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Cover Letter

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School of Dentistry
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Dear JoVE Editors,

On behalf of my co-authors, I appreciate the opportunity to submit revisions on our manuscript, **"Enhanced Viability for Ex Vivo 3D Hydrogel Cultures of Patient-Derived Xenografts in a Perfused Microfluidics Platform"** for re-consideration in JoVE. The reviewers' comments were thorough and fair, and we did our best to revise the manuscript in a way that responds to their suggestions and concerns.

In particular, we recognized multiple reviewers' concerns regarding some of the PDX calculations, and we even requested a few days of extension to attempt to best address those concerns. We revised Table 1, Figure 2, and the Steps for each, to provide our best clarification for users. We hope that these efforts suitably resolve the reviewers' concerns.

The revised documents are uploaded alongside this letter, and revisions to the text are indicated with a red font.

Please let me know if any further changes are needed in this revision. We look forward to hearing the decision from your reviewers and editorial board.

Sincerely,

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TITLE:

Enhanced Viability for Ex vivo 3D Hydrogel Cultures of Patient-Derived Xenografts in a Perfused Microfluidic Platform

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3D culture, hydrogels, microfluidics, patient-derived xenografts, prostate cancer, in vitro cancer models, high-throughput drug screening, perfusion

SUMMARY:

This protocol demonstrates methods to enable extended in vitro culture of patient-derived xenografts (PDX). One step enhances overall viability of multicellular cluster cultures in 3D hydrogels, through straightforward removal of non-viable single cells. A secondary step demonstrates best practices for PDX culture in a perfused microfluidic platform.

ABSTRACT:

Patient-derived xenografts (PDX), generated when resected patient tumor tissue is engrafted

directly into immunocompromised mice, remain biologically stable, thereby preserving molecular, genetic, and histological features, as well as heterogeneity of the original tumor. However, using these models to perform a multitude of experiments, including drug screening, is prohibitive both in terms of cost and time. Three-dimensional (3D) culture systems are widely viewed as platforms in which cancer cells retain their biological integrity through biochemical interactions, morphology, and architecture. Our team has extensive experience culturing PDX cells in vitro using 3D matrices composed of hyaluronic acid (HA). In order to separate mouse fibroblast stromal cells associated with PDXs, we use rotation culture, where stromal cells adhere to the surface of tissue culture-treated plates while dissociated PDX tumor cells float and self-associate into multicellular clusters. Also floating in the supernatant are single, often dead cells, which present a challenge in collecting viable PDX clusters for downstream encapsulation into hydrogels for 3D cell culture. In order to separate these single cells from live cell clusters, we have employed density step gradient centrifugation. The protocol described here allows for the depletion of non-viable single cells from the healthy population of cell clusters that will be used for further in vitro experimentation. In our studies, we incorporate the 3D cultures in microfluidic plates which allow for media perfusion during culture. After assessing the resultant cultures using a fluorescent image-based viability assay of purified versus non-purified cells, our results show that this additional separation step substantially reduced the number of non-viable cells from our cultures.

INTRODUCTION:

Over the past decade, the field of cancer research has demonstrated renewed enthusiasm for patient-derived xenografts (PDXs) as a tool for assessing cancer cell pathway reliance and drug susceptibility[1]. The most common PDX models are established by subcutaneous or orthotopic implantation of human tumor cells—a tumor fragment, a cluster of dissociated tumor-derived cells, or a sample of isolated circulating tumor cells (CTCs)—into a rodent host. If the tumor "take" is successful, the xenograft cells will proliferate, vascularize, and otherwise interact with the host tissue to create a tumor, which can be harvested at an optimal size, subdivided, and re-implanted into other hosts. Among their many advantages as a model system, PDXs typically retain a substantial portion of the native tumor cell population's heterogeneity and enable the assessment of human-specific pathways and cell responses[2,3]. The in vivo context enables tumor interaction with vasculature and other adjacent stroma and recapitulates tissue characteristics such as drug diffusion dynamics, oxygen tension, and extracellular matrix influence that biologically and mechanically impact tumor progression. A negative aspect of PDXs is their reliance on a rodent host, both for tumor expansion and ultimately for hypothesis testing. Because many PDXs cannot adapt to traditional two-dimensional (2D) culture on tissue culture polystyrene without losing many of their desirable characteristics, there has been minimal middle ground for researchers between this relatively controlled in vitro method, and the significant increase in expense, facilities, and time requirements for in vivo PDX use.

We have described multiple in vitro models that implement 3D cell culture within a supportive matrix, and recently expanded that work to demonstrate the ex vivo culture of multiple prostate cancer (PCa)-derived PDXs, both alone and in co-culture with bone marrow-derived fibroblasts[4,5]. Hyaluronic acid (HA)-based hydrogel matrices provide customizable and

biologically-relevant support for both cell types, with facile control over hydrogel characteristics and optical clarity for imaging through the hydrogel depth[6].

Mature PDX tumor tissues comprise a variable mixture of heterogeneous human cancer cells and mouse stroma (fibroblasts, endothelial cells, etc.). To study cell-type specific contributions to tumor progression in vitro, it can be advantageous to dissociate tumors, separate the cell populations, and experimentally incorporate them in an organized manner to dissect pathways of intercellular communication. The mixed cell populations within tissue digestates have differential compatibility with specific culture conditions. For example, tumor-associated fibroblast viability necessitates either surface adherence or 3D matrices functionalized with integrin ligands, while epithelial-derived PDX cells do not typically have these requirements, instead favoring cell-cell interactions. These differences can be exploited to achieve effective separation of PDX cells from contaminating mouse stromal cells. Rotation culture of tissue digestates allows stromal cell adherence to the tissue culture surface while cell-cell adhesions drive PDX cells floating above the rotating culture surface to form multicellular clusters in the supernatant in 24−48 h. The specific characteristics of these clusters vary with the PDX (e.g., large, tight, highly spherical clusters or smaller, looser aggregates resembling bunches of grapes), but are typically of biologically relevant sizes (50−250 µm diameter), sufficient for assessing cellular interactions that rely on intercellular contacts.

Tumor retrieval and processing inevitably results in some degree of collateral cell death, either due to short-term damage from mechanical/enzymatic disruption, or long-term incompatibility of subpopulations with the chosen culture conditions. Despite the utility of rotation culture as an initial bulk separation, dead or dying cells are inevitably transferred with the PDX clusters and can influence the resultant culture. These dead cells are often individual PDX cells that were not integrated into a cluster, mouse stromal fibroblasts that cannot survive in selected culture conditions, or particularly fragile endothelial cells. Such dying cells can influence experimental results from “survivors” and can substantially impact quantification, e.g., via fluorescent image-based viability screening assays. To improve the selection of live PDX cells from this method, we adapted centrifugation methods with density steps to easily remove individual dead/dying cells from PDX mixtures and retain predominantly live multicellular clusters.

