic Patient Derived 3D Spheroids for Anti-neoplastic Drug Screening to Target Cancer Stem Cells**", authored by Michael E. Bregenzer, Ciara Davis, Eric N. Horst, Pooja Mehta, Caymen M. Novak, Shreya Raghavan, Catherine S. Snyder, and Geeta Mehta, for consideration of publication in *JOVE* as an *original video research article*.

We appreciate the insightful comments supplied by the editors and reviewers, and have made substantial changes in the manuscript in response to their suggestions. Our detailed responses to the reviewer comments are appended.

We hope that the paper is now acceptable for publication and are grateful for the opportunity to publish in *JOVE*.

Sincerely yours,

Geeta Mehta, PhD

Response to Reviewers:

The authors would like to thank the editors and the reviewers for their feedback and comments. We have made significant revisions to the manuscript, and this document will reflect and outline the changes we have made, per your suggestions. Several changes have been made in the main manuscript, and are highlighted in red text in the tracked changes manuscript. A detailed list of edits and the author response to the reviewers' comments follow below.

Editorial comments:

General:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
2. Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.
3. Note that all text written using Word's equation editor (or Microsoft Equation) will be formatted differently from the rest of the text. Please rewrite inline variables, if possible, in the same font as the rest of the text, and ensure all equations are on their own line.
4. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc. Please use the micro symbol µ instead of u. Please abbreviate liters to L to avoid confusion.
5. Please include a space between all numerical values and their corresponding units (except %): 15 mL, 37 °C, 60 s, 15,000 x g; etc.
6. Please provide an email address for each author.
7. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of 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: alamarBlue, Fisher Scientific, Gibco, Celltreat, Parafilm, Excel, GraphPad, Millipore, Histogel, Life Technologies, Olympus.

Thank you for these suggestions! All general suggested changes have been made.

Protocol:

1. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique. The actions should be described in the imperative tense in complete sentences wherever possible. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly.

2. There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headers 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.

3. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.

4. For each protocol step/substep. 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. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Thank you for these suggestions! All suggested changes in the protocol have been made. The filmable content of 2.75 pages in the protocol has been highlighted.

Specific Protocol steps:

1. "Evaluation of drug toxicity in spheroids" and "Analysis of cancer stem cell populations with flow cytometry"-please make these numbered sections, with the relevant sections as subsections under them. Everything in the protocol (except for the introductory ethics statement) should be in a numbered step, header, or 'Note'.

The suggested change has been made.

Results:

1. In the 2nd paragraph, there is a reference to Figure 4 after a sentence discussing fixing and staining spheroids, but this figure only has flow cytometry results-should there be another Figure here?

This section has been modified.

Figures:

1. Figure 3A, B: Please separate numbers and units here, and use 'µ' instead of 'u'.

The suggested change has been made.

References:

1. Please do not abbreviate journal titles in the reference section.

The suggested change has been made.

Table of Materials:

1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

The Table of Materials has been updated.

Formatted per JoVE guidelines (see attached), the highlighted portion of the protocol is ~5 pages; our limit is 2.75 pages for filming and video length purposes. Please reduce the highlighted portion to 2.75 pages (including by consolidating some of your shorter steps).

This has been addressed.

Please remove the embedded figures and upload the latest versions of them as Figure files in your revised submission.

This has been addressed.

Figure 4B: 'Discrimmination' is a typo.

This has been addressed.

Introduction: 'Cancer remains (the) second largest cause of death'-where? Worldwide?

Worldwide – this section has been adjusted.

Reviewers' comments:

Reviewer #1:

Major Concerns:

Q1. The author did not describe how the spheroids were collected from the 384-well hanging drop plates. For example, in line 366-367, before flow cytometry analysis, spheroids were collected. Was it by pipetting from each well? Or other methods?

Details of spheroid collection method have been added to each section that involves spheroid harvesting where appropriate. Mostly spheroids are taken up by a 1000 microliter pipet through the top of the hanging drop well. For immunofluorescence following embedding in

agarose, spheroids are instead pushed through the bottom of the well with 20 microliters of PBS.

Minor Concerns:

Q1. It may be helpful to add notes regarding how to dissociate the spheroids into single cells besides mechanically disaggregating by pipetting.

