Journal of Visualized Experiments

Physiologic Patient Derived 3D Spheroids for Anti-neoplastic Drug Screening to Target Cancer Stem Cells

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59696R2
Full Title:	Physiologic Patient Derived 3D Spheroids for Anti-neoplastic Drug Screening to Target Cancer Stem Cells
Keywords:	Patient derived, cancer stem cells, drug screening, high throughput, hanging drop spheroids, precision medicine
Corresponding Author:	Geeta Mehta University of Michigan Ann Arbor, UNITED STATES
Corresponding Author's Institution:	University of Michigan
Corresponding Author E-Mail:	mehtagee@umich.edu
Order of Authors:	Micheal Bregenzer
	Ciara Davis
	Eric Horst
	Pooja Mehta
	Caymen Novak
	Shreya Raghavan
	Catherine Snyder
	Geeta Mehta
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Ann Arbor, MI, USA, 48109

MICHIGAN ENGINEERING
UNIVERSITY of MICHIGAN ■ COLLEGE of ENGINEERING

GEETA MEHTA
DOW CORNING ASSISTANT PROFESSOR
MATERIALS SCIENCE AND ENGINEERING,
BIOMEDICAL ENGINEERING
2800 PLYMOUTH RD, BLDG. 28, ROOM 3044W
ANN ARBOR, MI 48109-2800. Ph: 734-763-3957

March 26, 2019

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).

TITLE:

Physiologic Patient Derived 3D Spheroids for Anti-Neoplastic Drug Screening to Target Cancer Stem Cells

AUTHORS & AFFILIATIONS:

Michael E. Bregenzer[1, §], Ciara Davis[1, §], Eric N. Horst[1, §], Pooja Mehta[2, §], Caymen M. Novak[1, §], Shreya Raghavan[2, §], Catherine S. Snyder[2, §], Geeta Mehta[1, 2, 3, 4,#]

[1]Department of Biomedical Engineering, University of Michigan, Ann Arbor, USA
[2]Department of Materials Science and Engineering, University of Michigan, Ann Arbor, MI, USA
[3]Rogel Cancer Center, School of Medicine, University of Michigan, Ann Arbor, MI, USA
[4]Macromolecular Science and Engineering, University of Michigan, Ann Arbor, USA
[§]These authors contributed equally.

[#]Corresponding author:
Geeta Mehta

Email addresses:
Michael Bregenzer - mbregenz@umich.edu
Ciara Davis – ciaradav@umich.edu
Eric Horst – horster@umich.edu
Pooja Mehta – mpooja@umich.edu
Caymen Novak – cmnovak@umich.edu
Shreya Raghavan – shreyar@umich.edu
Catherine Snyder – cssnyder@umich.edu
Geeta Mehta – mehtagee@umich.edu

KEYWORDS:

Patient derived, cancer stem cells, drug screening, high throughput, hanging drop spheroids, precision medicine

SUMMARY:

This protocol describes generation of patient-derived spheroids, and downstream analysis including quantification of proliferation, cytotoxicity testing, flow cytometry, immunofluorescence staining and confocal imaging, in order to assess drug candidates' potential as anti-neoplastic therapeutics. This protocol supports precision medicine with identification of specific drugs for each patient and stage of disease.

ABSTRACT:

In this protocol, we outline the procedure for generation of tumor spheroids within 384-well hanging droplets to allow for high-throughput screening of anti-cancer therapeutics in a physiologically representative microenvironment. We outline the formation of patient derived cancer stem cell spheroids, as well as, the manipulation of these spheroids for thorough analysis following drug treatment. Specifically, we describe collection of spheroid morphology,

proliferation, viability, drug toxicity, cell phenotype and cell localization data. This protocol focuses heavily on analysis techniques that are easily implemented using the 384-well hanging drop platform, making it ideal for high throughput drug screening. While we emphasize the importance of this model in ovarian cancer studies and cancer stem cell research, the 384-well platform is amenable to research of other cancer types and disease models, extending the utility of the platform to many fields. By improving the speed of personalized drug screening and the quality of screening results through easily implemented physiologically representative 3D cultures, this platform is predicted to aid in the development of new therapeutics and patient-specific treatment strategies, and thus have wide-reaching clinical impact.

INTRODUCTON:

Worldwide cancer-related mortality reached a toll of 9.8 million deaths in 2018[1], highlighting the need for the development of improved therapeutics. Unfortunately, the cost of developing cancer drugs is increasing, with the development of a single drug costing approximately USD$650 million[2] indicating the need for improved strategies to develop new anti-cancer drugs. Cancer stem cells (CSCs), which are characterized by increased chemoresistance[3], the capacity to self-renew, and the ability to seed new tumors[4] are thought to be responsible for tumor recurrence[4], metastasis[5], and chemoresistance[4,6], which all contribute to the malignant capacity of the tumor and thus the high death toll. In ovarian cancer, these cells are found enriched in the malignant ascites fluid in the peritoneal cavity, a condition associated with poor clinical outcomes[1]. As a result of the malignant capabilities of CSCs, there has been a push to develop new CSC targeting drugs to use in conjunction with traditional chemotherapies. There are several challenges that accompany the development of CSC targeting drugs including: 1) difficulty in expanding and maintaining CSCs in vitro; 2) scarcity of patient samples; 3) physiological relevance of the culture platform; and 4) heterogeneity in drug sensitivity between patients. This protocol outlines the implementation of a high throughput 3D culture platform that can overcome each of these challenges. In particular, this system allows for rapid drug screening using small numbers of patient-derived ovarian CSCs, and is highly amenable to downstream analysis techniques. While ideal for studying ovarian cancer and CSCs, our platform is also valuable in studying other cancers and differentiated cell types in complex 3D environments.

Complex 3-dimensional (3D) models are critical in studying the tumor microenvironment (TME), which is a 3D niche made up of cancer cells, non-cancer supporting cells, and extracellular matrix (ECM) proteins[4]. This 3D environment results in unique cell morphology, cell-cell and cell-matrix interactions, cell differentiation, cell migration, cell density, and diffusion gradients compared to traditional 2D cell culture in vitro[4]. All of these factors culminate in differential drug response within 3D cultures, exhibiting increased drug resistance and physiological relevance[7,8]. Due to the role of the 3D TME in CSC differentiation and chemoresistance, it is vital to screen for CSC targeting drugs in physiologic microenvironments. Improving the physiological relevance of CSC drug screening platforms has the potential to improve patient specific drug screening, drug development, formulation of treatment strategies, and ultimately clinical outcomes. It is equally important that the platform used for drug screening be high-throughput and compatible with downstream analysis methods to minimize cost, time, and clinical translation time of promising drugs[9].

Currently, the complex TME is best maintained for drug screening applications through in vivo models such as murine syngeneic tumor models, cell line-derived xenografts, and patient-derived xenograft (PDX) models[12], as they provide physiologic conditions. However, the low-throughput nature of these models, as well as, the cost, time, and technical skill sets that they require limits their utility in rapid, high throughput drug screening applications[13]. As alternatives to these in vivo models, many in vitro 3D models utilizing hydrogels[8], culture within microfluidic devices or 'organ-on-a-chip' devices[10,14], and non-adherent cultures[3,8] have also been developed, due to their low barrier to entry in terms of cost, time, and required skillset.

Hydrogel culture platforms are advantageous in the fine control afforded over the matrix composition, mechanical properties, and matrix structure[15]; however, they can inhibit high density cell culture[14]. Additionally, harvesting cells from hydrogels can complicate downstream analysis, due to potentially harmful effects of harvesting methods[15]. Microfluidic devices, on the other hand, are microscale devices that allow for output detection within the same device and for cell culture at physiologically relevant scales with minimal consumption of reagents, decreased reaction time, minimized waste, and rapid diffusion[14]. These characteristics make them promising platforms for investigating drug toxicity, efficacy, and pharmacokinetics. However, the challenges of efficient, quantifiable, reproducible, and user-friendly 3D cell culture, as well as, bulky and costly pumping systems have restricted microfluidic applications in high-throughput research[10]. Efficient detection setups and potentially difficult implementation across fields have also hindered widespread adoption of microfluidic systems[10].

