Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by "(Table of Materials)" to draw the readers' attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Mimetas OrganoPlate, Percoll, HyStem, etc.

Instances of "OrganoPlate" have been replaced with "perfusable microfluidic plate" "microfluidic plate" or "plate" where appropriate. Similarly, "Percoll" has been replaced with "density gradient centrifugation solution" and "HyStem" has been replaced with "HA hydrogel".

3. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement for a specific item. To this end, we ask that you please reduce the number of instances of " OrganoPlate" within your text. The term may be introduced but please use it infrequently and when directly relevant. Otherwise, please refer to the term using generic language.

All but a few instances of "OrganoPlate" have been removed and replaced with the generic language described in the previous response.

4. All methods that involve the use of human or vertebrate subjects and/or tissue sampling must include an ethics statement. Please provide an ethics statement at the beginning of the protocol section indicating that the protocol follows the guidelines of your institution.

An ethics statement has been included (lines 151-153).

5. Please revise the Protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "NOTE." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

Noted and revised.

6. Please revise the Protocol steps so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary.

Noted and revised. Several steps (especially in Protocol Sections 3 and 4) have been split into less bulky steps.

7. Please list all centrifugation speeds in terms of centrifugal g-force (x g) instead of rpm.

Corrected (lines 200 and 240)

8. After you have made all the recommended changes to your protocol section (listed above), please highlight in yellow up to 2.75 pages (no less than 1 page) of protocol text (including headers and spacing) to be featured in the video. Bear in mind the goal of the protocol and highlight the critical steps to be filmed. Our scriptwriters will derive the video script directly from the highlighted text.

Concerning comments 8-10 concerning the highlighted text for the video script, the length and narrative flow have been adjusted to fit the requested parameters.

9. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. The highlighted text must include at least one action that is written in the imperative voice per step. Notes cannot usually be filmed and should be excluded from the highlighting.

10. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

11. Figure 1 and Table 1: Please remove commercial language: OrganoPlate.

Commercial language has been removed from this figure and table.

12. Table of Materials: Please remove any ™/®/© symbols. Please sort the materials alphabetically by material name.

Symbols have been removed and the materials have been sorted.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
The development and validation of alternative in vitro models, able to recapitulate the physiology and drug sensitivity features of a primary tissue sample, is currently of paramount interest and importance. The present work submitted by Lindsay et al. presents a novel approach to recreate 3D matrix-embedded organoids from prostate cancer in a gravity-perfused microfluidic chamber plate. The procedure will be of interest to the community.

Minor Concerns:
There are several places where the protocol contains relatively vague references rather than a detailed description.

We appreciate the reviewer's suggestion here and endeavored to address this within the text and as described below.

Line 171,187, Steps 2.2, 2.5: State the typical size the PDXs reach before resection from the mouse and if this parameter, together with the digestion time, depends on the origin of the PDX (i.e prostate-derived PDXs were used in the paper but, would the protocol differ when using another type of tumor?)

PDX tumors were resected when they reached 1.0-1.5 cm in diameter (now included in Step 2.2, line 176). This could potentially be varied within IACUC guidelines to provide more tissue for particularly low-yield tumors. Tumor characteristics vary between individual PDX lines even within the same tissue of origin (prostate, breast, etc.). Some PCa PDX lines are more fibrotic and difficult to isolate viable cells from than others which are more cellular. This is likely the case with all epithelial-derived cancer PDXs. This information has been added to the Discussion section.

Line 201, Step 2.7: Change centrifuge speed (rpm to rcf) and indicate temperature.

Centrifuge speed has been corrected. Step 1.4 states all steps should be conducted at room temperature unless otherwise specified.

Line 235-254, Steps 3.2-3.4: Temperature is critical for gradient preparation, please indicate for each step the required temperature.

Step 1.4 states all steps should be conducted at room temperature unless otherwise specified.

Line 257, Step 3.6: Specify the volume taken for re-dissociation and cell number determination. Indicate the amount of enzyme solution used.

A 50-100ul volume was removed and re-dissociated with an equal volume of enzyme solution. This was noted in the relevant Protocol Step (now Step 3.8, lines 260-261).

Line 268-288, Steps 4.1-4.5: This section is critical for the success of the whole technique, and despite the effort of the authors, the whole procedure is difficult to understand. I encourage a full revision of it, and the use of exact and clear indications for the hydrogel preparation and plate seeding. The comment also applies to Table 1.

This section and table were revised for clarity. (Now Steps 4.1 – 4.6) Figure 2 was revised to provide even more detail and clarity, by demonstrating an example calculation (in Figure 2F) and by linking steps and calculations to Table 1.

We attempted multiple versions of Table 1, including a reversal of its calculation (i.e. a right-to-left swap of the values), to match the workflow in Figure 2. However, upon review, we felt that most users would ultimately want to begin with a desired cell concentration per chip, and calculate back from there to derive an estimated starting number of cells. Our revised submission here represents our best attempt to clarify that workflow.

Line 354-364, Steps 5.3.1-5-4-1: I agree with the general indications given by the authors but providing the exact imaging settings would be useful information for the readers. Please state height of the z-stack as well as the z-step, objective, microscope type and instead the exact amount of time needed to image the whole plate. Similarly, provide the exact analysis tools used for the image processing, structure count and viability determination.

Details of imaging and analysis have been included in the relevant Protocol Steps (lines 348-364).

General comments.
To modify (or split) table 1 in such a way that the two processes contained there (sample preparation and organoid plate seeding) are grouped more efficiently, to make it more friendly to the readers.