To enhance the study of resultant PDX-derived clusters in 3D culture, we utilized a microfluidics-based perfusion culture platform, the OrganoPlate (**Figure 1**), which is a high-throughput organ-on-a-chip platform that allows for simultaneous culture of up to 96 individual perfused, 3D cultures on a 384-well microtiter plate-base (**Figure 1A**)[7,8]. In the 2-lane microfluidic plate, a single tissue chip is connected by two microfluidic channels (**Figure 1B**, gel channel: red, perfusion channel: blue) which span four wells in a row. The two microfluidic channels are separated by a short plastic ridge called a Phaseguide which prevents overflow of one channel into its adjacent neighbor channel, and simultaneously allows for a membrane-free interface between the contents of the gel and perfusion channel[9]. Because the bottom of the microfluidic plate is composed of microscope-grade glass, the cultures can be viewed in the observation window through the bottom of the plate with a standard or automated

microscope. Perfusion is established in the microfluidic plate with a programmable rocker, using gravity to drive media through the microfluidic channels, between reservoir wells (**Figure 1C**). The perfusion flow-mimic more closely recapitulates the tumor microenvironment than static culture, allowing for the incorporation of shear stress and enhanced distribution of gases and nutrients. The benefits of maintaining a perfused cancer cell culture in the microfluidic plate have previously been described as perfused breast cancer cultures exhibited optimal viability as compared to a static 3D culture of the same cells[7].

The present report describes an adapted density gradient centrifugation method for isolating live multicellular PDX clusters and demonstrates its utility in establishing 3D PDX cultures within perfusable microfluidic plates. Because an increasing number of research laboratories are seeking methods to facilitate PDX use, we anticipate that the protocols presented here will be of immediate utility.

PROTOCOL:

Tumor tissue was obtained with patient consent and according to an approved Institutional Review Board (IRB) protocol. Xenografts were implanted, grown, and harvested according to an accepted Institutional Animal Care and Use Committee (IACUC) protocol.

NOTE: All work is to be performed in a sterile biological safety cabinet to maintain sterility. All steps should be conducted at room temperature unless otherwise specified.

1. Preparation of materials for PDX processing

1.1. Autoclave forceps and scalpel handle or razor blades.

1.2. Thaw dissociation enzyme solution at 4 °C overnight or at room temperature the same day as tissue dissociation.

NOTE: Thawing at 37 °C is not advised, as this can inactivate some dissociation enzymes.

1.3. Prepare at least 100 mL of PDX culture medium (Dulbecco's modified Eagle medium-nutrient mixture F-12 [DMEM-F12] with 100 U/mL penicillin and streptomycin and 30% fetal bovine serum [FBS]), and at least 25 mL of PDX processing medium (DMEM-F12 with 100 U/mL penicillin and streptomycin and no FBS). Store at 4 °C until ready to use.

2. PDX dissociation and initial purification of stromal component

2.1. Gather autoclaved utensils, 70 µm cell strainers (2−3), 60 mm round tissue culture dishes (2), 6-well tissue culture plates, sterile 50 mL conical centrifuge tubes, and sterile 1x phosphate-buffered saline (PBS). Warm culture medium to 37 °C and allow dissociation enzyme solution to come to room temperature.

2.2. When PDX tissue has reached a diameter of 1.0−1.5 cm in a mouse host, surgically remove the tumor from the mouse by standard means (e.g., under accepted anesthesia) and store on ice in a 50 mL tube with PDX culture medium (**Figure 2A**). Process the tissue promptly, via the steps below, to maximize cell viability, preferably within 1−2 h after harvest.

2.3. Transfer tumor tissue to a pre-weighed sterile 50 mL conical tube. Rinse 6x with 30 mL of sterile PBS to remove blood and contaminants. Remove as much liquid as possible and weigh the tumor tissue.

2.4. Transfer tumor tissue to a 60 mm round tissue culture dish and mince into ~1 mm^3 pieces using a sterile razor blade or scalpel.

2.5. Add 5 mL of PDX processing medium to collect tumor slurry and transfer to a new sterile 50 mL tube. Rinse the culture dish with another 5 mL of PDX processing medium, then with dissociation enzyme solution (10 mL/g tumor, at least 5 mL), adding all rinses to the 50 mL tube.

2.6. Incubate 20 min at 37 °C with gentle shaking. Swirl the tube gently halfway through the incubation time.

2.7. Pipette up and down gently with a serological pipette to break up clumps. Filter cells with a 70 µm cell strainer placed over a new sterile 50 mL tube.

NOTE: More than one strainer may be necessary.

2.8. Centrifuge at 200 x *g* for 5 min to pellet cells. Remove supernatant and resuspend in 2−3 mL of PDX culture medium. Count cells using a hemocytometer or automated cell counter.

2.9. Use **Table 1** to estimate the required number of dissociated PDX-derived cells needed to achieve the desired cell density per chip.

NOTE: The values in **Table 1** are intended to be a starting point. Actual values will vary due to tissue viability/cellularity and cell loss from freezing/recovery. PDX tumors are individuals even within a given cancer type so these values should be adjusted empirically.

2.10. Of the number calculated in step 2.9, plate 1−2 x 10^6 cells in 5 mL of PDX culture medium per well of a 6-well tissue culture plate. Incubate (37 °C, 5% CO_2, 95% humidity) for 48 h with gentle shaking (50−55 rpm) to promote cluster formation (**Figure 2B**). After cluster have formed, proceed to section 3 for centrifugation.

2.11. Cryopreserve unused PDX cells from the initial dissociation in 50% FBS + 40% DMEM-F12 + 10% dimethyl sulfoxide (DMSO) or a commercially available primary cell freezing medium.

NOTE: Adherent mouse stromal cells may also be recovered from the tissue plate surface, if desired, by rinsing briefly with culture medium and expanding by standard means.

2.12. For later use of cryopreserved PDX tumor cells/mouse stroma, thaw cells in a 37 °C water bath for 2 min. Count and plate in a 6-well tissue culture plate as described in step 2.10 before proceeding to section 3. Increase the number of PDX cells by ~20% to accommodate viability losses from cryopreservation.

3. Density gradient centrifugation-based separation of PDX-derived clusters from single cells

3.1. Prepare 20 mL of 100% density gradient solution by thoroughly mixing 18 mL of density gradient centrifugation solution with 2 mL of sterile 10x Hanks' balanced salt solution (HBSS) in a sterile 50 mL conical tube. Make 10 mL each of 20%, 30%, 40%, and 55% density gradient solutions by diluting this 100% solution with sterile 1x HBSS and mixing well.

NOTE: These volumes are sufficient for two 15 mL gradients which can be used to separate ~15 x 10^6 cells each. If separating fewer than ~15 x 10^6 cells, the second gradient should be used as a balance for centrifugation.

3.2. Add 3 mL of 55% density gradient solution to the bottom of a 15 mL conical tube. Holding the tube at an angle, very gently layer 3 mL of 40% density gradient solution on top of the 55% layer, slowly dispensing the liquid onto the angled side of the tube to avoid mixing the layers. Repeat with the 30% density gradient solution.

3.3. Collect the supernatant of PDX rotation cultures with a 5 mL serological pipette, rinsing plate surface gently. Centrifuge at 200 x *g* for 2 min to pellet cells.

3.4. Remove supernatant and resuspend the cell pellet in 3 mL of 20% density gradient solution according to number of gradients needed to separate the cells. Carefully layer 20% density gradient solution with cells onto the top of the gradient(s). If only using one gradient tube for cells, top the balance gradient tube with cell-free 20% density gradient solution.

3.5. Cap the tubes and centrifuge in a swing bucket rotor centrifuge for 30 min at 4 °C, 2,000 x *g*, and 0 brake.

3.6. After centrifugation, fractions will be visible (**Figure 2C**). Collect 2−3 mL of fractions into fresh 15 mL tubes. Add 3−4 volumes of sterile 1x HBSS to each fraction and invert to mix thoroughly.