Contrarily, spheroids generated in non-adherent conditions in rotating mixers (nutators), ultra-low attachment plates, and hanging droplets do not include user-defined matrix components. These methodologies are especially relevant for studying ovarian cancer as the non-adherent conditions are representative of the conditions in which spheroids grow within the peritoneal cavity[5]. Within these non-adherent culture methods, nutator and hanging drop spheroids have been shown to exhibit higher compaction, remodeling, and chemoresistance compared to spheroids generated in ultra-low attachment plates, suggesting increased physiological relevance[16–19]. Due to increased capacity for high-throughput screening from smaller well sizes and minimal required cell numbers, spheroid generation in hanging drop plates is an ideal platform for drug screening. Here, we present a tunable 3D physiologic platform in 384-well hanging drop plates, that is easy to implement and highly amenable to downstream analysis, making it ideal for high throughput drug screening of ovarian cancer and ovarian CSCs.

Our 3D physiologic platform provides all of the advantages of 3D culture, including physiological cell-cell contacts, diffusion gradients, cell densities, and naturally produced ECM proteins, which may contribute to realistic drug responses[16–19]. Additionally, by generating these spheroids with patient-derived CSCs, we are able to determine patient specific responses to drugs[1] with many technical replicates simultaneously, to overcome heterogeneity that may be found with patient tumor sample[20]. Furthermore, 3D culture has been shown to enhance maintenance of CSC populations[3,16] and thus is representative of enriched CSC populations in the ascites[7]. This combined with easy downstream analysis, including flow cytometry analysis of viability and CSC

proportions allows for optimal evaluation of CSC targeting drug efficacy. Finally, this physiologic platform is compatible with imaging at multiple time points during the experiment, evaluation of cell death and proliferation, cell organization and morphology with immunohistochemistry, soluble signaling with ELISA on conditioned medium, cell phenotypes with flow cytometry, and gene expression following PCR.

PROTOCOL:

All patient samples are collected under an approved IRB protocol from consenting patients, whose samples are de-identified after tumor debulking and ascites collection.

1. Generation of spheroids from small cell numbers in 384-well hanging drop plates

1.1. Place the hanging drop plate in a sonicator filled with sterile deionized (DI) water and sonicate for 20 min.

1.2. With a gloved hand, remove the plate from the sonicator and wash it with running DI water.

1.3. Allow the plate to sit in a bath of 0.1% Pluronic acid for 24 hours to prevent protein adsorption and spheroid adherence to the wells.

1.4. Remove plates with a gloved hand and rinse both sides of the plate with running DI water thoroughly.

1.5. Vigorously tap or shake the plate inside a biosafety cabinet to remove water from the wells in a sterile environment.

1.6. Place the plate under UV light for 30-60 min on each side to sterilize the plates and minimize contamination.

NOTE: The plate can also be exposed to ethylene oxide gas chamber for sterilization.

1.7. Fill each well of a 6-well plate with 4–5 mL of sterile autoclaved DI water and sandwich the hanging drop plate between the lid and the bottom of the 6-well plate. Add 800-1,000 µL of sterile DI water around the rim of the hanging drop plate to provide a humid, stable, and sterile environment to minimize volume lost to evaporation (**Figure 1 A-C**).

1.8. For 2D grown cells, aspirate medium covering cells in their log phase of growth and wash with 1x phosphate buffered saline (PBS) to remove traces of fetal bovine serum (FBS) in the growth medium, as it hampers the action of trypsin.

1.9. Aspirate the PBS and pipet 1.0-1.5 mL of 0.25% trypsin-EDTA to the 100 mm tissue culture dish. Incubate cells for 5 min in an incubator set to 37 °C.

NOTE: Cells may detach at different rates, so plates should be checked on a benchtop light microscope to ensure cell detachment after 5 min. Adjust detachment protocol per vendor instructions when using a cell line.

1.10. Add 3–4 mL of cellular medium containing FBS or any serum to the dish to neutralize the trypsin, collect cells with a 10 mL serological pipet, and deposit them in a 15 mL conical tube.

1.11. Count cells by loading 10 µL of the cell suspension on each side of a hemocytometer and follow the associated counting protocol.

1.12. For patient-derived cells collected from primary or metastatic solid tumors or from ascites that have not been 2D cultured, prepare single cell suspensions in serum free medium (SFM) described previously[3].

1.13. Process tumor tissues as previously described and store single cell suspensions for later use in appropriate freezing medium[21,22].

1.13.1. For solid tumor tissue, mince mechanically with a razor blade and filter resulting solution through a 40 µm filter before isolating desired cells from a density gradient[21,22].

1.13.2. For ascites samples, concentrate cells by centrifugation, lyse red blood cells in ammonium-chloride-potassium (ACK) buffer, wash in 1x PBS, and then pass through a 40 µm filter and a 28G needle 4 times[22].

1.14. For isolation of ovarian CSCs, sort cells with flow cytometry as described below in detail.

1.15. Freshly isolated single cells are frozen for storage and thawed when needed for experimentation.

1.16. To prepare the cell suspension for plating, calculate the desired volume of cell solution required for plating: 20 µL ÷ drop X total number of droplets = total solution volume needed.

1.17. Dilute cell concentration to the desired cell concentration per 20 µL (i.e., 100 cells in a 20 µL drop).

1.18. Mix the cell suspension gently using a 1,000 µL pipet before plating to ensure homogeneous distribution of cells and improve uniformity between droplets.

NOTE: Overmixing of the cell suspension may lead to cell death and debris in the hanging drop spheroids.

1.19. Place the tip of the pipette in the well at an angle of approximately 45° and pipet 20 µL of the cell solution into each hanging drop well.

NOTE: Plating patterns can be adjusted depending on number of spheroids needed. Plating spheroids in every other well is safer when large quantities of spheroids are not needed in an experiment, because it prevents accidental merging of the droplets (**Figure 1D, E**). If large quantities of spheroids are needed for an experiment, plate every well leaving one row of border wells on all sides (**Figure 1F**).

1.20. Place the lid of the 6-well plate back on top of the hanging drop plate and use a stretchy thermoplastic strip that is insensitive to moisture loss, to seal the edges and prevent additional evaporation of droplets. Incubate in a standard CO_2 humidified incubator (5% CO_2, 37 °C).

1.21. Feed hanging drops once every 2-3 days to replenish cell culture medium for necessary nutrients by adding 2-3 µL to each spheroid containing well.

NOTE: After imaging, it is always advised to feed the hanging drops, as air exposure during imaging leads to evaporation.

2. Adding cell culture medium to hanging drop spheroid plates

2.1. Remove the thermoplastic strip and lid within a biosafety cabinet and add 2-3 µL of the appropriate culture medium to each well containing a hanging drop.

NOTE: The volume added will depend on drop size and the amount of time between feedings.

2.2. After feeding, cover the plate with the top lid and apply fresh thermoplastic strip to the outside edges before putting the plate back in the incubator.

3. Phase contrast imaging of spheroid morphology

3.1. Remove the thermoplastic strip from around the edges of the plate in a biosafety cabinet.

3.2. Carefully remove the 384-well hanging drop spheroids plate from the biosafety cabinet with the lid still in place and place it in the microscope tray at an epifluorescent microscope.

3.3. Use the live imaging option in the imaging software at 4x, 10x or 20x to observe the hanging drop spheroids and take desired images.

3.4. After saving the images, take the plate back to the biosafety cabinet and feed cells as described above.

3.5. Reseal plate with a fresh thermoplastic strip and place the sealed plate back in the incubator.

4. Quantification of proliferation and viability within spheroids

4.1. Plate spheroids in a sufficient number of wells to obtain >10 technical replicates for each time point (i.e., day 1 and day 7) that is to be examined.

NOTE: Wells that are used for this assay are generally not used again due to potential contaminants from the resazurin dye.

4.2. Add 2 µL of filtered resazurin-based solution to the wells designated for proliferation analysis as if feeding those wells and incubate for a pre-determined incubation period.

NOTE: This incubation time can vary based on cell type and the number of cells in the spheroid. It is advised to determine the required incubation time needed prior to beginning proliferation experiments by beginning measurements after 1 hour incubation and then re-measuring every 30 min until the signal readouts in control wells plateau. Assay readings can be taken as many times as desired. Incubation is typically 4 hours for spheroids initiated with 100 cancer cells.

4.3. Turn on the microplate reader first followed by the associated plate reader software at least 15 min prior to first reading to allow for the machine to warm up and secure the internal temperature at 22 °C as temperature can affect the readings.

4.4. Open a 384-well plate protocol set with 530/25 nm excitation and 590/35 nm emission wavelengths with **Optics** set to **Bottom**, **Gain** set to **35**, **Read Speed** set to **Normal**, and read type set to **Fluorescence**.

4.5. After the incubation period, open the hanging drop sandwich in a biosafety cabinet and bring the 384-well plate with the lid still in place to the plate reader.

4.6. Place the 384-well plate with the lid still in place in the plate reader tray, which will be extended once the machine is warmed up and click **Ok** to read the plate.