A note has been added regarding enzymatic digestion to facilitate generation of a single cell suspension while maximizing viability and single cell yield[1] in section 5 of the protocol.

Reviewer #2:

Major Concerns:

Q1. The authors describe limitations to the culture method mostly centered on the maintenance of individual hanging drops. However, little to no discussion of the limitations of the analysis methods is included while these methods comprise a significant portion of this manuscript. Also, the methods for isolating patient cells are somewhat opaque, as the string of as-described-in-[reference]s ends in a paper that doesn't actually describe the method.

A paragraph describing spheroid specific limitations has been added to the discussion. One such limitation is increased droplet size during longer cultures, which decreases droplet stability and makes capture of clear images difficult. This is ameliorated by replacement of medium during feeding (remove an equal amount of old medium to the amount of new medium that will be added), as opposed to simple addition of fresh medium. An additional limitation is changes in viability due to method of spheroid disaggregation. Mechanical dissociation and/or enzymatic dissociation is required for spheroid dissociation, which can affect cell viability[1]. Finally, histological analysis of spheroids can be difficult due to challenges locating the spheroids during the sectioning process. Improvement is achieved with practice.

The incorrect citation has been updated and a brief description of methods to isolate patient cells was been added in section 1 of the protocol.

Q2. Are the 384-well hanging drop plates commercially available? They are not listed in the materials table. If the plates are a custom product, this should be indicated. If a custom product, how can this work be reproduced in other laboratories?

This information has been added in the Table of Materials.

Q3. A brief description of the isolation method for patient-derived cells would be helpful to include in this manuscript along with the reference for more details.

A brief description of the isolation methods has been added, and references have been provided in section 1 of the protocol.

Q4. How long can tumor spheroids be maintained in the hanging drop system? Are longer-term cultures possible?

In our previous and unpublished work, we have maintained spheroids for 42 days on the hanging drop plates. We routinely perform long term culture of spheroids for 3 weeks in our lab, prior to experiment termination. This data has not been published. Multiple other sources have published longer-term cultures of spheroids. Once such study performed 12 day cultures and showed greater than 90% viability within spheroids made with 1500 cells per drop on day 12[2]. Because our spheroids are typically made with less than 100 cells per well, they can be cultured for longer periods of time. Alternatively, modified hanging drop plates with micro-ring structures have been shown to stabilize droplets to allow for stable droplets for 14 - 24 days in culture[3]. It should be noted, and is mentioned in the discussion that longer term cultures require replacement of medium during feeding steps as opposed to simply adding some new medium, since the droplets may become too voluminous, compromising stability and quality of images.

Q5. Control samples should be treated with cell culture medium containing an equal concentration of vehicle (e.g. DMSO) compared to the treated samples.

The example provided in this manuscript is drug treatment with cisplatin, which is solubilized in water. Given that a small volume of cisplatin is added to medium to create the drug solution applied to spheroids, a suitable control is culture medium without drug. That said, drugs solubilized in other solvents, like DMSO, will require a cell culture medium control that has the same concentration of DMSO that the experimental condition has. This has been clarified in the manuscript in section 5 of the protocol.

Q6. Does the alamarBlue reagent completely penetrate larger tumor spheroids during the incubation periods indicated?

One of the advantages of our culture system is the fact that spheroids can be formed reliably with small cell numbers (<10 cells/well)[4]. Spheroids formed with small cell numbers may not need as long of an incubation with alamarBlue as larger spheroids. Additionally, diffusion may depend on cell type, how compact the spheroid is, and spheroid formation method and thus may require longer incubation times[5]. For this reason, it is recommended in our protocol to first determine the appropriate incubation time with alamarBlue by reading fluorescence measurements every 30 minutes until the measurement starts to plateau. The time at which the measurement plateaus, is the time point at which future experiments should be read. This has been noted in section 4 of the protocol. Recent studies, as well as our previous publication utilize 24 hour alamarBlue incubation periods, as opposed to the standard 4 hours mentioned in this manuscript [4,6].