3.7. Centrifuge at 1,000 x *g* for 3 min to pellet cells. Remove supernatant and resuspend the cell pellet in 1−2 mL of PDX processing medium.

NOTE: Viable PDX cell clusters are typically found at the 40−55% density gradient solution interface (**Figure 2D**) with single dead/dying cells accumulating at the 20−40% density gradient solution interface for most PDXs tested.

3.8. Remove a small, representative aliquot (50–100 µL) for re-dissociation with an equal volume of dissociation enzyme solution to assess cell number in the clustered cell suspension. Count cells with a hemocytometer or automated cell counter.

4. Hydrogel preparation and microfluidic plate seeding

4.1. Reconstitute HA hydrogel solutions (thiol-modified HA, HA-SH; thiol-reactive polyethylene glycol diacrylate, PEGDA) according to the manufacturer's instructions.

4.2. Using a multichannel pipette, add 50 µL of HBSS to all wells in observation window columns (column 3, 7, 11, 15, 19, 23) of a 2-lane microfluidic plate to maintain culture humidity and optimal imaging conditions.

4.3. Calculate the volume of cell suspension from section 3 needed for 50 µL of hydrogel at the desired cell density (i.e., 5,000 cells/µL). For seeding one microfluidic plate, aliquot the calculated volume into each of 4 sterile 1.5 mL centrifuge tubes.

NOTE: HA hydrogels have a fixed time to gelation. Adjust the volume of gel solution aliquots for user efficiency at dispensing if premature gelation occurs.

4.4. Adjust the pH of the HA-SH solution to 8.0 with 1 N NaOH immediately prior to use. Perform a test gelation by mixing 40 µL of HA-SH with 10 µL of PEGDA and monitoring gelation over time. Gelation typically begins 5–8 min after mixing HA-SH with the PEGDA crosslinker.

4.5. Centrifuge cell suspension aliquots for 2 min (200 x *g*, room temperature) to pellet cells. Carefully remove the supernatant and resuspend cells in the appropriate volume of HA-SH.

NOTE: Final hydrogel is a 4:1 HA-SH:PEGDA solution (by volume) so cells should be resuspended in 40 µL of HA-SH for a 50 µL final volume.

4.6. Add 10 µL of PEGDA to one aliquot of cells in HA. Mix well and wait 1–3 min (depending on gelation time from step 4.4) before seeding the microfluidic plate.

NOTE: Allowing the gelation reaction of start before seeding helps to minimize cell settling.

4.7. Affix a tip for dispensing 1.5 µL volumes to a single channel repeating pipette and load with cells in HA hydrogel solution. Remember to keep the hydrogel aliquot well-mixed to ensure even cell distribution.

4.8. To seed the microfluidic plate, align pipette tip perpendicular to the plate while gently placing the tip in the center of the gel inlet (columns 1, 5, 9, 13, 17, 21) to ensure contact but no pressure when dispensing the hydrogel solution. Working quickly to prevent premature hydrogel solidification, dispense 1.5 µL of gel solution into each gel inlet.

4.9. Observe the fill status of the microfluidic channels by viewing from the top of the plate, bottom of the plate, or by microscope, and assess loading, using **Figure 3** as a guide (successful loading in **Figure 3A**, pipet positioning guidance in **Figure 3B**, missed loading in **Figure 3C**, not filled to end in **Figure 3D**, overflow in **Figure 3E**). Identify any necessary adjustments in technique that may improve filling success for the next round of chips (see discussion for troubleshooting tips).

4.10. Repeat steps 4.6–4.9 with the remaining 3 aliquots of cells in HA solution. Invert the plate while preparing the next aliquot (~1 min).

NOTE: The 1 min wait time and inversion of the plate improve the 3D distribution of the cells by reducing cell settling as gelation occurs.

4.11. After all chips are filled, incubate the plate at 37 °C in a humidified incubator until gelation is complete (~45 min).

4.12. Using the manual provided, ensure the perfusion rocker is installed in the cell culture incubator with the correct perfusion settings (14° angle, 4 min intervals).

4.13. Add 50 µL of PDX culture medium to all medium inlets (columns 2, 6, 10, 14, 18, 22) and check if the channels filled properly by flipping the plate. Gently tap the plate against a surface to encourage the liquid to fill the microfluidic channels.

4.14. Add 50 µL of DMEM-F12 (10% FBS) for all medium outlets (column 4, 8, 12, 16, 20, 24). If any air bubbles are trapped in the perfusion channel, remove by gently tapping the plate against a surface.

4.15. Using a microscope and plate layout form (**Supplemental Figure 1**), record chip filling success. Exclude improperly filled chips from further experimental use.

4.16. Place plate on a tilting rocker set to a 14° tilt and a 4 min cycle to begin perfusion. Replace PDX culture medium every 2 days (first 50 µL in inlet, then 50 µL in outlet).

5. Cell staining, imaging, and image quantification

5.1. Prepare a cell viability assay solution containing three fluorescent dyes (Hoechst 33342, ethidium homodimer-1, calcein acetoxymethyl [AM]).

5.1.1. Prepare stock solutions of each dye as follows: Hoechst 33342 at 1.6 mM (1 mg/mL) in deionized water; calcein AM at 4 mM in anhydrous DMSO; ethidium homodimer-1 at 2 mM in DMSO/H_2O (1:4, v/v).

NOTE: The stock calcein AM and ethidium homodimer-1 solutions are provided in the noted kit in **Table of Materials**.

5.1.2. Prepare a single working solution in HBSS or phenol red-free medium, containing all three dyes. Optimize final working concentration for each cell type and matrix, within the ranges of 1.6−8.0 µM for Hoechst 33342, 0.1−10 µM for calcein AM, and 0.1−10 µM for ethidium homodimer-1.

5.2. Remove culture medium and apply the working viability dye solution to desired microfluidic chips (75 µL in inlet, 25 µL in outlet) and place back on the perfusion rocker in the cell culture incubator for 1 h.

5.3. Image the observation windows of the stained cultures using a manual or automated confocal microscope (**Table of Materials**) with fluorescent filters (listed as excitation/emission wavelengths, in nm) to observe all nuclei (Hoechst 33342, 350/461), dead cell nuclei (ethidium homodimer-1, 528/617), and live cell cytoplasm (calcein AM, 494/517).

5.4. Capture 140 µm Z-stacks with a step size of ≤1 µm using a 20x air objective. Three fields of view are needed to image the entire microchannel with a small amount of overlap. To avoid double sampling, image only two fields of view per chip.

NOTE: Imaging conditions should be optimized to ensure proper NyQuist sampling. In authors' experience, a fast imaging system, based either on deconvolution of a conventional epi-fluorescence source or resonance scanning mode on a confocal, is necessary to fully assay a complete Z-stack, with three laser colors, across 96 chips on a plate within a reasonable amount of time (roughly 3.5 h with automated imaging, including setup).

5.5. Using image analysis software, assay the Z-stack images for the desired quantified data, such as morphology, aggregation state, or other features. To quantify cell viability, count the number of dead cells (red) and total cell nuclei (blue).