4.7. Return the well plate to the 6-well base and place it in the incubator. If all time points have been read for the day, reseal the plate before placing it back in the incubator.

4.8. Save the experiment in the pop-up window and then click **Yes** when prompted **Do you want to execute PowerExports for Plate 1** to output data from the plate reader software into a spreadsheet for organization.

4.9. Average fluorescence values for each condition and normalize by the average of the control condition to report fold change in proliferation in various experimental conditions.

4.10. When comparing day 1 to day 7, normalize by dividing by day 1 average fluorescence to obtain fold change in proliferation over time.

4.11. Calculate error bars using standard error of the mean and determine statistical significance between experimental groups with an appropriate statistical test, like the student's two-tailed T test.

5. Evaluation of drug toxicity in spheroids

5.1. Drug administration

5.1.1. At any time following spheroid formation, deliver drug diluted to the desired concentration such that a 2 µL dose contains 10x the desired final drug concentration.

NOTE: This is assuming an evaporation of 2 µL from the droplet so that the ending volume post drug treatment is 20 µL. For example, a 50 µM dose of cisplatin will have a prepared solution of 500 µM. If droplets contain more than 20 µL, the concentration of drug and/or the volume added should be adjusted accordingly.

5.1.2. Treat control samples with 2 µL of cell culture medium.

NOTE: Cisplatin is solubilized in water. Therefore, controls are 2 µL of cell culture medium; however, if the drug vehicle is a different solvent (e.g., DMSO), then controls should be cell culture medium with an equal concentration of DMSO as used in the drug treatment.

5.1.3. Continue to monitor the drug treated and the control spheroids throughout the drug incubation period with phase microscopy to have a visual record of the effect of drug toxicity as well as with resazurin dye as described above to monitor cellular viability.

NOTE: Typically, the drugs are added after 5-7 days in the hanging drops and toxicity measured after 48-72 hours but this can be varied depending on the experiment. Drugs can be added as soon as stable spheroids have formed, usually between 1-4 days.

5.2. Drug toxicity quantification by cell counting

5.2.1. At the end time point of drug treatment, collect 10 spheroids each from control and drug treated wells using a 1,000 µL pipet and deposit each set of spheroids into its own microcentrifuge tube.

5.2.2. Break down the spheroids into single cells by repeated pipetting.

NOTE: To reduce potential cell damage due to repeated pipetting, enzymatic digestion with an enzyme such as trypsin can be performed to facilitate generation of single cell solutions prior to pipetting[23]. Minimization of cell death due to pipetting will depend on the cell type and should be optimized accordingly. If concerned about death due to disaggregation, refer to analysis with resazurin dye and the cytotoxicity assay for alternative methods that do not require spheroid disaggregation.

5.2.3. Centrifuge the tubes for 5 min at 400 x *g* to collect cells at the bottom of microcentrifuge tubes and aspirate the supernatant.

5.2.4. Add 20 µL of fresh media or 1x PBS to each tube and mix well.

5.2.5. Add Trypan blue at a ratio of 2 µL of Trypan blue stain to 20 µL of cell suspension.

5.2.6. Load 10 µL of cells and Trypan blue suspension into each chamber of the hemocytometer and count cells under a light microscope, with blue stained cells representing dead cells and unstained cells representing live cells.

5.2.6.1. Calculate the live cell % = Number of live cells ÷ total number of cells x 100.

6. **Spheroid characterization with histological techniques**

NOTE: There are two mold options 3D printed in a biocompatible polymer to replicate spheroid array molds made by Ivanov et al.[24]: 1) 20 well mold that can hold 28 µL per well and 2) 63 well mold that can hold 9 µL per well (**Figure 2D**).

6.1. Sterilize histology mold and its border by wiping them down with 70% ethanol and attach the border firmly around the mold and lay the mold upright. Both molds fit approximately 3 mL of fluid (3.203 mL for the 20 spheroid array and 3.196 mL for the 63 spheroid array).

6.2. Lightly coat the spheroid array with 10,000 cSt Si oil using a pointed cotton swab to facilitate removal of the specimen processing gel cast in subsequent steps.

6.3. Warm up specimen processing gel until liquefied via microwave for 10-20 seconds with the cap loosened.

6.4. Add between 2.6 and 2.8 mL of molten processing gel into the mold until approximately level with the top of the border.

6.5. In a few minutes, once solidified, remove the processing gel cast by separating the border from the mold and then inverting the array mold.

6.6. Insert tweezer tip between mold and the border to carefully separate them and then use a cell scraper to help dislodge the cast from the mold if it does not detach right away.

6.7. Harvest the spheroids from the hanging drop plate by pipetting the contents of a single well onto a 100 or 150 mm cell petri dish with a 1,000 µL pipet, and isolate the spheroid visually.

6.8. Pipet 5 µL of the well contents including the identified spheroid into one of the array wells, until the array is filled.

6.9. Wait 3 min to ensure spheroids have settled to the bottom of the array before gently pipetting molten processing gel into each of the wells being careful not to disturb the spheroids.

6.10. Add additional processing gel to level off the top of the array and once cooled, place the solidified spheroid array into a labeled cassette for processing.

6.11. Submerge labeled cassette in 4% formalin overnight to fix the spheroids at 4 °C and then store in 70% ethanol at 4 °C until ready for processing.

6.12. Process with standard 1 h paraffin program and then embed the spheroid arrays such that the processed arrays are facing upright with the bottom of the wells closest to the block face.

6.13. Ensure that the arrays are embedded as flat as possible, with the bottom face flush with the bottom of the block and place blocks on ice.

6.14. Load the block onto the microtome and adjust the block such that the blade is parallel with the loaded block face.

6.15. Cut array (sample depth = 15 µm) until the bottom of the array wells are reached making sure that the circular wells are visible on cut samples.

6.16. Switch to a 5 µm sample depth and continue to slice and collect ribbons. Check under the microscope every few slices to determine if spheroid depth has been reached. Once spheroids are reached collect each subsequent slide until the spheroids are no longer visible.

6.17. Stain each slide with standard H&E protocol.

7. **Live dead cytotoxicity assay**

7.1. To each hanging drop, add the live cell dye, calcein-AM to the final concentration of 2 µM, and the dead cell dye, ethidium homodimer-1 to the final concentration of 4 µM, keeping in mind that the drop volume is 20 µL.

NOTE: Try to keep volumes added to a minimum, and typically add 2 µL of the dyes combined.

7.2. Place plates back in incubator sandwiched in a 6-well plate with a lid and incubate the spheroids for 45 min at 37 °C.

NOTE: Depending on the size of spheroids, this incubation time significantly varies. For example, spheroids under 400 µm typically only require 30-45 min of incubation time, whereas larger spheroids closer to 800 µm have required up to 90 min of incubation time. Incubation times must be optimized for an individual's experiment.

7.3. For subsequent imaging, harvest spheroids with a 1,000 µL pipet in a biosafety cabinet and deposit each spheroid onto pre-cleaned glass microscope slides.

7.4. Image spheroids through the glass, on an inverted confocal microscope.

NOTE: Depending on the proximity of the confocal microscope to the lab in which spheroids are harvested, the spheroids can either be imaged in the original droplet of medium placed on the slide or encased in 2% agarose if more stability is required for spheroid transport.

7.5. Using the **Multidimensional Acquisition** mode in MetaMorph, locate the spheroid using DIC illumination at 10x magnification and then scan the z-plane to identify the heights encompassing the spheroid.

7.6. Click on **Z Series** and set the upper and lower limit of the z-scan slightly higher than the top of the spheroid and slightly lower than the bottom of the spheroid.

7.7. Excite the spheroids at 488 nm for calcein-AM (live cells; green) and 561 nm for ethidium homodimer-1 (dead cells; red) with the step size recommended by the software, maximal gain and minimal exposure for each color.

7.8. Click **Acquire** to obtain a composite z-stack image using for live and dead cells within the spheroid.

7.9. Quantify live/dead proportions using ImageJ to quantify a percentage of pixel intensity from the channel corresponding to live cells versus the percentage of pixel intensity from the channel corresponding to dead cells from the composite z-projection images.

NOTE: Due to the limitations in quantifying a 3D structure with a 2D projection, an alternative method to quantify live versus dead fluorescence, within the hanging drop plates is to use a plate reader following the protocol included with the calcein-AM and ethidium homodimer-1 kit.

8. **Immunofluorescence**

8.1. Heat a low melting 2% agarose solution, so the solution is viscous and just above melting point and place on a microscope slide to create a soft bed of agarose

8.2. Harvest spheroids by pushing 20 µL of PBS through the drop onto the soft bed of 2% agarose.

NOTE: This should be done quickly so that agarose does not solidify before spheroids are embedded.