Q7. What is the cell viability loss after mechanical dissociation? Normalizing the data to the untreated control accounts for this but it decreases the sensitivity of the assay to include this background of cell death. Have other methods of dissociation been explored? Discussion of the limitation of this assay method should be included.

While it is true that mechanical dissociation can cause cell death, we have not quantified this value as it will likely depend on the individual performing the experiment and cell type(s). That said, there are alternative methods to facilitate spheroid disaggregation including incubation with enyzmes, such as Trypsin, Accutase, or dispase, for example[1]. It is recommended that each lab tailor their dissociation method to optimize cell viability and single cell yield based on their cell types and downstream analysis. It is also recommended that trypan blue results be corroborated by alternative methods of analyzing cell viability, such as alamarBlue and live/dead staining, which are both methods that do not require spheroid disaggregation and have shown agreement in our previous work[4]. This has been addressed in section 5 of the protocol.

Q8. Are live/dead quantifications done using maximum intensity projections or single z-slices? Max intensity projections are a poor way to quantify 3D objects.

Live/dead quantifications are done using composite images generated at the confocal microscope. Composite images are then split into different color channels in ImageJ and the percentage of live versus dead cells was determined based on the fluorescence measured in the live color channel and the dead color channel. We have published this work previously and showed using this analysis method that results correlate well with alternative methods of determining cell viability (alamarBlue) suggesting that this analysis method produces accurate results[4]. That said, we recognize reviewer's point that max intensity projections are not the best way to quantify 3D objects so we have also referred the readers to a microplate fluorescence protocol provided with the Live/Dead Assay kit as an alternative. This has been acknowledged in section 7 of the protocol.

Minor Concerns:

Q1. Sections of this manuscript would benefit from light editing for grammar and spelling.

The manuscript has been reviewed and edited for grammar and spelling.

<u>Reviewer #3:</u>

Minor Concerns:

Q1. Line 55: 'chemoresistant cells are also called the cancer stem cells' is inaccurate as it is not necessary that all chemoresistant cells are cancer stem cells.

This has been addressed in the introduction. Thank you for catching that.

Q2. Line 58: Comma after 'chemoresistance.'

This has been addressed.

Q3. Line 175: More details on tissue processing and flow cytometry will be useful.

Details have been added throughout the flow cytometry section and readers are referred to two recent publications for more details[7,8].

Q4. Line 176: Should be 'flow cytometry' and not 'low cytometry.'

This has been addressed.

Q5. Line 178: Not clear what is meant by 'Freshly isolated single cells are frozen-thawed patient derived cells.'

This has been clarified in section 1 of the protocol. We meant to say that freshly isolated cells are frozen for storage and later thawed for use in experiments. In other words, isolated single cells were not always used immediately.

Q6. Line 189: Please provide details of the hanging drop plate in the Table of Materials.

Details on the hanging drop plate have been added in the Table of Materials.

Q7. Lines 195-7: Parafilm will prevent gas exchange and that can affect buffering by the bicarbonate buffer system in the medium. Please comment/discuss.

It is likely that the Parafilm does not completely block off the interior of the 6-well plate sandwich with the 384-well plate. Our medium contains phenol red, which has a red/pink color at pH of 8.2 and yellow/clear at pH of 6.8. If CO2 could not escape the sandwich structure, we would expect the pH to drop as a result of the buffer system. While we do see the hanging droplets transition to a yellow/clear color, it does not appear to be different than the change in color of the medium in 2D culture dishes, which are not sealed by Parafilm. Therefore, we suspect that any change in pH is a natural consequence of cell culture. That said, the product information sheet for Parafilm M, claims that it is permeable to gases, including CO2, but not to moisture.

Q8. Line 253 and Line 265: There is a discrepancy '1 and 4 days' vs. '5-7 days.'

This has been clarified in section 5 of the protocol. It usually takes between 1 and 4 days for a stable spheroid to form. Any time after stable spheroids form and are equivalent size across all replicates, the drug treatment can be started. Therefore, if the spheroids have all formed by day 1, they could theoretically be treated on that day. However, for consistency, and to ensure that the spheroids are always completely formed and are same size by the start of drug treatment, we usually treat with drugs after 5-7 days.

Q9. Line 259. It will be more appropriate to use the solvent in which the drug is dissolved as a vehicle control instead of culture medium.