REPRESENTATIVE RESULTS:

A programmable perfusion rocker was prepared in a standard water-jacked cell culture incubator, and two-lane microfluidic plates were prepared in a standard biosafety cabinet for loading (**Figure 1**). An MDA-PCA-118b PDX tumor was expanded in vivo, harvested when it had reached a maximum size, and dissociated as described in protocol section 2 to create a slurry suspension of cells, at approximately a single-cell state (**Figure 2A**). The slurry was dispensed into 6-well tissue culture plates, and placed on an XY rotator as described, for 48 h. Mouse stroma attached to the bottom of the tissue culture plate, as has been previously described, and the human PDX cells remained floating in the medium, assembled into clusters (**Figure 2B**). Unassembled single cells were visible throughout the solution as well.

A step density gradient was established in a centrifuge tube using the methods in protocol section 3. The supernatant suspension of PDX cells was aspirated gently from the multiwell plate, loaded onto the step gradient, and centrifuged as described. After centrifugation, a hazy band, containing the PDX clusters, was visible near the interface between fractions 2 and 3, and

single cells were identified at the interface between fractions 1 and 2 (**Figure 2D**). Fractions were collected as described, diluted further in HBSS, and centrifuged again to remove the density gradient solution. The supernatant was aspirated, and the resultant cell pellets were resuspended in PDX processing medium. A small aliquot of each processed fraction was assessed by microscope, to confirm that the expected fraction contained PDX cluster, and that the separate fraction contained primarily single cells.

HA hydrogel solutions were reconstituted as described in protocol section 4, and PDX clusters were resuspended in the hydrogel solution. PDX solutions were loaded into chips on the microfluidic plate and assessed for successful loading (**Figure 3**). In most cases, >90% of chips were loaded successfully after some practice with the technique and adjustment of loading volumes. After loading the desired number of chips, and incubating for gelation as described, PDX culture medium was added to the microfluidic plate, and the plate was cultured with perfusion.

Using fluorescent dyes and the methods in protocol section 5, and confocal microscopy, 3D microfluidic PDX culture viability and morphology were evaluated in both unseparated and density gradient centrifugation separated conditions (**Figure 4A**). Images of these plates were recorded as Z-stacks on the confocal microscope and assessed for cell viability in image analysis software. On day 1, those cultures which underwent the separation method exhibited 10-fold fewer single, dead cells (red), compared to unseparated cultures (**Figure 4B**). Importantly, the separated clusters primarily consisted of live cells (green). No statistically significant difference was identified for the cluster size distribution (**Figure 4C**).

Cultures were further maintained in the microfluidic plate for seven days (**Figure 5A**). Samples were assessed periodically, using the same imaging and quantification methods as above. The total number of live cells remained consistent (**Figure 5B**) and clusters retained ~80% viability (**Figure 5C**) over the life of the culture. The cell density in the unseparated condition is roughly one-third of the separated condition because single cells are alive during cell counting, but quickly die within the hydrogel. There is also more variability in cluster size without the density gradient separation.

FIGURE AND TABLE LEGENDS:

Figure 1: The 2-lane perfusable microfluidic plate. (**A**) The 2-lane microfluidic plate is a standard 384-well microtiter plate with a modified bottom consisting of microfluidic channels embedded between glass plates. Each plate consists of 96 tissue chips for 3D cell culture. (**B**) As viewed from the top of the plate, a single 2-lane microfluidic chip is made up of 4 wells in a row connected by the gel channel (red) and perfusion channel (blue); the gel inlet, perfusion inlet, observation window, and perfusion outlet. (**C**) Perfusion is achieved in the microfluidic plate with a programmable rocker which uses gravity to drive media between perfusion inlet and outlet wells which are media reservoirs.

Figure 2: PDX cluster isolation. (**A**) Dissect primary PDX tissue and dissociate into a single cell

suspension containing both human PCa cells and mouse tumor stroma. Either cryopreserve dissociated cell mixture or (**B**) add to rotation culture to recluster tumor cells and allow mouse stromal cells to adhere to the tissue culture plate (48 h). (**C**) Use density gradient centrifugation to remove residual dead single cells, and (**D**) collect viable PDX clusters at the interface between density gradient layers 40% and 55% (red dashed box). (**E**) Recover PDX clusters, resuspend in hydrogel precursor solution, and dispense onto the plate with a repeating pipette. (**F**) Example calculations, corresponding to **Table 1**, demonstrate the necessary initial cell numbers required to reach a final desired cell concentration for all chips across a plate.

Figure 3: Monitoring microfluidic plate loading. Loading success and errors, can be assessed by eye (in top and bottom views), with confirmation by microscope. (**A**) Successful loading is identified by an open (bright) perfusion lane in the top and bottom views, and a slightly darker gel lane in bottom view. Visualization by microscope confirms that the gel lane was filled completely with cells and gel precursor, without spilling over into the adjacent lane. (**B**) Cartoon demonstrating correct pipet tip placement during gel dispensing, with correct placement being just above the inlet port (left) and incorrect placement being off-centered (middle) or applying force to the inlet port (right). (**C**) Bright lanes in all views indicate a missed loading, suggesting that the pipet tip was not correctly placed within the gel inlet. (**D**) A partial fill of the gel lane (not filled to end) is identified by the red arrow, which shows where the advancing gel solution front was interrupted, either by a trapped air bubble, or a premature pause in loading. The red arrow in the microscope view identifies the same location. (**E**) The contents of the gel lane overflowed the PhaseGuide, spilling into the perfusion lane and ultimately blocking it. This overflow is visible in bottom view by the dark appearance of both gel and perfusion lanes. Scale bar = 200 μm.

Figure 4: Cell staining and viability assessment in the microfluidic plate. (**A**) Using fluorescent stains, viability was assessed in unseparated and separated PCa cell clusters seeded in the microfluidic plate (all nuclei: Hoechst 33342, blue; dead cells: ethidium homodimer-1, red; and live cells: calcein AM, green). Scale bar = 50 μm. (**B**) Total dead cells were quantitated on day 1 of the culture and separated MDA-PCa-118b PDX cultures demonstrated a significant decrease in the number of dead cells. (**C**) Quantification of cluster size for PCa PDXs, in separated and unseparated cultures, showed a slight increase but no statistically significant difference. Bars and error bars in panel B represent the mean and standard deviation of 4 images per condition (2 chips with 2 image/chip). Asterisk (*) represents statistically significant differences ($p < 0.05$) compared to the unseparated condition using a student's t-test. For the box and whisker plot in panel C, the cross icon indicates the mean, and the horizontal line represents the median, of 4 images per condition (2 chips with 2 image/chip). The box contains 50% of the data while the whiskers extend to the minimum and maximum cluster diameter for each condition.

Figure 5: Characterization of separated MDA-PCa-118b in the microfluidic plate. (**A**) Separated (left) and unseparated (right) PCa cancer cell clusters were seeded in the microfluidic plate and perfused for up to seven days. Cultures were stained with three dyes specific for all nuclei (Hoechst 33342, blue), dead cells (ethidium homodimer-1, red), and live cells (calcein AM, green). Scale bar = 50 μm. (**B,C**) Quantitation of number of cells and culture viability from

images over one week of culture at a seeding density of 8,000 cells/µL. Bars and error bars represent the mean and standard deviation of four images per condition.

Table 1: Approximate initial and final PDX material required to load a single 2-lane microfluidic plate.

Supplemental Figure 1: The 2-lane microfluidic plate layout. After seeding the microfluidic plate, record individual chip loading success (successful, missed loading, not filled to end, or overflow) using the 2-lane plate layout.