8.3. Once the agarose cools and gels with the harvested spheroid trapped within (under 5 min), add 4% neutral buffered formalin to fix the spheroids. Alternately, add ice cold methanol, and fix the agarose-embedded spheroids at -20 °C for 30 min.

8.4. Wash 3x for 5 min each with 1x PBS, discarding PBS after each wash.

8.5. Block for 1 h at RT with 10% serum (i.e., horse serum) and 0.15% soap solution (Triton X-100) in 1x PBS.

8.6. Wash 3x for 5 min each with 1x PBS, discarding PBS after each wash.

8.7. Stain spheroids with desired fluorescent antibody at recommended or pre-determined antibody dilution (i.e., fluorescently labeled phalloidin at a 1:100 dilution), incubating for at least 90 min at room temperature, covered from light.

NOTE: Protocols will vary depending on antibody and target and antibody dilution may need to be optimized depending on the experiment.

8.8. Visualize fluorescently stained spheroids with the inverted confocal microscope using methods outlined in the previous section for imaging spheroids.

8.9. Composite z-stack images will demonstrate 3D morphology in the spheroids.

9. **Collection and analysis of cancer stem cell populations with flow cytometry**

9.1. Preparing spheroids for flow cytometry analysis

9.1.1. Collect spheroids from each well in the hanging drop plates using a 1,000 µL pipet and deposit them into a 15 mL centrifuge tube for disaggregation with repeated pipetting.

9.1.2. Count viable cells on a hemocytometer using Trypan Blue to determine cell number and concentration as outlined above.

9.1.3. Aliquot cell suspension into five microcentrifuge tubes such that each contains a minimum of 50,000 cells.

9.1.4. Centrifuge all tubes at 400 x *g* for 5 min in a microcentrifuge.

9.1.5. Aspirate the supernatant from each tube and resuspend pellets in 100 µL of buffer.

9.1.6. Label tubes “Unstained”, “DAPI”, “APC-iso”, “DEAB”, and “ALDH/CD133”, respectively.

9.1.7. Add 0.5 µL of APC-isotype antibody to the APC-iso tube and 1 µL of CD133 antibody to the ALDH/CD133 tube as determined by serial dilution and manufacturers recommendation.

9.1.8. Add 5 µL of DEAB Reagent and 0.5 µL of ALDH to the DEAB tube, and 1 µL of ALDH to the ALDH/CD133 tube.

9.1.9. Vortex all tubes for approximately 2 s and incubate at 37 °C for 45 min.

9.1.10. Vortex all tubes again and centrifuge at 400 x *g* for 5 min in a microcentrifuge.

9.1.11. Label FACS tubes “Unstained”, “DAPI”, “APC-iso”, “DEAB”, and “ALDH/CD133” and fill an insulated foam container to set aside.

9.1.12. Aspirate supernatant and resuspend the “unstained” control in 400 µL FACS Buffer (1x PBS with 2% FBS) and all other tubes in 400 µL of FACS DAPI Buffer (FACS Buffer with 300 µM 4’,6-diamidino-2-phenylindole).

9.1.13. Place tubes in container with ice until analyzed on a flow cytometer.

NOTE: To sort for ovarian cancer stem cells, collect all cells that the cytometer measures to be ALDH+ and CD133+ with gates set to include 0.5% non-specific APC signal and 0.15% non-specific ALDH+ signal. At least 10,000 cells need to be analyzed for reliable results. Additional details can be found in recent publications[3,17].

9.2. Analysis of ALDH+/CD133+ populations in FlowJo

9.2.1. Double click the **FlowJo** icon to open the program and drag .fcs files obtained from flow cytometry software into the workspace.

9.2.2. Double click the **unstained** file and set the y-axis to side scatter height (SSC-H) and the x-axis to forward scatter height (FSC-H).

9.2.3. Click the **T** button next to each axis to adjust the scale and transformation to maximize the separation between different cell populations.

9.2.4. Click on the **Polygon** gating button and draw a polygon gate around the cell population and label the population ‘Cells’.

9.2.5. Double click the **Cells** population in the workspace to view only the cells within the ‘Cells’ population and then click on the FSC axis to change the axis channel to FSC-width and then click on the SSC axis and change the axis channel to the FSC-H.

9.2.6. Choose the **Rectangle** gate tool and draw a rectangle around the left-most dense population of cells spanning the entirety of the y-axis to exclude potential doublets toward the right of the window and label this gate ‘Single Cells’.

9.2.7. Right click and copy the **Cells** and the nested **Single Cells** gate and paste them under each sample in the workspace.

9.2.8. Double click the **Single Cells** gate nested under the DAPI sample in the workspace to view the single cell population from that sample tube and click on the FSC axis and change the axis channel to the DAPI - Area channel.

9.2.9. Click on the SSC axis and change the axis channel to **Histogram**.

NOTE: The live cells will be towards the right, as they exclude DAPI, while dead cells will take in the DAPI and appear towards the right of the graph.

9.2.10. Click on the **T** button next to the **DAPI** axis and click on **Customize Axis** to adjust the scale to maximize separation between DAPI positive and DAPI negative peaks.

NOTE: A window will pop up with scaling options. Often times, setting the **Scale** field to Biex and adjusting the Extra Negative Decades and Width Basis will yield the greatest separation.

9.2.11. Click **Apply** in the pop-up window to apply scaling changes, choose the **Range** gate button, and spread it over the DAPI negative peak, corresponding to the live cell population.

9.2.12. Label this gate 'Live Cells' and right click to copy the 'Live Cells' gate to paste it under the 'Single Cells' gate under the 'APC-iso, 'DEAB', and 'ALDH/CD133' tubes to select the same portion of live cells in each sample tube.

9.2.13. Then double click the **Live Cells** population nested under the APC-iso sample file and switch the x-axis to the ALDH - Area channel and the y-axis to the APC - Area channel.

9.2.14. Again, adjust the axis scale by clicking the **T** button and **Customize Axis** of each axis so that the events are localized in the bottom left region of the plot and select the **Quad** gate option and click the plot window to establish a quadrant gate.

9.2.15. Adjust the intersection of the gate such that approximately 0.5% of the population lies in the upper left of the plot window or 'APC positive' quadrant.

NOTE: This 0.5% represents the non-specific staining of the APC isotype.

9.2.16. Right click each quadrant label individually in the workspace and rename them appropriately. Control or Command click each quadrant gate label in the workspace and copy them.

NOTE: 'Q1' represents CD133+ cells; 'Q2' represents CD133+ and ALDH+' cells; 'Q3' represents ALDH+ cells; and 'Q4' represents CD133- and ALDH- cells and control or command click each quadrant gate label in the workspace and copy them.

9.2.17. Copy and paste the quadrant gates onto the **Live Cells** population nested under the 'DEAB' file. Adjust the vertical line such that approximately 0.15% of the cell population lies within the 'ALDH+' quadrant taking care not to move the horizontal line.

NOTE: This 0.15% represents non-specific ALDH signal.

9.2.17.1. To ensure that the horizontal line did not change position, copy the new quadrant gates nested under the **DEAB** file and paste them onto the **Live Cells** under the APC iso file. Select **Yes** when asked to replace existing quadrant gate.

NOTE: When asked to replace the existing quadrant gate with the new gates which have the same name - select **Yes**.

9.2.17.2. Verify in the workspace that the 'CD133+' percent is still approximately 0.5 under the 'APC-iso' file.

9.2.18. Copy and paste the quadrant gates to the 'ALDH/CD133' file's 'Live Cells' population.

NOTE: The percentage of the viable cell population present in the top right quadrant will be the percentage of ALDH+ and CD133+ double positive CSCs within the sample.

REPRESENTATIVE RESULTS:

Spheroids formed with cell lines or patient-derived CSCs can be formed with a range of small cell numbers within hanging droplets (**Figure 2A**). Spheroids form reliably with as few as 10 cells per well, which allows for conservation of rare patient samples. Cells within these spheroids are surrounded by other cells in 3 dimensions as they would be in vivo, allowing for physiologic cell-cell contacts and diffusion rates. Tumor cells within the spheroids proliferate causing the spheroids to expand in size over time (**Figure 2B**). As more aggressive patient cells or cell lines grow faster than their counterparts, it is important to quantify the proliferation capacity of each sample and examine how drug treatment affects the proliferation of each sample. To do this, a metabolic activity assay, such as resazurin based fluorescence assay, can be easily performed with the 384-well physiologic platform, without any requirement for harvesting spheroids, the results of which can be seen in **Figure 2C**. Multiple spheroids can also be harvested, fixed, sectioned, and stained with hematoxylin and eosin or immunofluorescent antibodies at the same time to identify cell morphologies and organization within spheroids, as well as, the distribution of cell types and ECM proteins (**Figure 2D, E**).

