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TITLE:

Handling and Assessment of Human Primary Prostate Organoid Culture

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SUMMARY:

Here, we present a protocol to guide human primary prostate organoid handling then suggest endpoints to assess phenotype. Seeding, culture maintenance, recovery from matrix gel, morphologic quantification, embedding and sectioning, FFPE sectioning, whole-mount staining, and application of commercial assays are described.

ABSTRACT:

This paper describes a detailed protocol for three-dimensional (3D) culturing, handling, and evaluation of human primary prostate organoids. The process involves seeding of epithelial cells sparsely in a 3D matrix gel on a 96-well microplate with media changes to cultivate expansion into organoids. Morphology is then assessed by whole-well capturing of z-stack images. Compression of z-stacks creates a single in-focus image from which organoids are measured to quantify a variety of outputs, including circularity, roundness, and area. DNA, RNA, and protein can be collected from organoids recovered from the matrix gel. Cell populations of interest can be assessed by organoid dissociation and flow cytometry. Formalin-fixation-paraffin-embedding (FFPE) followed by sectioning is used for the histological assessment and antibody staining. Whole-mount immunofluorescent staining preserves organoid morphology and facilitates observation of protein localization in organoids *in situ*. Commercial assays that are traditionally used for 2D monolayer cells can be modified for 3D organoids. Used together, the techniques in

45 this protocol provide a robust toolbox to quantify prostate organoid growth, morphologic
46 characteristics, and expression of differentiation markers.

48 **INTRODUCTION:**

49 Organoids are a valuable tool to study organogenesis and disease. They provide a less expensive
50 alternative to animal models, and patient-derived organoids are evolving as a strategy for
51 personalized medicine¹⁻³. This three-dimensional (3D) culture system involves seeding stem or
52 progenitor cells (harvested from tissue or induced pluripotent stem cells) into a gel of
53 extracellular matrix components (matrix gel)⁴. The cells proliferate and differentiate, resulting in
54 organotypic structures that recapitulate the cellular hierarchy and morphology of the organ of
55 interest's functional unit. Organoids have been grown using cells originating from a variety of
56 organs, including the salivary gland, stomach, intestine, liver, prostate, lung, and brain⁴. Although
57 there are numerous protocols describing steps to establish prostate organoids^{5,6}, it is challenging
58 to find sufficiently detailed methods on how to achieve quantitative endpoints from organoids.
59 This paper summarizes methods developed for human primary prostate cells and details a series
60 of suggested endpoints to assess the organoid phenotypes. These techniques were optimized for
61 prostate organoids and may be applicable to other 3D cell cultures.

63 Prostate organoid cultures have recently emerged as a valuable *in vitro* model that lacks the
64 limitations of monolayer cultures using established immortalized cell lines. Benign prostate
65 epithelium and prostate cancer is challenging to model *in vitro* using immortalized cell lines. The
66 number of benign cell lines is limited, and all have undergone transformation with oncogenes⁷.
67 Primary prostate epithelial cells in monolayers do not differentiate into luminal cells and lack
68 androgen receptors⁸. The majority of prostate cancer cell lines do not have functional androgen
69 receptors, a significant mediator of early disease state, and lack key genetic changes that have
70 been found in patient tumors⁹. Prostate organoid cultures can be grown easily from benign
71 epithelium and are valuable in studying stem cell properties, progenitor cells, differentiation, and
72 effects of experimental changes in the microenvironment^{4,5}. Prostate cancer organoids can be
73 grown as part of a precision medicine approach to modeling patient disease and response to
74 therapies¹⁰.

76 This protocol has been compiled from existing protocols that use various cell types, but it has
77 been optimized here for use in human primary prostate cells. It compliments protocols outlined
78 by Sawyers, Clevers, and Shen^{5,6} that describe growth, passaging, bright-field imaging,
79 cryopreservation, and RNA and DNA isolation of prostate mouse and human organoids. The
80 whole-mount protocol is modified from Mahé *et al.*¹¹, who used gastrointestinal epithelial
81 organoids and describes live imaging and frozen and paraffin embedding. Borten *et al.*¹²
82 described the analysis of breast, colon, and colorectal cancer organoid morphology from bright-
83 field imaging. Additionally, Richards *et al.*¹³ used a method for the morphologic assessment of
84 prostate organoids. Finally, Hu *et al.*¹⁴ described a method of adhering prostate organoids to a
85 chamber slide overnight prior to the immunofluorescent staining to observe single cells and
86 dispersion of spheres for flow cytometry analysis.