DISCUSSION:

Here we describe a method for processing and culturing viable PDX-derived tumor cells in a high-throughput, perfused 3D culture system. While this protocol utilizes PCa PDX tissue, it is equally effective for other epithelial-derived 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 while others are more cellular. The tumor size noted here can be varied within IACUC guidelines to provide more tissue for particularly low-yield tumors. Additionally, tumor fragments can be collected after step 2.7, re-digested, and pooled with the initial digestion to increase yield. This is especially helpful for more extracellular matrix-heavy tumors. Additional rounds of digestion past two typically result in low viability and low yield and are not recommended.

The purification of PDX-derived populations, to remove dead/dying cells or contaminating host cells, is beneficial for the extended 3D culture and quantification of the desired cancer cells. The first step of the purification described here—suspension culture in a relatively large volume of media along with XY rotation at moderate speeds—promotes 1) the selective extraction of highly adhesion-dependent cells, typically mouse stromal fibroblasts, and 2) the formation of cell aggregates to supply pro-survival cell-cell contact. The efficiency of extracting host-derived mouse fibroblasts during the 48 h XY rotation step is very high, as described in Fong et al.[5]. Intentional PCa-fibroblast co-cultures are certainly feasible, as we have demonstrated, but require a tuned matrix (to support fibroblast adhesion to matrix) and a larger proportion of fibroblasts. The occasional PDX, usually those with more mesenchymal characteristics, will adhere more readily to tissue culture surfaces during the 48-hour rotation culture step. PDX lines should be closely monitored the first time this protocol is performed, and immunostained for HNA, to identify the proportion of human cells in the adherent and non-adherent populations. Non-tissue culture treated plates should be used for cluster formation with these PDXs. Mouse stromal cells will still adhere eventually to the surface, but separation will not be as efficient.

Following rotation culture, the supernatant containing the non-adherent population is processed through the described density gradient centrifugation method. The protocol reported here can be simplified to exclude at least one density step (e.g., using only the 20%, 40%, and 55% solutions), but all four should be used when establishing this method with each new PDX. Multicellular clusters are easily separated from individual cells by this method, and

largely retain their clustered phenotype and other characteristics. The discarded single-cell population represents either (a) cells that were dead/dying prior to the 48 h rotation step, and thus never integrated into a cluster, or (b) cells that remained alive after the 48 h rotation, but still did not integrate into clusters. It is important to note that group (b) may include cells that some investigators find desirable; e.g., some reports have described primary cell cultures containing early stem/progenitor cells, or drug-resistant cells, which float within supernatant as poorly-adherent single cells above an otherwise adherent population[10]. Relevant to cancer studies, circulating tumor cells (CTCs), tumor initiating cells (TICs), or other similar metastasis-promoting cell types could be part of this single cell population. Therefore, investigators using our protocol should analyze carefully the discarded single cell population to ensure that any cells of interest have not been lost. In our hands, the vast majority of these single cells are either a small fraction of cancer cells that are dead/dying due to the initial tissue dissociation protocol, or rodent fibroblasts that are already proceeding through anoikis.

It is strongly recommended to practice the technique of seeding the microfluidic plate with less precious cells to ensure success when working with limited and valuable PDX material. Be aware of tip placement during dispensing, as improper technique is likely to result in 1) overflow if pressure is applied during loading, or 2) empty or partially filled channels if the tip is not centered over the inlet port. Premature gelation will also cause channels to not fill to the end. If this occurs, decrease the wait time between addition of the PEGDA crosslinker and dispensing the gel solution, or work with smaller aliquots of gel solution at a time. The dispensing volumes used in this protocol are well-optimized for success with HA hydrogels. Use of more viscous solutions such as Matrigel in this system requires an increase in the dispensing volume to ~2 µL for proper filling of the microfluidic channels. Note also that the choice of 50 µL increments in step 4.3, the preparation of four cell pellets in this same step, and the repeat resuspensions in step 4.10 are deliberate; although they yield more material than necessary, they also provide overage to account for losses from the repeating pipette.

It should be noted that each PDX is likely to have a unique morphology and size distribution within this 3D culture model. In one sense, this is expected, due to the diversity of patient disease, tissue history, matrix parameters, and many other factors. An expansive assessment of multiple prostate cancer lines in Matrigel demonstrated this potential diversity[11]. Still, investigators may be surprised to find a wide variation in the above parameters. In a manuscript in preparation, we present a broad look at several PDXs on this culture platform; the cells maintain a consistent response within each PDX type, but can vary substantially across specimens, resulting in clusters that are, for example, large with high cell density per cluster, or smaller, with looser intercellular connections. The specific behavior of each PDX should be assessed by investigators over multiple trials, to confirm a predictable and characteristic morphology. Investigators can expect specimens to follow the general behaviors outlined in this paper.

In summary, the presented protocol offers researchers a new method for employing PDXs ex vivo in a platform that allows for the fine tuning of culture conditions and incorporation of relevant 3D environmental cues such as tissue specific extracellular matrix (ECM) and perfusion

flow, enabling extended cell survival over several days or weeks. Detailed characterization of these cultures was achieved by the staining and morphological analyses demonstrated here. In addition, if needed, cultures can be submitted to plate reader-based assays, immunofluorescent labeling, or used for the preparation of lysates enabling further molecular characterization. Although we have published previously some elements of PDX 3D culture within hydrogels, this application of multistep purification is new, easy to employ in a standard laboratory, and successful in retaining high-viability representative cultures. The OrganoPlate platform, in its 2-lane and 3-lane varieties, offers further complexity through its model of tissue perfusion, and a key opportunity to use even fewer cells per experiment. Compared to our previous models, the microfluidic platform reduced cell requirements by a factor of ~30, per experiment, which is an enormous benefit, given the scarce availability of PDX tumor tissue in most laboratories. The microfluidic system enables extended imaging, immunostaining, and facile imaging, across a cell population that is large enough (n = ~2,000−4,000 cells per imaging window) to permit quantification of phenotype within the context of spatial organization.

ACKNOWLEDGMENTS:

This work was supported by National Institutes of Health National Cancer Institute SBIR Phase I (HHSN26120700015C) and P01CA098912.

DISCLOSURES:

The authors have nothing to disclose.

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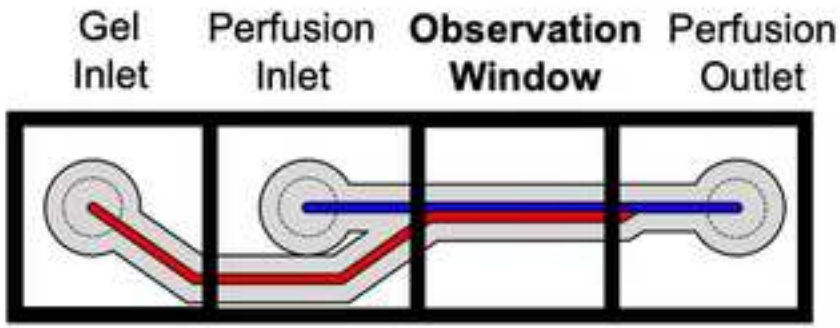
Figure 1