To examine the effect of drug treatment on spheroid morphology, spheroids can be easily visualized by phase contrast imaging (**Figure 3A).** More quantitatively, the effect of drug treatment on tumor cell or CSC proliferation can also be measured by resazurin dye fluorescence readings in control untreated spheroids compared to in the drug treated spheroids (**Figure 3B**). As a validation of cell death following drug treatment, viability within control and drug treated spheroids can easily be determined via the addition of calcein-AM and ethidium homodimer-1 to

multiple spheroids for each condition (**Figure 3C**). Following incubation time, stained spheroids can be harvested with a pipette and imaged on a confocal microscope.

Finally, by harvesting spheroids and dispersing them into single cell suspensions, the presence of CSCs and other cell phenotype markers can be analyzed with flow cytometry (**Figure 4**). Comparison of viable CSCs between different drug treatments within the same patient, as well as, between patients can help to discern the effectiveness of various CSC targeting drugs in a patient specific manner.

FIGURE LEGENDS:

Figure 1: 3D high throughput 384 hanging drop spheroid plate storage and plating layouts. (**A**) Hanging drop plate is placed on the bottom of a 6 well plate partially filled with water. (**B**) Lid of 6 well plate is placed on top of hanging drop plate to create sterile hydration chamber. (**C**) A thermoplastic strip is stretched to surround the edges of a hanging drop and 6 well plate stack to seal in moisture and protect from contaminants. (**D**) Alternating plating pattern for hanging drop plate. Pink squares indicate wells filled with cells and medium mixture. Blue areas indicate water chambers for hydration. Gray boxes indicate blank wells that act as a border between hydration chambers and spheroid droplets. (**E**) Live image of hanging drop plate plated with the alternating well pattern. (**F**) All well plating pattern layout utilized for high throughput hanging drop spheroids experiments. Pink squares indicate cell plated wells and blue areas indicate water filled chambers for increased hydration. Gray squares indicate border wells that act as boundary between hydration sections and cell culture areas.

Figure 2: Patient derived CSC spheroids morphology and proliferation within 3D hanging drops. (**A**) Live image of prepared 384-hanging drop spheroid plate as viewed from the bottom. (**B**) Progressive light microscope images of patient derived CSC spheroid growth after 2, 3, and 5 days in a hanging drop. Scale bars are 100 µm. (**C**) Resorufin fluorescence intensity shows significant increase after 7 days of hanging drop culture, correlating to proliferation and growth of the hanging drop patient derived CSC spheroids (Unpaired two tailed t-test, $p < 0.0001$, $n > 10$). (**D**) Picture of spheroid array mold used to create a cast for collection of spheroids for histology sectioning. (**E**) H&E image of a spheroid cultured with primary ovarian cancer stem cells, mesenchymal stem cells, endothelial cells, and donor peripheral blood mononuclear cells collected in the spheroid array. Scare bar is 100 µm.

Figure 3: Drug treatment analysis of patient derived CSC spheroids hanging drops. (**A**) Patient derived CSC spheroids seeded at 50 cells/drop treated with increasing concentrations of paclitaxel after 5 days of growth. Representative images taken 48 hours after drug treatment. (**B**) Quantification of cellular viability via resorufin fluorescence at increasing concentrations of paclitaxel treatment. All samples have a significant reduction in viability compared to control. (One-way ANOVA, $p < 0.0001$, $n > 8$). (**C**) Confocal imaging of live (calcein-AM) and dead (ethidium homodimer-1) cells within hanging drop spheroid. Green color indicates live cells and red color indicates dead cell population. Scale bar = 100 µm.

Figure 4: Quantification of CSCs in patient derived CSC spheroids generated and maintained on the 384-hanging drop platform. (**A**) In analyzing the flow cytometry data, the cell population is first selected using a polygon gate to eliminate any events attributable to debris. (**B**) The single cells are then selected to eliminate potential doublet signals which may obscure results. (**C**) All of the single live cells are then selected based on DAPI exclusion. (**D**) The vertical axis of a quadrant gate is adjusted in the DEAB control to allow for about 0.15% non-specific ALDH staining. (**E**) The horizontal axis of the quadrant gate is adjusted in the APC ISO control to allow for about 0.5% non-specific APC staining. (**F**) The quadrant gate is then used to determine the percent of CD133+, ALDH+, and CD133/ALDH+ cells are present in our live cell populations in the experimental CD133 + ALDH condition.

DISCUSSION:

The 384-well hanging drop plate platform for 3D spheroid formation is an easily implemented tool for any cell biology or cancer biology labs. This physiologic platform enables the study of cell lines, as well as, primary patient samples within physiologically relevant 3D cultures while allowing for high throughput drug screening. The platform also ensures that the culture conditions are highly tunable, enabling tight control over plating densities, cellular co-culture ratios, extracellular components, and medium composition. Furthermore, this physiologic platform allows experiments to be highly amenable to downstream analysis techniques requiring large or small cell counts such as qRT-PCR, FACS, and various imaging methods. While ease of utilization comes with experience, new trainees become successful quickly, once speed and ease of pipetting are mastered. Thus, this physiologic platform is highly applicable for personalized 3D drug screening, CSC biology and chemoresistance investigations.

Some points of concern when newly implementing this platform include plate transfer and drug treatment. When transferring plates from one location to another as required for routine feeding, imaging, and analysis, careful precautions should be taken to avoid unnecessary jostling. Grip the plates from the outer edges keeping them as level as possible and take care to avoid jarring movements when placing the plate down. This helps to avoid droplet loss or merging of neighboring drops. Similarly, vigilant attention should be given to the task of drug treating hanging drop spheroids, as to avoid incorrect dosing of any one hanging drop spheroid. As with any technique, confidence and accuracy with these tasks arrive with practice.

A few limitations are innate to this 3D physiologic platform. First, droplet instability may be of issue at long term culture time points, if care is not taken to maintain correct total droplet volume. Furthermore, as mentioned above, transport and storage of plates must be done carefully to avoid loss or merging of droplets. Additionally, the size for this 3D environment is dictated by innate stable droplet size of 20 µL, though many replicates can be produced for enhanced cell counts.

Modifications to this 3D physiologic platform can be utilized to increase throughput and alter physiological characteristics. For instance, droplet layout can be altered to include every well within the 384 well plate to increase throughput. In addition to increasing throughput, the 3D

hanging drop platform is highly conducive to co-culture investigations by simple inclusion of multiple cell types in each well. To generate and maintain spheroids successfully, cell culture medium and initial cell seeding density can be easily modulated, to tightly regulate spheroid size. With these highly tunable variables countless investigations are possible within the realm of 3D physiological patient derived spheroids.

While most analysis methods described are widely used in scientific research, there are a few limitations specific to analyzing hanging drop generated spheroids with some of these methods. For example, when spheroids are cultured for long periods of time in hanging drop plates, droplet size can increase significantly causing droplets to shake during phase contrast imaging, potentially compromising image quality. This can be ameliorated by taking an equivalent amount of medium out of each well prior to adding fresh medium. Additionally, techniques such as flow cytometry and counting individual viable cells may be affected by the technique used to break apart spheroids, which may be harmful to cells. As such, it is important for each lab to optimize spheroid disaggregation techniques based on their cells and experiment to minimize cell damage while maximizing single cell density. Finally, histological analysis of spheroids can be complicated by their small size and requires practice to obtain successful sections.

Overall, the 3D hanging drop spheroid platform is widely adaptable within cancer and non-cancer research. The system is easy to learn and provides a 3D physiologically relevant environment for cell culture in a high throughput format. Initiation time of this 3D physiological platform is minimal, with few, if any, technical analysis hurdles to overcome. The versatility of this system provides a means for patient specific screening of effective chemotherapeutics for precision medicine, in a more physiologic environment than ever before.

ACKNOWLEDGMENTS:

This work is supported primarily by DOD OCRP Early Career Investigator Award W81XWH-13-1-0134(GM), DOD Pilot award W81XWH-16-1-0426 (GM), DOD Investigator Initiated award W81XWH-17-OCRP-IIRA (GM), Rivkin Center for Ovarian Cancer and Michigan Ovarian Cancer Alliance. Research reported in this publication was supported by the National Cancer Institute of the National Institutes of Health under award number P30CA046592. CMN is supported by the National Science Foundation Graduate Research Fellowship under Grant No. 1256260. MEB is supported by the Department of Education Graduate Assistance in Areas of National Need (GAANN) Fellowship.