The goal of this protocol is to demonstrate in sufficient detail these technically challenging methods as one protocol, including cell seeding; media and matrix gel maintenance; collection of cells for flow cytometry; RNA, DNA, and protein extraction; morphologic assessment from bright-field z-stack analysis; embedding, processing and sectioning for histological staining; and whole-mounting for immunofluorescence or fluorescent probe assays. The biological relevance and interpretation of these various endpoints will vary among experimental design and antibodies used for analysis. Through utilization of this protocol, users should feel prepared to set up an experiment with a toolkit of endpoints.

Human primary prostate epithelial (PrE) cells were established in the lab by the protocol described by Peehl^{15,16} and maintained to a limited number of passages (up to four) as previously described¹⁷, but they are also available from commercial vendors. Hepatocyte serum-free media-based⁶, KSFM-based¹³, and R-spondin 1-conditioned⁵ media are all published as having successfully produced organoids. KSFM-based requires the fewest number of additives, so it is described here.

PROTOCOL:

The following protocol was optimized using human primary prostate epithelial cells harvested from patient tissue at the University of Illinois at Chicago Hospital. All human tissues used for these experiments were acquired *via* an Institutional Review Board-approved protocol and/or exemption at the University of Illinois at Chicago. While the culture conditions will vary depending on the cell type of interest, the endpoints can be applied to organoids of other tissues.

1. Seeding of Epithelial Cells into Matrix Gel and Changing Media

1.1 Collect necessary materials: human primary prostate epithelial cells (PrE), matrix gel on ice, 96-well microplate, keratinocyte serum-free media (KSFM) on ice, charcoal stripped fetal bovine serum (FBS), and dihydrotestosterone (DHT).

1.2 Prepare KSFM-based organoid media by combining 47.5 mL of KSFM with 2.5 mL of charcoal stripped FBS and dihydrotestosterone (DHT) to a final concentration of 10 nM DHT, then reserve on ice.

1.3 Plate cells into a 3D matrix in a cell culture hood using sterile technique.

1.3.1 Gently mix cold KSFM-based organoid media with ice-cold matrix gel to obtain a 50% matrix gel mixture by slowly pipetting up and down, avoiding bubbles.

NOTE: Matrix gel should be thawed overnight at 4 °C and kept on ice whenever possible. It solidifies quickly at room temperature (RT).

1.3.2 Pre-wet a pipette tip in cold media and transfer 40–50 µL of 50% matrix gel onto the bottom of each well to be used on a 96-well plate. Coat the bottom of the well to form a base layer.

NOTE: Do not fully dispense to the second stop on pipette, as this can create bubbles.

1.3.3 Place the 96-well plate into 37 °C incubator for 30–45 min to solidify the base layer.

NOTE: Do not plate epithelial cells onto the base layer until it has solidified, or cells may fall through the matrix onto the bottom of the plate and grow as a monolayer.

1.3.4 Mix epithelial cells suspended in KSFM-based organoid media with matrix gel to achieve a final concentration of 100–1000 cells/100 µL and 33% matrix gel. Plate epithelial cells on top of base layers using 100 µL of 33% matrix gel/cell mixture per well.

NOTE: Previous experiments have shown that seeding epithelial cells too densely in 3D will restrict the size of organoid growth, while seeding too sparsely can result in very low yield¹³. It is important to optimize seeding conditions for the cell type of interest, based on the patient-specific organoid formation efficiency.

1.4 Place the 96-well plate in a 37 °C incubator for 30–45 min to allow the matrix gel to solidify, then gently add 100 µL of KSFM-based organoid media on top of cells by pipetting slowly against the edge of the well.

1.5 Change the media every 2–3 days. Pipetting against the side of the well with space between the media and matrix gel, carefully remove 50 µL of media and replace with 50 µL of fresh media.

NOTE: For a long-term culture, the matrix gel needs to be changed every 2–3 weeks. If the matrix gel culture appears unstable and displays translucent wrinkles under the microscope, then the matrix gel has broken down and needs to be replaced.