(A) 2-lane Microfluidic Plate

(C) Programmable Perfusion Rocker

(B) Single 2-lane Microfluidic Tissue Chip



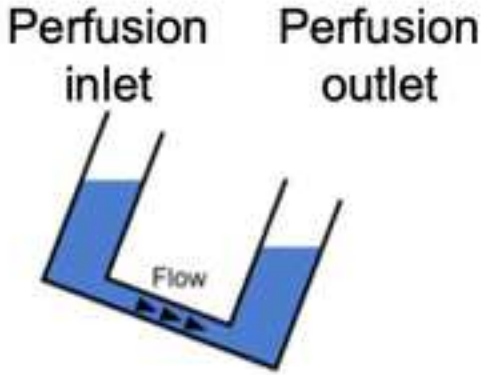
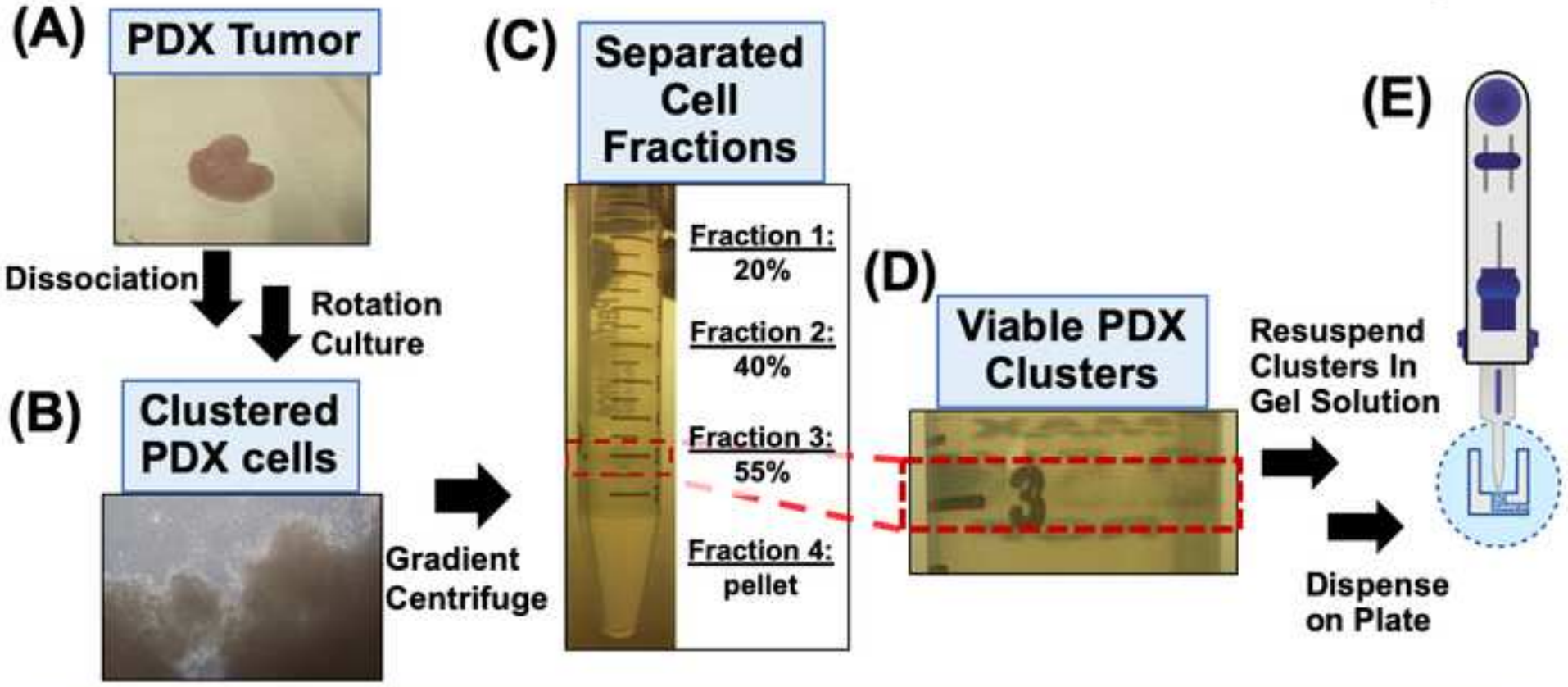