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. http://gco. iarc.fr/today/data/factsheets/cancers/39-All-cancers-fact-sheet.pdf. All cancers

Source: Globocan 2018. (2018).

2. Prasad, V., Mailankody, S. Research and Development Spending to Bring a Single Cancer Drug to Market and Revenues After Approval. *JAMA Internal Medicine.* **177**, 1569–1575 (2017).
3. 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).
4. Jordan, C. T., Guzman, M. L., Noble, M. Cancer Stem Cells. *New England Journal of Medicine.* **355**, 1253–61 (2006).
5. Ishiguro, T. et al. Establishment and Characterization of an In Vitro Model of Ovarian Cancer Stem-like Cells with an Enhanced Proliferative Capacity. *Cancer Research.* **76**, 150–160 (2016).
6. Páez, D. et al. Cancer Dormancy: A Model of Early Dissemination and Late Cancer Recurrence. *Clinical Cancer Research.* **18**, 645–653 (2012).
7. Ahmed, N., Stenvers, K. L. Getting to Know Ovarian Cancer Ascites: Opportunities for Targeted Therapy-Based Translational Research. *Frontiers in Oncology.* **3**, (2013).
8. Pradhan, S., Clary, J. M., Seliktar, D., Lipke, E. A. A three-dimensional spheroidal cancer model based on PEG-fibrinogen hydrogel microspheres. *Biomaterials.* **115**, 141–154 (2017).
9. Varna, M., Bertheau, P., Legrès, L. Tumor Microenvironment in Human Tumor Xenografted Mouse Models. *Journal of Analytical Oncology.* **3**, 159–166 (2014).
10. Huang, S. B. et al. An integrated microfluidic cell culture system for high-throughput perfusion three-dimensional cell culture-based assays: Effect of cell culture model on the results of chemosensitivity assays. *Lab on a Chip.* **13**, 1133–1143 (2013).

11. Zang, R., Li, D., Tang, I.-C., Wang, J., Yang, S.-T. Cell-Based Assays in High-Throughput Screening for Drug Discovery. *International Journal of Biotechnology for Wellness Industries.* (2012).
12. Murphy, B. et al. Evaluation of alternative in vivo drug screening methodology: a single mouse analysis. *Cancer Research.* **76**, 5798–5809 (2016).
13. Hidalgo, M. et al. Patient-derived Xenograft models: An emerging platform for translational cancer research. *Cancer Discovery.* **4**, 998–1013 (2014).
14. Damiati, S., Kompella, U. B., Damiati, S. A., Kodzius, R. Microfluidic devices for drug delivery systems and drug screening. *Genes.* **9**, (2018).
15. Caliari, S. R., Burdick, J. A. A Practical Guide to Hydrogels for Cell Culture. *Nature Methods.* **14**, 69–81 (2016).
16. Mehta, G., Hsiao, A. Y., Ingram, M., Luker, G. D., Takayama, S. Opportunities and challenges for use of tumor spheroids as models to test drug delivery and efficacy. *Journal of Control Release.* (2012).
17. Mehta, P., Novak, C., Raghavan, S., Ward, M., Mehta, G. Self-Renewal and CSCs In Vitro Enrichment: Growth as Floating Spheres. in *Cancer Stem Cells: Methods and Protocols* (eds. Papaccio, G., Desiderio, V.), *Methods in Molecular Biology,* 61–75 (Springer New York, 2018).
18. 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).

19. Raghavan, S. et al. Comparative analysis of tumor spheroid generation techniques for differential in vitro drug toxicity. *Oncotarget.* **7**, 16948–16961 (2016).
20. Kim, S. et al. Evaluating Tumor Evolution via Genomic Profiling of Individual Tumor Spheroids in a Malignant Ascites. *Scientific Reports.* **8**, 1–11 (2018).
21. Silva, I. A. et al. Aldehyde dehydrogenase in combination with CD133 defines angiogenic ovarian cancer stem cells that portend poor patient survival. *Cancer Research.* **71**, 3991–4001 (2011).
22. Pulaski, H. L. et al. Identifying alemtuzumab as an anti-myeloid cell antiangiogenic therapy for the treatment of ovarian cancer. *Journal of Translational Medicine.* **7**, 49 (2009).
23. 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).
24. Ivanov, D. P., Grabowska, A. M. Spheroid arrays for high-throughput single-cell analysis of spatial patterns and biomarker expression in 3D. *Scientific Reports.* **7**, 41160 (2017).