2. Collection of Organoids from Matrix Gel

NOTE: Collection of organoids is necessary for matrix gel changes, passaging or endpoints of RNA extraction, DNA extraction, protein extraction, and flow cytometry.

2.1 Warm neutral protease and Hanks' balanced salt solution (HBSS) in a 37 °C water bath.

2.2 Carefully remove media from wells without disrupting the matrix gel.

2.3 Add ~200 µL of neutral protease to each well at a ~1:2 ratio of matrix gel:neutral protease and incubate for 20 min at 37 °C.

2.4 Mechanically dissociate the matrix gel:neutral protease mixture by pipetting up and down.

2.5 Incubate for 20 min at 37 °C.

2.6 Transfer the neutral protease-matrix gel-cell mixture to a microcentrifuge tube or conical tube, depending on the volume. Centrifuge at 300 x *g* for 3–5 min to pellet cells.

NOTE: If no cell pellet appears, the matrix gel may not be completely dissociated and can be visualized as a translucent phase at the bottom of the tube distinct from the pink supernatant. Remove the supernatant and add more neutral protease at a 1:2 ratio to matrix the gel pellet, incubate for another 20–40 min at 37 °C, and repeat centrifugation at 300 x *g*.

2.7 When an organoid pellet is acquired, remove the supernatant. Proceed with passaging (see step 2.7.1), organoid lysis for RNA, DNA, or protein extraction (see step 2.7.2), processing single cells by flow cytometry, and single-cell sequencing (see step 2.7.3).

2.7.1 For long-term culture, gently resuspend intact organoids in 33% fresh matrix gel and plate by following steps described in steps 1.1–1.5. To dissociate organoids and replate as single cells, resuspend the pellet in 1 mL of warm cell-dissociation enzymes and incubate at 37 °C for 10–15 min, wash once with 5 mL of HBSS, and re-plate in the fresh matrix gel following steps 1.1–1.5.

2.7.2 Resuspend the organoid pellet in an appropriate lysis buffer for RNA extraction, DNA extraction, or protein extraction as listed in the **Table of Materials** and follow the manufacturer's protocol.

2.7.3 Resuspend organoids in 1 mL of warm cell-dissociation enzymes and incubate at 37 °C for 10–15 min to dissociate the organoids into single cells. Wash once with 5 mL of HBSS and proceed with the protocol for flow cytometry⁵ or single-cell sequencing¹⁸.

NOTE: To dissociate into single cells at low concentrations of matrix gel, the neutral protease may not be needed, and cell-dissociation enzymes can be applied directly to the well after step 2.2.

3. Whole-well Image Acquisition and Analysis of Organoid Morphology

3.1 Acquire whole-well z-stack images using a transmitted light inverted microscope with a motorized X/Y scanning stage (workflow illustrated in **Figure 1A**).

3.1.1 With the focal position adjusted to the bottom of the well, begin focusing upward until the first organoid is encountered, which will be in the center of the well due to the meniscus from the base layer. Set the bottom z-plane at this position.

3.1.3 Continue focusing upward until the last organoid is in focus, which will be in the periphery of the well. Set the top z-plane at this position.

NOTE: After setting the top and bottom of the z-stack, ensure that these positions capture the organoids within all remaining wells and adjust the top/bottom as needed. This allows for the z-range to be used for a single scan that includes every organoid within each well.

3.1.4 Capture 15–35 z-planes per well, using a larger number for greater resolution. Capture the entire well image, merge and collect quadrants or subsections if needed.

NOTE: It is recommended to take multiple z-planes as opposed to a single z-plane, as illustrated in **Figure 1B** in which organoids are out of focus when capturing only one z-plane.

3.1.5 Save z-stack images for downstream analysis.

3.2 Analyze images using image software with freeform drawing capabilities.

3.2.1 Open a single z-plane image set from step 3.1.4.

3.2.2 If needed, tile the images taken at each individual z-plane into a single, whole-well image. Save the new image as a separate file. Repeat this process for each z-plane acquired.

3.2.3 Using image software, open the whole-well image for every z-plane from a single well and create an extended depth of field (EDF) image from the stack of z-planes.

NOTE: This type of image will select the best focus region of each z-plane to create a single in-focus image of the well.