The example provided in this manuscript is drug treatment with cisplatin, which is solubilized in water. Given that a small volume of cisplatin is added to medium to create the drug solution applied to spheroids, a suitable control is culture medium without drug. That said, drugs solubilized in other solvents, like DMSO, will require a cell culture medium control that has the same concentration of DMSO that the experimental condition has. This has been clarified in the manuscript in section 5 of the protocol.

Q10. Line 271: What is the size of the tips used. How to minimize cell death due to shearing?

The size of the tips used has been clarified on where appropriate throughout the manuscript. Minimization of cell death could be accomplished in combination with enzymatic digestion such as with Accutase, Trypsin, and/or dispase to facilitate spheroid dissociation[1]. This has been mentioned in section 5 of the protocol.

Q11. Lines 272, 372 and 380: 17,000g is too high a centrifugal force for cell pelleting. They might rupture under such a high force.

We acknowledge that this has been reported and have changed the protocol to 400 x g for cell pelleting as used in culture of ovarian cancer cells and tumor initiating cells by House et al[9].

Q12. Line 339: Please provide more details of the sample preparation for confocal microscopy since these are spheroids.

Additional details have been added to section 8 of the protocol.

Q13. Line 458: While describing the results, it is desirable to have 3A before 3B in the sequence.

This has been addressed in the results section.

Q14. 466: 'solutions' should be changed to 'suspensions.'

This has been addressed.

Q15. Can this method be used to identify difference(s) between cancer stem cells from solid tumors and ascites?

Yes. Forming spheroids from cancer stem cells derived from the two different sources would allow for comparison between the two groups of cancer stem cells. Whole spheroids could be

sequenced to examine the overall difference in spheroids generated from each group of CSCs. Alternatively, single cell sequencing techniques, such as drop sequencing[10] could be used to identify the specific genetic signature of each CSC group. We have previously demonstrated that CSCs can be isolated from primary tumors as well as ascites to make spheroids[7,8].

References:

1. Jager, L. D. *et al.* Effect of enzymatic and mechanical methods of dissociation on neural progenitor cells derived from induced pluripotent stem cells. *Advances in Medical Sciences* **61**, 78–84 (2016).
2. Tung, Y.-C. *et al.* High-throughput 3D spheroid culture and drug testing using a 384 hanging drop array. *The Analyst* **136**, 473–478 (2011).
3. Hsiao, A. Y. *et al.* Micro-Ring Structures Stabilize Microdroplets to Enable Long Term Spheroid Culture in 384 Hanging Drop Array Plates. *Biomedical Microdevices* **14**, 313–323 (2012).
4. Raghavan, S. *et al.* Formation of stable small cell number three-dimensional ovarian cancer spheroids using hanging drop arrays for preclinical drug sensitivity assays. *Gynecologic Oncology* **138**, 181–189 (2015).
5. Raghavan, S. *et al.* Comparative analysis of tumor spheroid generation techniques for differential in vitro drug toxicity. *Oncotarget* **7**, 16948–16961 (2016).
6. Eilenberger, C. *et al.* Optimized alamarBlue assay protocol for drug dose-response determination of 3D tumor spheroids. *MethodsX* **5**, 781–787 (2018).
7. Mehta, P., Novak, C., Raghavan, S., Ward, M. & Mehta, G. Self-Renewal and CSCs In Vitro Enrichment: Growth as Floating Spheres. *Methods in Molecular Biology* **1692**, 61–75 (2018).
8. Raghavan, S. *et al.* Personalized Medicine Based Approach to Model Patterns of Chemoresistance and Tumor Recurrence Using Ovarian Cancer Stem Cell Spheroids. *Clinical Cancer Research* **23**(22):6934-6945 (2017).
9. House, C. D., Hernandez, L. & Annunziata, C. M. In vitro Enrichment of Ovarian Cancer Tumor-initiating Cells. *JoVE (Journal of Visualized Experiments)* e52446 (2015). doi:10.3791/52446
10. Macosko, E. Z. *et al.* Highly Parallel Genome-wide Expression Profiling of Individual Cells Using Nanoliter Droplets. *Cell* **161**, 1202–1214 (2015).