Figure 1

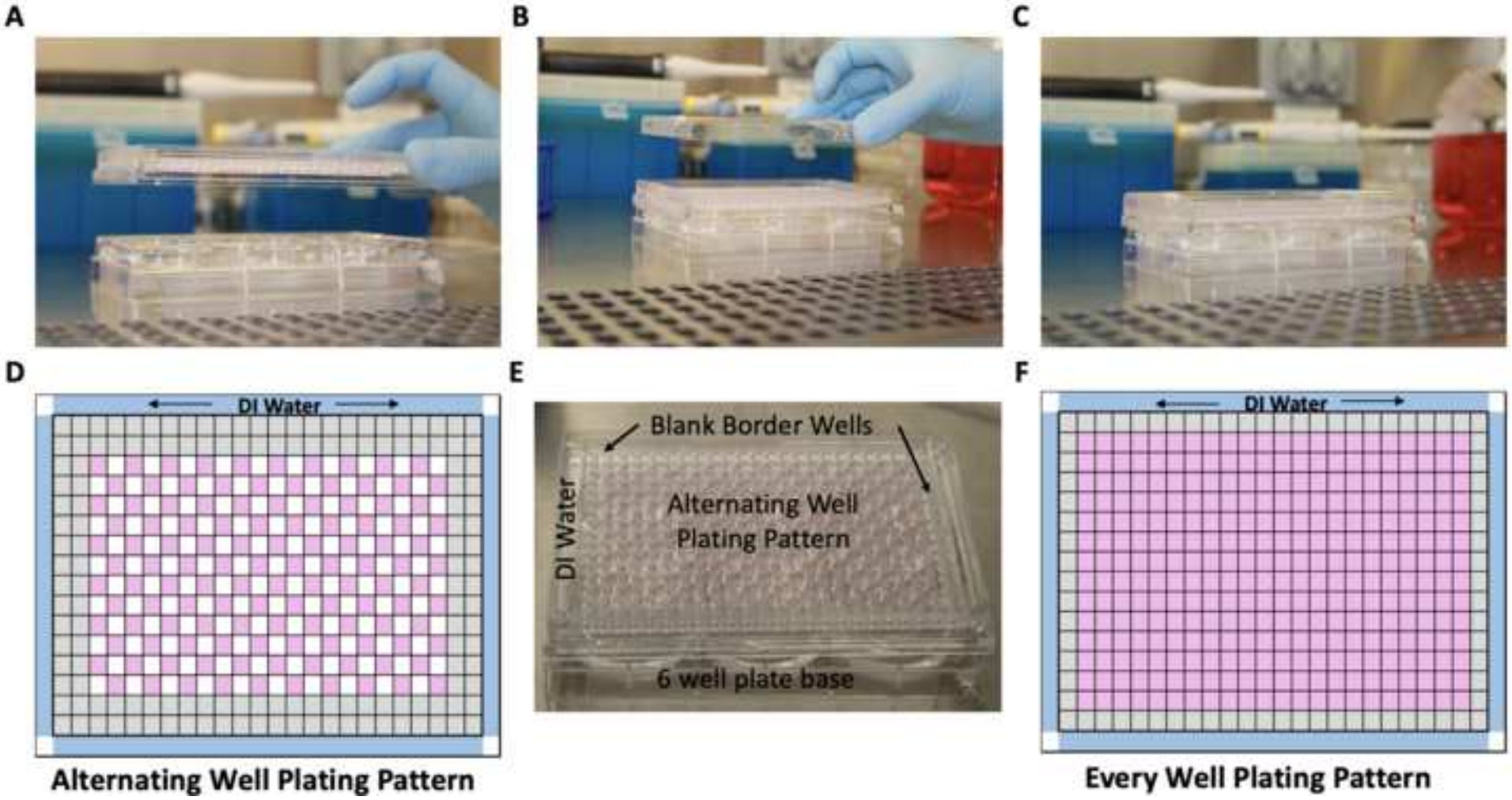


Figure 2 revised

Click here to access/download;Figure;Figure 2 revised.tiff

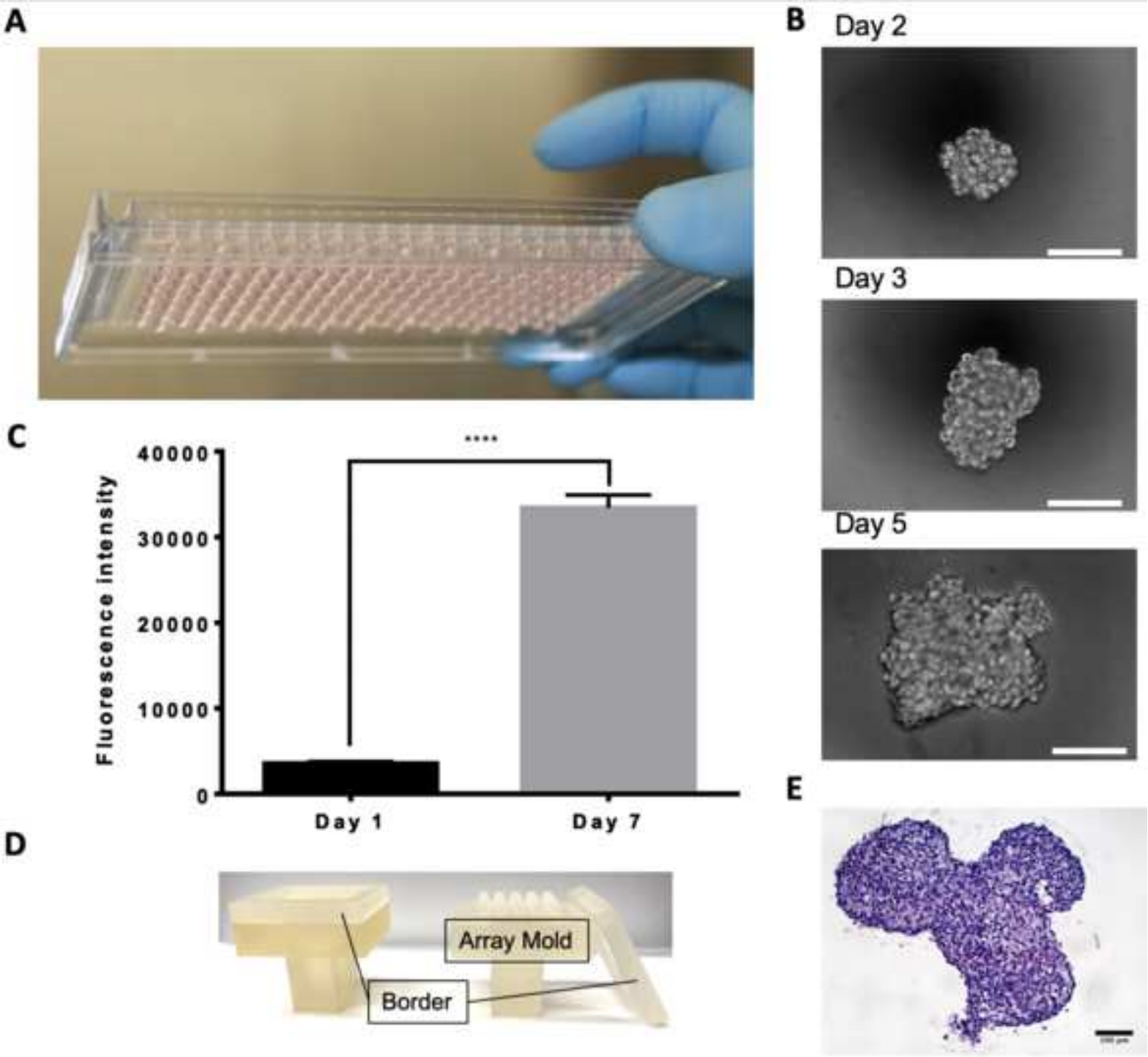


Figure 3

Click here to access/download;Figure;Figure 3 revised.tiff

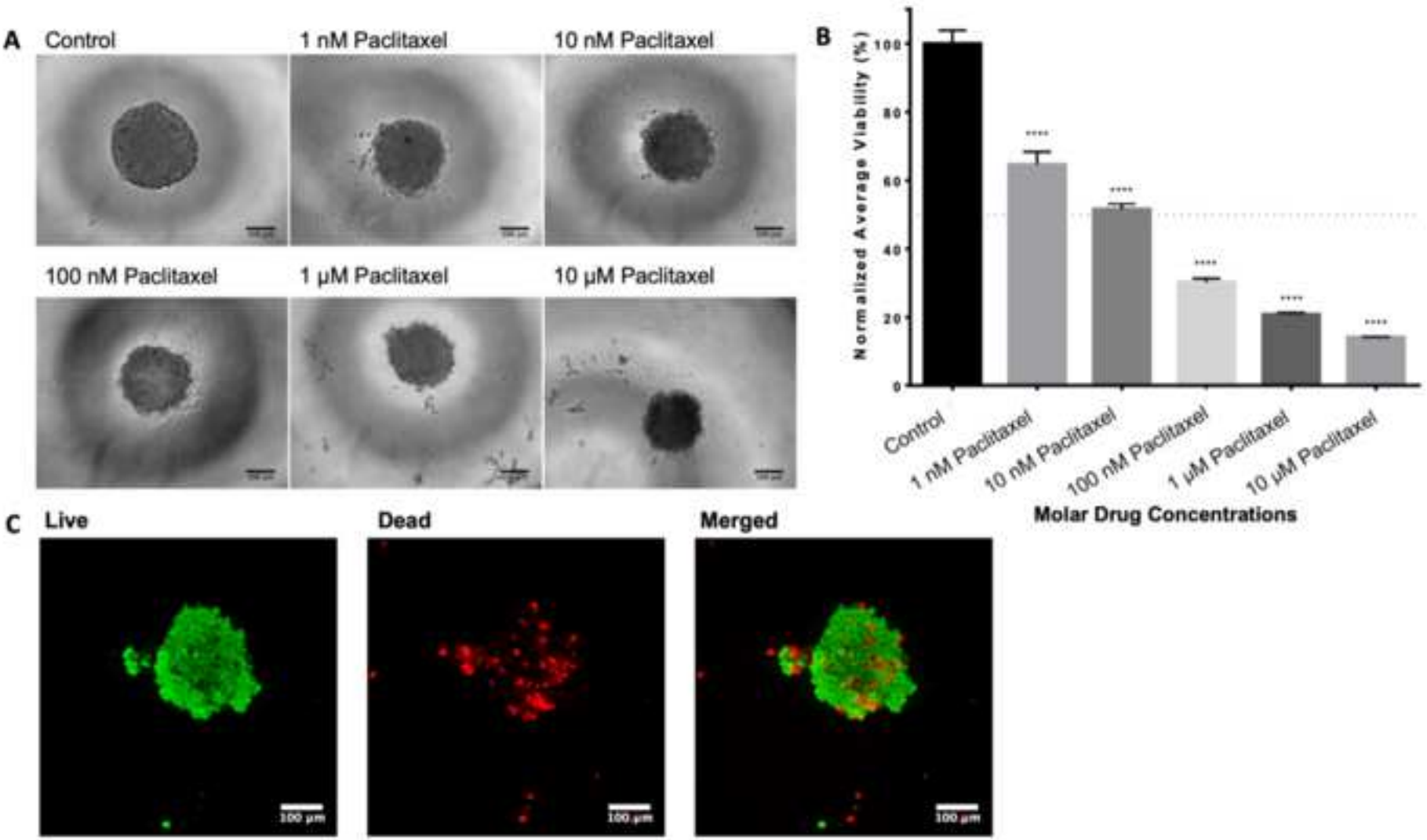
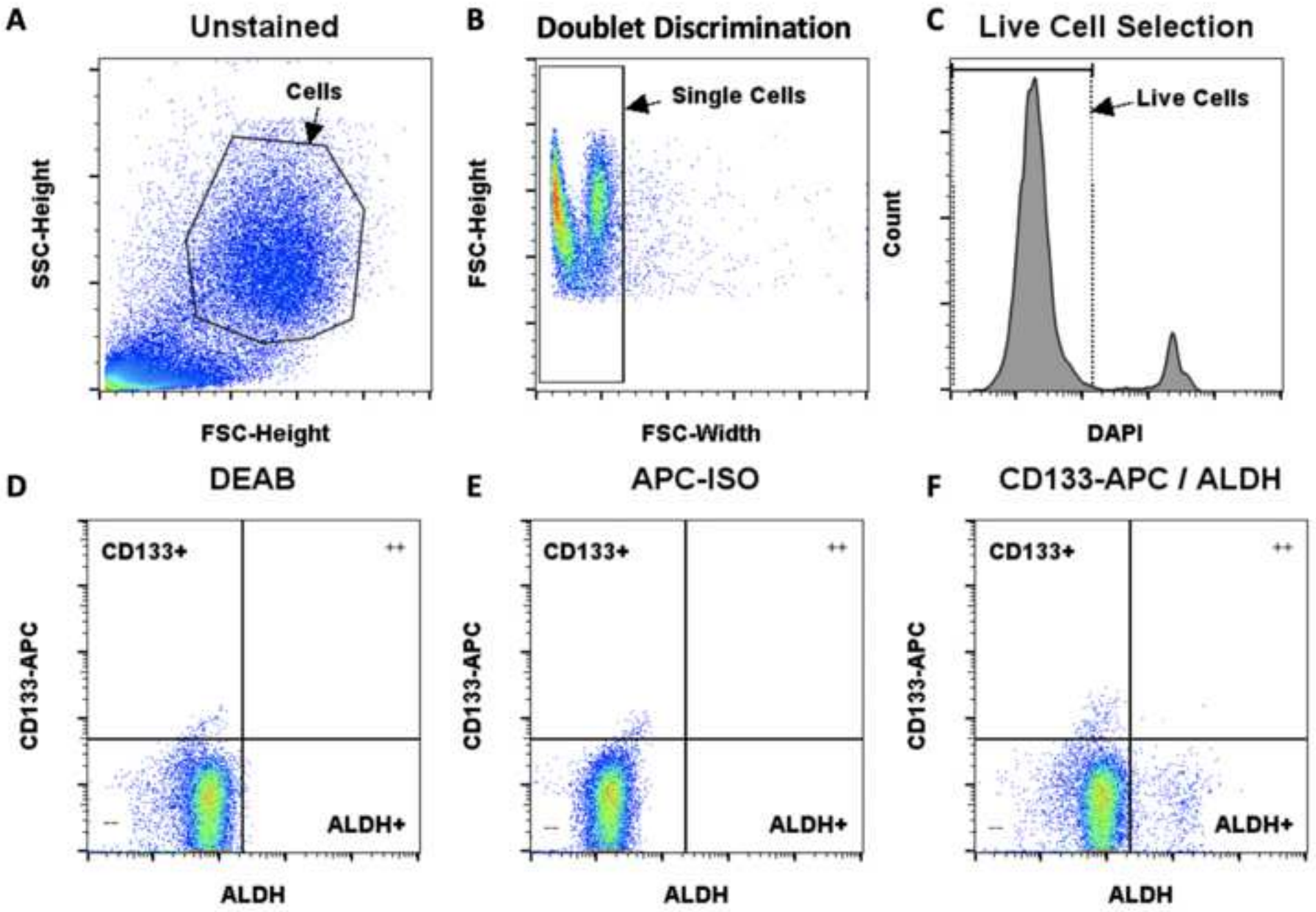