3.2.4 Set the pixel scale of the software to the scale bar of the image that is being measured.

3.2.5 Using the freeform drawing tool of the image software, trace the perimeter of every organoid in the well.

3.2.6 Obtain the measurement metrics for traced organoid perimeters from the image software.

NOTE: Recommended parameters are area, circularity, and maximum/minimum radius ratio. Representative results illustrating the application of these metrics are shown in **Figure 1C**. Area is calculated from the polygon defined by the organoid's perimeter. Circularity is the ratio of the area of the organoid to the area of a circle with a diameter equal the organoid's maximum ferret, where 0 is less circular and 1 is more circular. Maximum/minimum radius ratio is the ratio between the maximum radius and minimum radius of the traced organoid.

$$\text{Circularity} = \frac{(4)(\text{Area})}{\pi (\text{MaxFerret}^2)}$$

$$\text{Radius ratio} = \frac{\text{Maximum radius of traced polygon}}{\text{Minimum radius of traced polygon}}$$

4. Formalin Fixation and Paraffin Embedding of Organoids for Histological Endpoints

4.1 Prepare embedding supplies: HBSS, 2% agar solution, histological marking dye, histology gel, pipette tip mold, tape, plunger from a 1 cc insulin syringe, ice pack, cassettes, 10% neutral buffered formalin (NBF), pencil, and 75% histological grade ethanol (EtOH).

4.1.1 Prepare two microcentrifuge tubes of 2% agar solution. Incubate in a water bath or on a heat block at 100–110 °C to maintain agar in liquid form. Add 1–2 drops of histologic marking dye to one of the 2% agar solutions, which will denote the top of the agar plug and assist in maintaining orientation during embedding. Mix well.

4.1.2 Warm the histology gel in a water bath or heat block at 55–65 °C.

4.1.3 Using a 1000 µL pipette tip, create molds by cutting out a small section of the pipette tip to make a cylinder that holds ~50 µL. Create a second, wider mold that fits around the first mold.

4.1.4 Create a cold block by wrapping an ice pack with tape (adhesive side up) and adhering molds to the tape.

4.1.5 Create a plunger by removing the plunger from a 1 cc insulin syringe.

4.2 Embed the organoids in a histology gel plug for processing, following the workflow depicted in **Figure 2A**.

4.2.1 Dissociate the matrix gel and pellet organoids by following steps 2.1–2.7.

4.2.2 Wash the organoid pellet once with 1 mL of HBSS and re-pellet by spinning for 3–5 min at 300 x g and removing the supernatant.

4.2.3 Re-suspend the organoid pellet in 30–50 µL of liquid histology gel. Mix gently by pipetting up and down slowly. Transfer the histology gel/organoid mixture into a mold on the cold block and allow the specimen to cool and solidify into a plug.

4.2.4 Use a plunger to push the plug out of the small mold and into the larger mold. Pipette clear 2% agar around the plug to fill the larger mold.

4.2.5 Pipette histologic dye-tinted 2% agar on top of the plug to denote the top of the sample.

4.2.6 Once cooled, use a plunger to transfer the plug into a histology cassette. Label the cassette using a pencil and place cassette into 10% NBF for fixation overnight.

4.2.7 Transfer the cassette from 10% NBF to 70% histological grade EtOH.

4.3 Process the histology gel plug using a pre-set biopsy protocol on a processor, if available. If preset is not available, input the following sequence and lengths: 70% EtOH for 6 min, 70% EtOH for 10 min, 80% EtOH for 10 min, 95% EtOH 2 times for 10 min, 100% EtOH 3 times for 10 min,

305 xylene 3 times for 15 min, and paraffin 3 times for 15 min. Deviating from the recommended
306 processing sequence can result in ruptured organoids (**Figure 3A**).

307
308 4.4 Embed the histology gel plug with the colored portion facing up, as this will allow the
309 discolored portion containing the organoids to be sectioned into first.

310 4.5 Section the FFPE histology gel blocks:

311
312
313 4.5.1 Collect necessary supplies: FFPE histology gel blocks, histology pen, tissue float water bath,
314 ice bucket with ice, microtome, microtome blades, embedding workstation, positively charged
315 microscope slides, and laboratory oven.

316
317 4.5.2 Fill water bath until very full and set to 40 °C, then pre-warm laboratory oven to 45–50 °C.

318
319 4.5.3 Prepare an ice bucket, then fill with ice and add water to create an ice-water slurry. Chill
320 the blocks on ice until they are very cold.