Figure 2



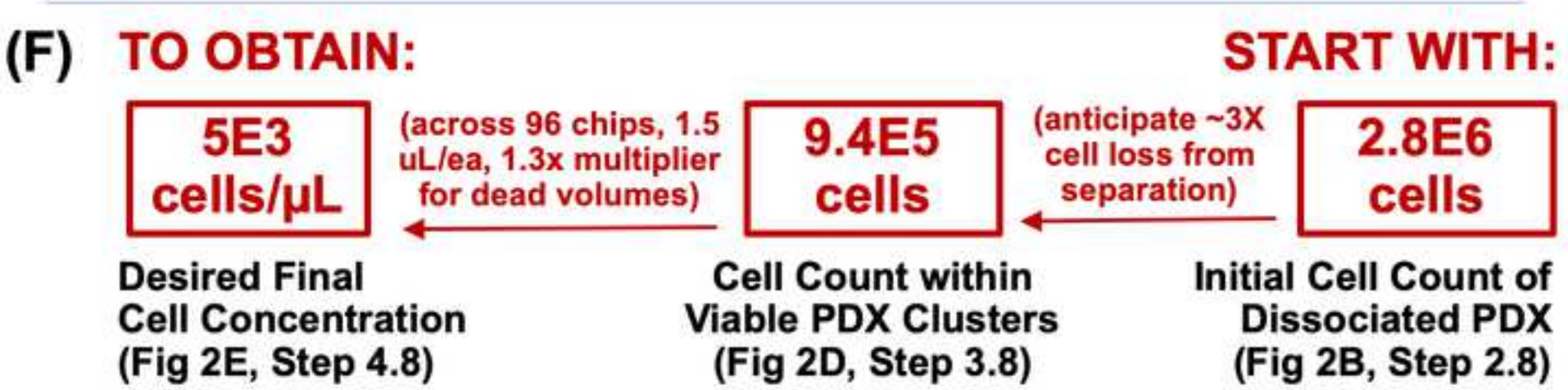


Figure 3

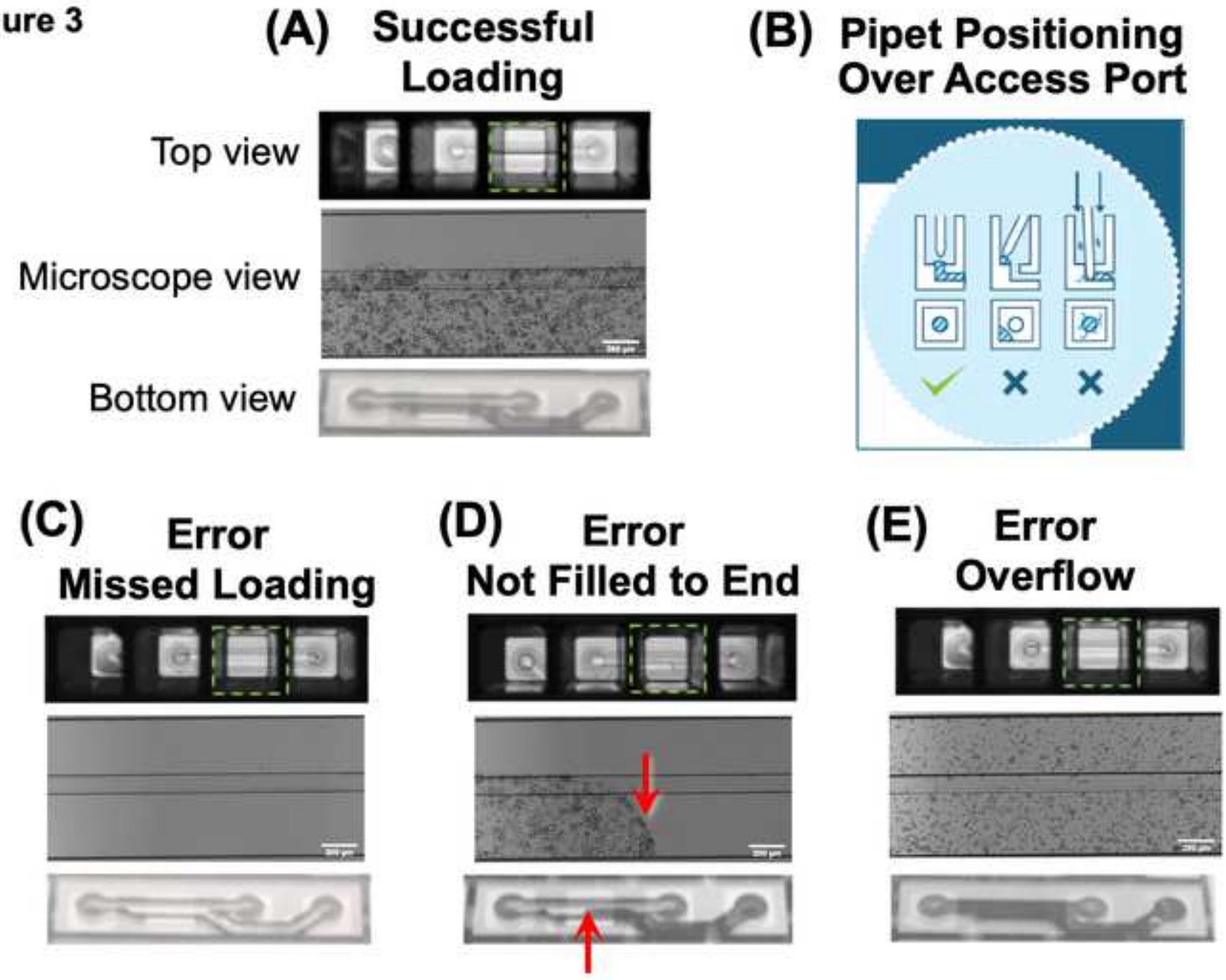


Figure 4

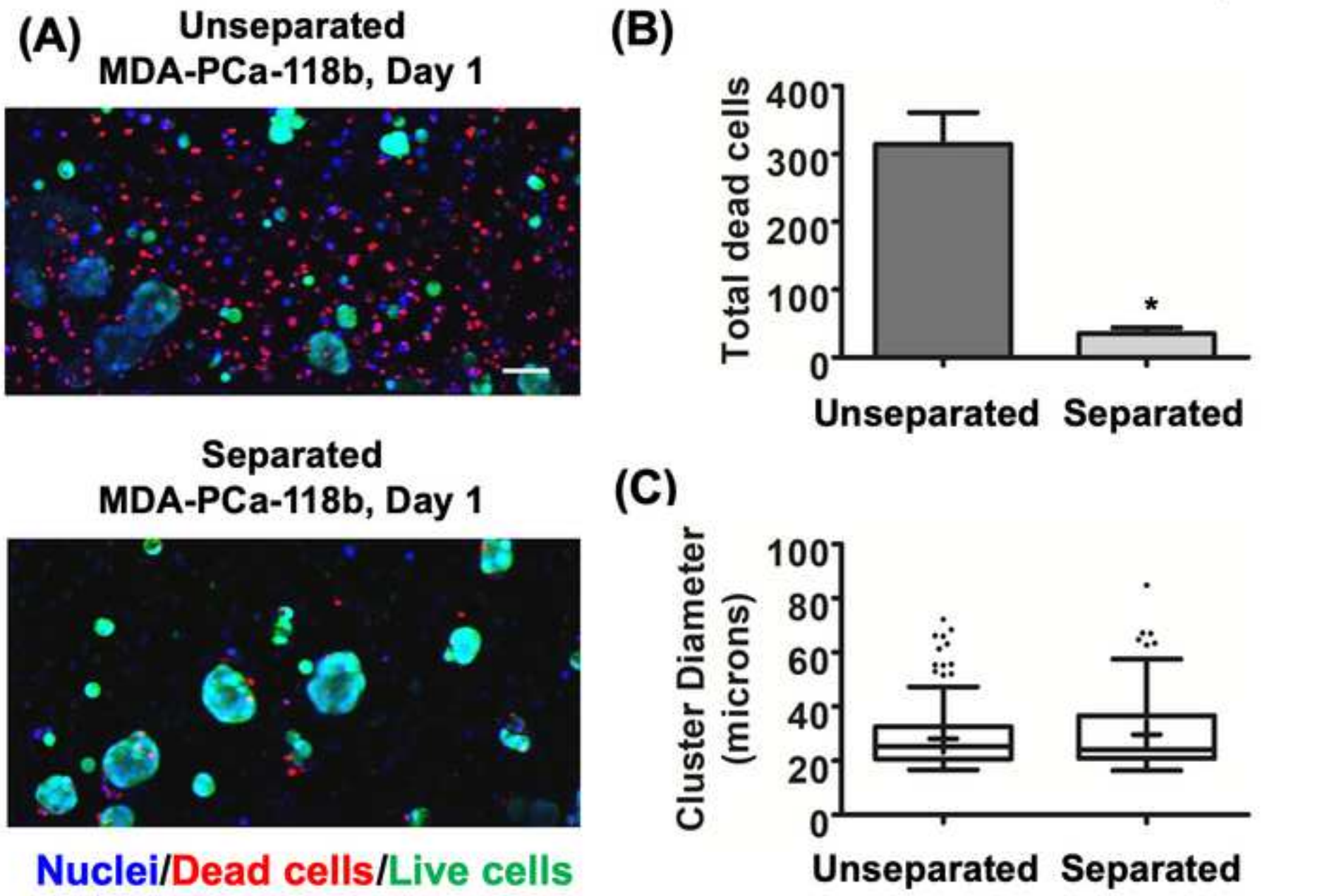


Figure 5

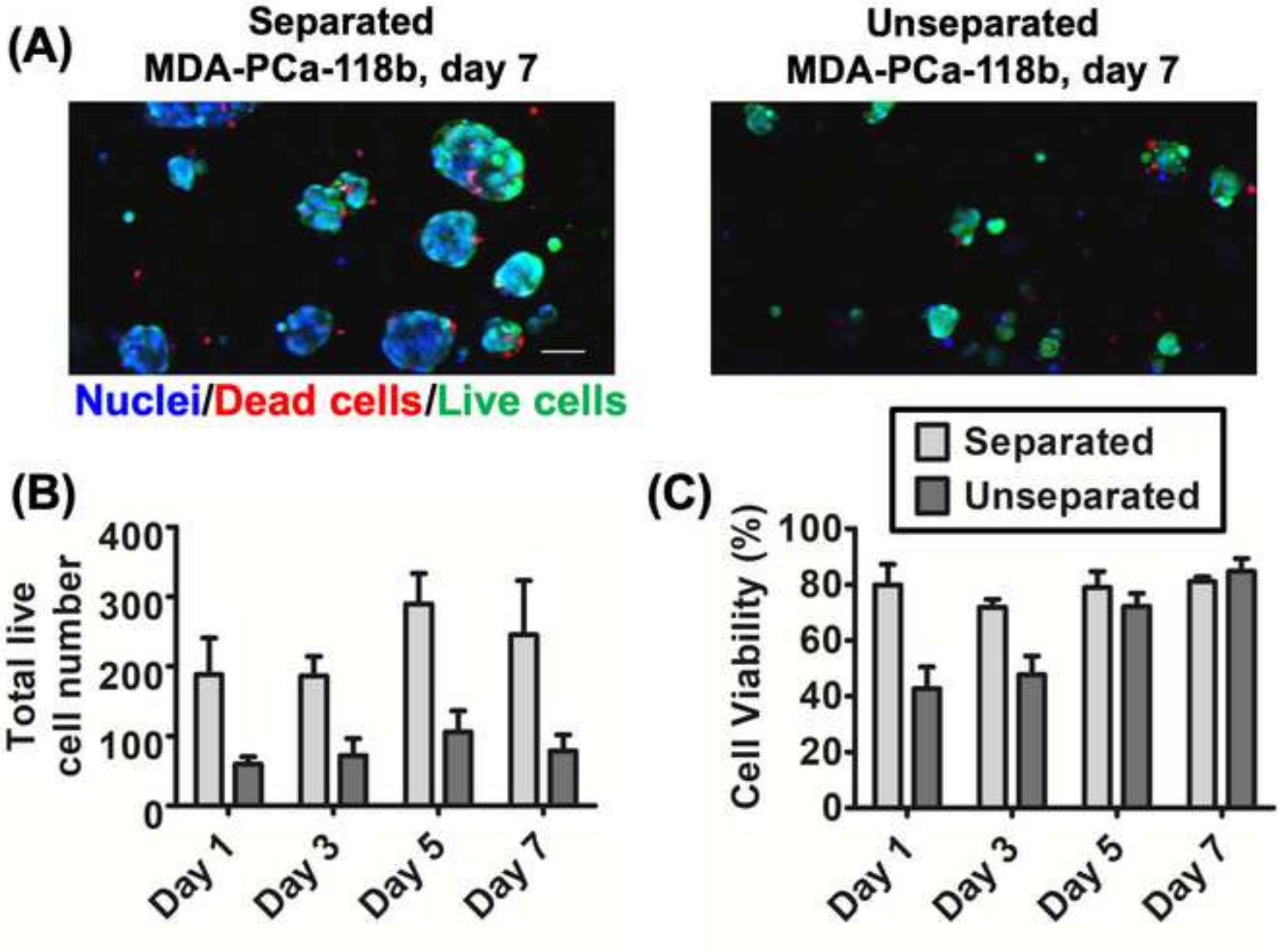


Table 1

Final desired cell density in single chip (cells/µL)	Volume loaded per chip (µL)	Number of chips on microfluidic plate	Mutiplier for extra volume	Figure 2C: Number of separated cells required for a full microwell plate (step 3.8)	Multiplier for cell losses (stroma removal, cell death)	Figure 2B: Starting # of dissociated PDX cells for protocol (step 2.8)
2,500	1.5	96	1.3	4.7E+05	3	1.4E+06
5,000	1.5	96	1.3	9.4E+05	3	2.8E+06
10,000	1.5	96	1.3	1.9E+06	3	5.7E+06
20,000	1.5	96	1.3	3.7E+06	3	1.1E+07

Name of Material/Equipment	Company	Catalog Number	Comments/Description
1N NaOH			any suitable tissue culture grade
60 mm round tissue culture dishes			any suitable
6-well tissue culture plates			any suitable
70 µm cell strainers	Corning	431751	or equivalent
Centrifuge	Eppendorf	5810R with suitable rotor and buckets for 15/50 mL conical centrifuge tubes	or equivalent
Density gradient centrifugation solution	Millipore Sigma	P1644	Percoll
Dimethylsulfoxide			any suitable tissue culture grade
Dissociation enzyme solution	StemCell Technologies	07921	ACCUMAX
DMEM-F12	ThermoFisher Scientific	11039021	or equivalent
Forceps			any suitable
HA hydrogel kit	ESI BIO	GS311	HyStem (Hyaluronic acid-SH and PEGDA)
Hanks Balanced Salt Solution	Lonza	10-527F	or equivalent
Heat-inactivated fetal bovine serum	Atlanta Biologicals	S11150	
Hemocytometer	Fisher Scientific	02-671-51B Hausser BrightLine	or equivalent
Hoechst 33342	ThermoFisher Scientific	H1398	or equivalent
Image processing software	Oxford Instruments	Imaris 9.3	or equivalent
LIVE/DEAD Cell Viability/Cytotoxicity Kit (Calcein-AM/Ethidium Homodimer-1)	ThermoFisher Scientific	L3224	or equivalent
Microfluidic culture plate	Mimetas	9603-400-B	2-lane OrganoPlate
Microscope	Nikon	A1R	or equivalent

Multichannel pipette	Eppendorf	3125000036	or equivalent
PDX-derived tumor tissue			obtained under IRB approval for human tissue and IACUC approval for animal host