Figure 4 revised

Click here to access/download;Figure;Figure 4 revised.tiff



Name of Material/ Equipment	Company
0.25% trypsin-EDTA	Gibco
10 mL serological pipet	Fisher Scientific
10,000 cSt Si oil	Millipore Sigma
100 mm tissue culture dish	Thermo Scientific
15 ml conical tube	Celltreat
1X DMEM for Serum Free Medium	Gibco
1X F12 for Serum Free Medium	Gibco
1X phosphate buffered saline (PBS)	Gibco
4',6-diamidino-2-phenylindole (DAPI)	Thermo Fisher
40 µm filter	Fisher Scientific
6-well plate	Fisher Scientific
Accutase	Innovative Cell Technologies Inc.
ACK Lysing Buffer	Thermo Scientific
alamarBlue	Invitrogen
ALDEFLUOR assay kit	Stem Cell Tech
ALDEFLUOR Diethylaminobenzaldehyde (DEAB)	Stem Cell Tech
Andor iXon x3 CCD Camera	Oxford Instruments
Antibiotics and Antimycotics	Gibco
APC-isotype IgG2b	Miltenyi biotec
B27 Supplement	Gibco
basic Fibroblast Growth Factor	Stem Cell Technologies
BD Lo-Dose U-100 Insulin Syringes	Fisher Scientific
BioTek Synergy HT Microplate Reader	BioTek
CD133-APC	Miltenyi biotec
cellSens Dimension Software	Olympus
Cisplatin	Sigma-Aldrich
DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride)	Invitrogen
Epidermal Growth Factor	Gibco
EVOS XL Core Cell Imaging System	Life Technologies

Fetal Bovine Serum - premium (FBS)	Atlanta Biologicals
Ficoll 400	Sigma-Aldrich
Hemacytometer	Hausser Scientific
Histogel	Thermo Scientific
Human Adipose-Derived Mesenchymal Stem Cells	Lonza
Human Microvascular Endothelial Cells	Lonza
Insulin-Transferrin-Selenium Supplement	Gibco
Live/Dead viability kit	Invitrogen
MEM Non-essential Amino Acids	Gibco
MetaMorph 7.8 Software	Molecular Devices
Olympus IX81 Inverted Confocal Microscope	Olympus
Olympus IX83 Research Inverted Microscope	Olympus
Parafilm M	Thomas Scientific
Perfecta 3D 384 Well Hanging Drop Plates	3D Biomatrix
phalloidin AlexaFluor488	Invitrogen
ProJet 3500 HD Max	3D Systems
Sterile DI water	Fisher Scientific
Trypan Blue	Gibco
VisiJet M3 Crystal	3D Systems
Yokogawa CSU-X1 Confocal Scanner Unit	Yokogawa

Catalog Number	Comments/Description
ILT25200056	
13-678-11E	
63148-62-9	Used to coat spheroid array mold to facilitate removal of tissue processing gels, like Histogel, from the mold.
130182	
FL4021	
11965-092	
11765-054	
ILT10010023	
D1306	
22363547	
353046	
1449	A gentle cell detachment enzyme composed of proteolytic and collagenolytic enzymes.
A1049201	
DAL1025	Resazurin dye used to measure viability and proliferation of cells based on their ability to reduce resazurin to resorufin, which is highly fluorescent.
1700	Kit to identify stem and progenitor cells that express high levels of aldehyde dehydrogenase , an indicator of cancer stem cells. The kit is composed of ALDEFLUOR Reagent, DEAB, Hydrochloric Acid, Dimethylsulphoxide, and ALDEFLUOR Assay Buffer.
1705	Diethylaminobenzaldehyde (DEAB) is an inhibitor of ALDH isozymes, used to determine non-specific ALDH staining.
-	
15240-062	
130-092-217	Isotype control to quantify non-specific staining of IgG2b antibodies.
17504044	
78003.1	
14-826-79	
7091000	
130-113-184	Fluorescent antibody targeting CD133, a cancer stem cell marker.
P4394	Platinum based chemotherapy agent that functions as an alkylating agent that disrupts DNA.
D1306	
PHG0311	
AME3300	

S11150	
F4375	
1490	
HG-4000-012	Tissue processing gel that can penetrate and hold the specimen within the gel while preventing discoloration around the specimen upon staining.
PT-5006	
CC2543	
51500-056	
L3224	Kit for the fluorescence based detection of live (calcein-AM) and dead cells (Ethidium Homodimer-1).
11140-050	
-	
-	
7315D35	Thermoplastic polymer strips that serve to limit droplet evaporation in hanging drop plates while still allowing for gas exchange.
HDP1384-8	Available through Sigma-Aldrich
A12379	Phalloidin is a peptide to fluorescently label F-actin in fixed cells.
-	3D printer
353046	
15250061	Azo dye used to differentiate between live and dead cells based on its ability to pass through the damaged membrane of dead cells, but not the intact membrane of live cells.
-	A biocompatible polymer material for 3D printing.
-	

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	*Physiologic Patient Derived 3D Spheroids for Anti-neoplastic Drug Screening to Target Cancer Stem Cells*
Author(s):	*Michael E. Bregenzer, Ciara Davis, Eric N. Horst, Pooja Mehta, Caymen M. Novak, Shreya Raghavan, Catherine S. Snyder, and Geeta Mehta*

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☑ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

☑ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	*Geeta Mehta*
Department:	*Materials Science and Engineering, Biomedical Engineering*
Institution:	*University of Michigan*
Title:	*Dow Corning Assistant Professor*
Signature:	
Date:	*1/15/2019*

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

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 CO_2 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 CO_2, 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).