321
322 NOTE: Blocks can be set on ice for up to 1 day, but if left overnight, the blocks will become
323 hydrated and will need to be reprocessed.

324
325 4.5.4 Insert a block into microtome cassette clamp, add the blade to the knife holder, and unlock
326 any protective locking measures on the machine.

327
328 4.5.5 Begin by trimming the face of the paraffin block (facing off) at a 10 µm thickness. Once a
329 section emerges that contains the plug area, stop trimming, lock the microtome rotor, and
330 transfer the block back onto the ice to chill. If several blocks are to be sectioned, face-off each
331 block before moving to step 4.5.6.

332
333 4.5.6 Move the blade to a new, unused portion and transfer the block back to the clamp. Unlock
334 the machine and begin trimming at 5 µm per section.

335
336 NOTE: 5 µm is recommended for best results, but 3–10 µm is also acceptable.

337
338 4.5.7 Using tweezers, transfer the section to the 40 °C water bath and allow the section to spread
339 out. Transfer the section onto a slide by dipping vertically into the water.

340
341 NOTE: Dipping at an angle can transfer bubbles from the bottom of the bath to the section and
342 cause distortion of the sample.

343
344 4.5.8 Visualize the section under microscope (**Figure 2B,C**).

345
346 NOTE: If an organoid present, it will appear similar to the red arrow in **Figure 2B**. Bubbles (**Figure**
347 **2B**, blue arrow) can appear similar to organoids and are easier to discern on a fresh-slide as dry

organoids appear translucent (**Figure 2C**). If no organoids are present, section further into the block.

4.6 When slides containing organoids have been acquired, circle the area around the section on the back of slide with a histology pen and bake overnight at 45–50 °C.

4.9 Collect slides and proceed to H&E (see step 4.9.1), immunohistochemical (see step 4.9.2), or immunofluorescent staining (see step 5).

NOTE: Representative results are shown in **Figure 3B**.

4.9.1 To perform H&E staining, obtain a kit from the materials list and follow the manufacturer's specifications.

4.9.2 To perform immunohistochemical staining, obtain a kit and primary antibody from the materials list and follow the manufacturer's specifications.

5. Immunofluorescent Staining of FFPE and Sectioned Organoids

5.1 Collect supplies: baked slide from step 4, xylene, 100% EtOH, 95% EtOH, 70% EtOH, deionized water (DI H₂O), coplin jars, 1x antigen retrieval solution (sodium citrate buffer or EDTA-Tris buffer), phosphate-buffered saline (PBS), 5% normal horse serum (NHS) with 0.1% non-ionic detergent in PBS, 1% bovine serum albumin (BSA) with 0.3% non-ionic detergent in PBS, staining rack, staining dish, decloaking chamber, hydrophobic barrier pen, humidity chamber, 1° and 2° antibodies of interest, and counterstains of interest.

5.2 Deparaffinize and rehydrate the slides by dipping into coplins filled with the following solutions: xylene (3 dips, 5 min each), 100% EtOH (2 dips, 3 min each), 95% EtOH (2 dips, 3 min each), 70% EtOH (2 dips, 3 min each), and DI H₂O (2 dips, 3 min each).

5.3 Fill the staining dish with antigen retrieval solution and pre-heat decloaking chamber to 100 °C.

5.3 Perform antigen retrieval by immersing slides into the pre-heated staining dish and incubate for 5–10 min at 100°C. Once the cycle is complete, allow the decloaking chamber to sit for 20 min before opening the lid.

5.4 Once the slide dish has cooled to RT, place the staining dish under running DI H₂O to rinse the slides for 5 min.

5.5 Remove the slides from the staining dish, draw a circle around the sample with a hydrophobic barrier pen, and lay the slide on the humidity chamber.

5.6 Block any non-specific antibody staining by applying 5% NHS with 0.1% non-ionic detergent in PBS to the hydrophobic circle around the sample (about 30 μ L is needed to cover the sample).

5.7 Wash slides in coplins filled with PBS 3 times for 5 min each.

5.8 Add the 1° antibodies (**Table of Materials**) diluted in 1% BSA with 0.3% non-ionic detergent in PBS to the hydrophobic circle around the sample (about 30 μ L should cover the section), then incubate for 1 h at RT or overnight at 4 °C in humidity chamber.