We appreciate that Table 1 was still challenging to interpret in our first submission. As noted above, we have revised Table 1 to tie particular cell counts to discreet Steps in the Protocol, and to link portions of Table 1 back to Figure 2. We added a sample calculation as Figure 2F, using the cell concentration that would be the best starting point for most users.

The presented protocol reduced the number of dead cells/debris by using classical Percoll gradient separation (Figure 4A, B) however the authors did not demonstrate enhanced viability of the resulting organoids. Could you provide related data? (Similar to Figure 5 but comparing organoid viability in Percoll-separated vs traditional method)

The requested data has been integrated into Figure 5.

Reviewer #2:

Manuscript Summary:

This paper describes the processing of PDX tissues and their subsequent seeding/culture in a commercial 3D microfluidic culture platform (Mimetas OrganoPlates). The pre-processing of the PDX tissues were specifically designed to remove contaminating mouse stromal cells from the tumor cells as well as purifying live PDX tumor cells that tend to be in small aggregates from non-viable single cells. As there are increasing interests to use patient-derived tumor samples for assessing personalized cancer treatment regimes, this protocol is timely and useful for the scientific community.

Major Concerns:

Step 2.7-2.8: The enumeration of cell density from the PDX tissues was performed immediately after PDX tissue digestion, and the cell seeding density into the Mimetas plate was calculated based on this cell density (Table 1). First, how practical is it to determine the cell density as many of the cells are existing as cell aggregates and not single cells? Would it make sense to determine the cell density and do calculations for Table 1 after the stromal cell removal and percoll gradient steps?

Table 1 provides an estimate of the number of freshly dissociated cells required at the beginning of the process to achieve the final seeding densities listed. The actual seeding density is calculated at the end of Section 3 by re-dissociation of a small volume of cell aggregates. (Lines 261-263)

Regarding the loading of the cells / hydrogel into the microfluidic channel, how uniform is the cell distribution along the microfluidic channel? Is there preferential clustering of cells near the inlet of the microchannel, and therefore resulting in non-uniform cell density along the microfluidic channel? It will be helpful to comment on the likelihood of this occurrence. This may potentially affect the survival of the PDX cells since their survivability may be sensitive to autocrine signaling from neighboring cells.

While there is some inherent variability in cell density over the length of the microchannel because of the use cell aggregates rather than single cells, clumping in any part of the channel is only seen in PDXs that form very large clusters that are unsuitable for culture in microfluidics. PDX cell requirement for signaling from neighboring cells is provided for by the use of cell aggregates.

Step 5.2: The assay describes staining of live / dead cells and the reagents only need to be incubated for 1 hour to get uniform staining throughout the PDX spheroids in the microfluidic channels. It will be useful to have optimized immunostaining protocols with antibodies to achieve uniform staining for the PDX spheroids in situ on the Mimetas plate to assess other phenotypes.

We appreciate that the Reviewer has highlighted the issue of immunostaining. Although we have conducted immunostaining within the microfluidic culture system, no such data is presented here so we have removed all references to immunostaining in this protocol.

Minor Concerns:
Step 5.3.1: It was mentioned that a 20X long working distance objective was optimal for image acquisition. Using this objective, how many images (field of views) are required to image the entire microchannel? Is it necessary to sample along the entire microchannel?

Three fields of view are needed to image the entire microchannel with a small amount of overlap. To avoid double sampling, two fields of view were imaged per chip. This has been noted in the relevant Protocol Step.

Line 357: "insure" should be "ensure".

Corrected

Reviewer #3:

Manuscript Summary:

The article by Sablatura et al provides a step-by-step protocol to improve viable tumor cell counts from PDX tissues cultured in a 3D OrganoPlate platform. The main premise of the work is important given the need for more relevant preclinical tumor models. Overall the protocol is clear and easy to follow. However, the utility of this method may be limited given the very specific 3D platform conditions described. It also appears the protocol improvements are incremental compared to previous publications. Despite these concerns, the quality of the work is solid. There are some minor edits to the protocol and figures that could improve the manuscript.

Major Concerns:

Figure 5 - the authors should show the time course data for the unseparated condition as a comparison.

This data has been integrated into Figure 5.

Minor Concerns:

1. Line 174 - it would be helpful for the authors to specify a range (or at least a minimum) of PDX tumor sizes that are appropriate for yielding the number of cells required for this 3D culture system.

PDX tumors were resected at 1.0-1.5 cm in diameter. Depending on tumor characteristics, this typically provides cells in excess of what is required for one microplate, even if processing only half the tumor with this protocol. Tumor size has been included in Protocol Step 2.2 (line 176-179) and discussion of tumor yield has been added to the Discussion Section.

2. Line 240 - add "balance for centrifugation" to make it clear what the 2nd tube is being used for.

Done.

3. Line 379 - reference to "Critical Conditions" seems like an error in the sentence.

Yes, removed.

4. Line 454 - please clarify whether "4 images" means 4 separate chips per condition or 4 different imaging fields of the same chip.

2 chips with 2 images/chip. This has been corrected in the caption for Figure 4.

5. Line 530 - please include the citation for the publication mentioned in this sentence.

This separate manuscript is in preparation. This has been noted in the text.

6. Figure 2 - it would be helpful to show a picture of the mouse stromal cells in the workflow. Also, the image quality for figure 2 is not great (at least when printed).

No work was done with the mouse stroma for this publication so, unfortunately, we cannot provide the requested images. However, these images have been documented in previous publications from our group (see Reference 4, Fong, *et al.* 2016). Concerning the image quality of Figure 2, we have done all we can to provide quality images; the separated cell layers are difficult to photograph. As noted in Representative Results (Lines 381-384) users should look for a hazy layer containing PDX cells at the interface of fractions 2 and 3. This was the focus of the red dashed box in Figure 2.