Penicillin-streptomycin	ThermoFisher Scientific	15140-122	or equivalent
Perfusion rocker	Mimetas	OrganoPlate Perfusion Rocker Mini	
pH strips (pH 5-9)			any suitable
Phosphate-buffered saline solution	Lonza	17-516F	or equivalent
Razor blades			any suitable
Rotating xy-shaker	VWR	Advanced 3500 Orbital Shaker	or equivalent
Scalpel handle			any suitable
Single channel repeating pipette	Eppendorf	22260201	
Sterile, 15mL conical centrifuge tubes			any suitable
Sterile, 50mL conical centrifuge tubes			any suitable

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.

Supplemental Figure 1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplemental Figure 1.pdf

Serial plate number

Experiment title/description	

Cells, ECM, etc.	Supplier	Catalog nr.	Lot nr.	Other notes

Rocker serial #:	Incubator #:	Using a microscope, navigate to the observation windows of each chip (observation window locations in gray below) and record OrganoPlate loading: ✓ = successful loading M = missed loading NTE = not filled to end O = overflow

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
	1				2				3				4				5				6			
A	A03				A07				A11				A15				A19				A23			
B	B03				B07				B11				B15				B19				B23			
C	C03				C07				C11				C15				C19				C23			
D	D03				D07				D11				D15				D19				D23			
E	E03				E07				E11				E15				E19				E23			
F	F03				F07				F11				F15				F19				F23			
G	G03				G07				G11				G15				G19				G23			
H	H03				H07				H11				H15				H19				H23			
I	I03				I07				I11				I15				I19				I23			
J	J03				J07				J11				J15				J19				J23			
K	K03				K07				K11				K15				K19				K23			
L	L03				L07				L11				L15				L19				L23			
M	M03				M07				M11				M15				M19				M23			
N	N03				N07				N11				N15				N19				N23			
O	O03				O07				O11				O15				O19				O23			
P	P03				P07				P11				P15				P19				P23			

Notes: _____

Terminated on: _____ ☐ Fixated ☐ Discarded

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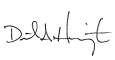
Signature:	
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