5.9 Wash the slides in coplins filled with PBS 3 times for 5 min each.

5.10 Add the 2° antibody diluted in 1% BSA with 0.3% non-ionic detergent in PBS to the hydrophobic circle around the sample (about 30 μ L will cover the area), then incubate for 1 h at RT in humidity chamber.

5.11 Wash the slides in coplin filled with PBS 3 times for 5 min each.

5.12 Add DAPI, diluted to the manufacturer's specifications, in 1% BSA with 0.3% non-ionic detergent in PBS to the hydrophobic circle around the sample, then incubate for 2–5 min at RT in the dark (30 μ L).

5.13 Wash the slides in coplins filled with PBS 3 times for 5 min each.

5.14 Mount the slides with antifade mounting media and coverslip, then visualize the results under a microscope.

6. Whole-mounting of Organoids for Immunofluorescent Staining

6.1 Collect supplies: chamber slide or confocal dish, phosphate-buffered saline (PBS), fixative, 50 mM NH_4Cl in PBS, 0.1% non-ionic detergent in PBS, 5% NHS with 0.1% non-ionic detergent in PBS, 1% BSA with 0.3% non-ionic detergent in PBS, 1° and 2° antibodies of interest, and counterstains of interest.

6.2 Carefully remove as much media as possible without disrupting the organoid culture by pipetting against the side of well, leaving space between media and matrix gel.

6.3 Using a trimmed, pre-wet with media or PBS, 1000 μ L pipette tip, draw up one drop (25–50 μ L) of matrix gel that contains the organoid(s) of interest and dispense onto the middle of a chamber slide well or confocal dish.

NOTE: 33% matrix gel is not a solid. It is a gelatinous fluid that handles similar to a Jello that has not fully set. A trimmed pipette tip with a large opening will prevent organoids from breaking during transfers.

6.4 If organoids remain in the parent culture, replace media with warm KSFM-based media.

6.5 With the naked eye or dissecting scope, aspirate the excess media and matrix gel from the chamber slide with a fine-tip pipette (10–200 μ L).

NOTE: It is optional to pretreat the chamber slide with an adherence reagent.

6.6 Plate an equal volume of matrix gel into an empty well of the chamber slide as an optional control to observe autofluorescence of leftover matrix.

6.7 Place the chamber slide in a 37 °C incubator for 30–45 min to promote the organoid to adhere to the glass and prevent loss of samples during wash steps.

6.8 Pipetting slowly to prevent detachment from slide, wash organoids with 200 μ L of 1x PBS for 5 min at RT while gently shaking.

6.9 Remove the 1x PBS and fix with 200 μ L of 4% paraformaldehyde for 30 min at RT while gently shaking. Ensure that the matrix gel appears clear after this step.

NOTE: Methanol or formalin fixation are also possible. Fixation method should be optimized for specific antibodies as certain fixation methods may crosslink the antigens of interest or quench fluorescent-labeling-proteins of interest.

6.10 Remove the fixative and wash twice with 200 μ L of 1x PBS for 5 min while gently shaking.

NOTE: The length of washing steps should be optimized depending on organoid size. Five minutes is a sufficient starting point, but the washing step can be lengthened as needed.

6.11 Quench autofluorescence with 200 μ L of 50 mM NH_4Cl in 1x PBS for 30 min at RT while gently shaking.

NOTE: This step will quench autofluorescence from debris in the lumen of the organoid and from fluorescent protein expression (such as GFP) that may present from experimental design. It should be included if it is desired to use a fluorophore in the 488 channel but can be skipped if desired to preserve GFP signal.

6.12 Remove NH_4Cl and wash twice with 200 μ L of 1x PBS for 5 min while gently shaking.

6.13 Permeabilize organoids in 200 μ L of 0.1% non-ionic detergent-100 in 1x PBS for 30 min at RT while gently shaking.

6.14 Remove the 0.1% non-ionic detergent-100 in 1x PBS and wash twice with 200 μ L of 1x PBS for 5 min while gently shaking.

6.15 Block with 5% NHS with 200 μ L of 0.1% non-ionic detergent X-100 in PBS and incubate for 60 min at RT while gently shaking.

6.16 Remove the blocking buffer and wash twice with 200 μ L of 1x PBS for 5 min while gently shaking.

6.17 Dilute the 1° antibody in 1% BSA with 0.3% Triton X-100 in 1x PBS and add to 200 μ L of the chamber slide, then incubate for 1–3 days at 4 °C.

6.18 Remove the 1° antibody and wash twice with 200 μ L of 1x PBS for 5 min while gently shaking.

6.19 Dilute the 2° antibody in 1% BSA with 0.3% Triton X-100 in 1x PBS and add to 200 μ L of the chamber slide, then incubate for 1–3 days at 4 °C in the dark.

NOTE: The length of incubation times should be optimized for organoid size and antibody. Some will require more time to penetrate through the organoid. Antibody concentration will also need to be optimized. In general, 2x the concentration used for FFPE sections is appropriate for 1° antibodies and the same concentration for FFPE sections is appropriate for 2° antibodies.

6.20 Remove the 2° antibody and wash twice with 200 μ L of 1x PBS for 5 min while gently shaking.

6.21 Counterstain the actin with phalloidin and the nucleus with DAPI or Hoescht, diluted according to the manufacturer's recommendations in 1% BSA with 0.3% Triton-X in 1x PBS. Add 200 μ L to chamber slide, then incubate at RT for 40 min in the dark.

6.22 Mount in 200 μ L of fresh 1x PBS or mounting media and image immediately (fluorescence should be preserved for up to 5 days, if not longer). Representative results are shown in **Figure 3C**.

NOTE: Sodium azide can be added to samples to prevent contamination if confocal imaging is not readily available. Adding a clearing agent can improve imaging.

7. Whole-mounting of Organoids for Assays and Fluorescent Probes

NOTE: There are commercially available assays and fluorescent probes/dyes (examples are included on the materials list) amendable for use on whole-mounted organoids to observe a variety of useful endpoints¹⁹. The following protocol is for the fluorescently-labelled EdU proliferation kit, however any kit can be modified for use with a whole-mounted sample.

7.1 Carefully remove 50 μ L of media and replace with 50 μ L of 20 μ M 2x working EdU solution by pipetting against the side of well, leaving space between media and matrix gel.

7.2 Incubate at 4 °C for 1–3 days (the desired length of time for EdU incorporation should be optimized for cell type of choice).

7.3 Follow steps 6.2–6.8 to transfer the organoids of interest onto a chamber slide or confocal dish.

7.4 Remove PBS and fix with 200 μ L of 3.7% paraformaldehyde for 30 min at RT while gently shaking. Ensure that the matrix gel appear clear after this step.

7.5 Remove the fixative and wash twice with 200 μ L of 1x PBS for 5 min while gently shaking. Remove PBS and add 200 μ L of 0.5% non-ionic detergent-100 in 1x PBS for 30 min at RT while gently shaking.

7.7 Prepare a 1x fluorescent reaction cocktail according to the manufacturer's specifications.

7.8 Remove 0.5% non-ionic detergent-100 and wash twice with 200 μ L of 3% BSA in 1x PBS for 5 min while gently shaking.

7.9 Add reaction cocktail and incubate for 30 min at RT in the dark.

7.10 Remove the fluorescent reaction cocktail and wash once with 200 μ L of 3% BSA in 1x PBS for 5 min while gently shaking. Counterstain and mount by following steps 6.21–6.22 (**Figure 3D**).

REPRESENTATIVE RESULTS:

Upon successful culture of human primary prostate organoids, morphology and differentiation can be assessed using bright-field image analysis and FFPE and whole-mount staining techniques.

The process of bright-field image capture is illustrated in **Figure 1A**. Organoids are grown in a 3D matrix and dispersed across different focal planes. To observe organoids in focus across many planes, it is suggested to use an EDF image (**Figure 1B**, right) instead of a single z-plane (**Figure 1B**, left). In the lab, organoids grown under different experimental conditions may result in changes in morphology. Using area, circularity (defined by how similar the organoid is to a circle) and max/min radius (measure of length) give users an indication of organoid size and shape and provide a quantifiable readout of morphology. To emphasize the usefulness of these measures, representative images of organoids with similar area but differing morphology are shown in **Figure 1C**, in which a long organoid will be less circular and have a larger maximum/minimum ratio than a spherical organoid.

Embedding organoids for histology is a useful endpoint to observe the interior of samples and ensure that necrosis is not present within the core of an organoid. A workflow for this process and representative results of the unstained, sectioned samples under a bright-field microscope are provided in **Figure 2A,B,C**. **Figure 3A** shows a mishandled organoid (left) compared to properly handled organoids in **Figure 3A** (right) and **Figure 3B**. To determine the differentiation-state of the sample, it is recommended to look at basal cell markers (cytokeratin 5 and p63)⁸ along with luminal cell markers (cytokeratin 8)⁸. If it is desired to stain organoids *in situ*, whole-

mount staining is a convenient alternative, and these representative results are provided in **Figure 3C,D**.

FIGURE AND TABLE LEGENDS:

Figure 1: Morphology assessment of organoids in 3D culture. (A) Image collection and compression workflow for human primary prostate organoids acquired by a transmitted light inverted microscope with a motorized X/Y scanning stage and companion software. (B) Representative images of a single z-stack (left) vs. an EDF image (right) of a whole-well sample of human primary prostate organoids, showing that more organoids are in focus when multiple z-stacks are combined into a single projected image (scale bar = 1000 μ m). (C) Representative images for area, circularity, and max/min ratio of morphologically dissimilar human primary prostate organoids which have the same area (scale bar = 200 μ m).

Figure 2: Organoid embedding workflow and representative sectioning results. (A) Human primary prostate organoid embedding workflow. (B) Representative image of an unstained, fresh slide containing human primary prostate organoids under a bright field microscope depicting agar (green arrow), histology gel (black arrow), bubble (blue arrow), organoids (red arrows) (scale bar = 1000 μ m). (C) Unstained freshly cut slide (left) vs. unstained dry slide (right), organoids (red arrows) appear translucent when dry (scale bar = 500 μ m) and are harder to discern under a bright-field microscope.

Figure 3: Histological staining on sectioned and whole-mounted organoids. (A) H&E staining of a broken human primary prostate organoid from mishandling (aggressive pipetting, wrong processing protocol, left) next to a well-handled organoid (right) (scale bar = 100 μ m). (B) Human primary prostate organoid that has been formalin-fixed, paraffin-embedded, sectioned, and stained with basal cytokeratin 5 or luminal cytokeratin 8 (top) or basal p63 and luminal cytokeratin 8 (bottom) and imaged with confocal microscope (scale bar = 100 μ m). (C) A whole-mounted human primary prostate organoid stained with basal cytokeratin 5 or luminal cytokeratin 8 and counterstained with phalloidin and DAPI, imaged with confocal microscope (scale bar = 100 μ m). (D) A whole-mounted human primary prostate cell organoid stained with fluorescently labeled EdU and counter stained with Hoescht, imaged with confocal microscope (scale bar = 500 μ m)

DISCUSSION:

Organotypic culture is an exciting new method for recapitulating tissue with the convenience of an *in vitro* environment. Currently, labs grow organoids from many types of tissues for various endpoints. The methods described in this paper summarize useful endpoints and highlight new techniques to fully characterize 3D primary prostate cell cultures.

There are a variety of media recipes for cultivating human primary prostate cell organoids^{5,6,13}. While all achieve viable and comparable results, KSFM-based media¹³ uses minimal additives so it is described here. Additionally, papers have been published using multiple concentrations for

matrix gel, from 10% to 75% to culture prostate organoids^{5,6,13}. Because matrix gel is an expensive reagent, and 33% matrix gel has been shown to be sufficient to cultivate long-term (2–3 weeks), viable, unclumped organoids¹³, this is the recommended concentration. However, matrix gel can vary in protein concentration between lot numbers, so this should be taken into account when plating. It is also recommended to purchase matrix gel in bulk for use across multiple experiments to reduce inconsistency between lots. Other labs have published different formats for plating matrix gel, such as droplets instead of coating a 96-well plate well⁵. Both methods form viable organoids, but the format described here allows for cells to be plated more sparsely, which has been shown to promote expansion of cells into larger organoids¹³. However, plating density should be optimized based off patient-specific formation efficiency. Matrix gel is available in many formats including growth factor-reduced, phenol red-free, high concentration, *etc.* Growth factor-reduced is recommended for defined culture conditions, and phenol red-free is recommended when working with cells that express GFP or in experiments that involve z-stack image capture. It is critical that any matrix gel plating steps be performed with ice-cold reagents, as matrix gel solidifies quickly at room temperature.

Organoids grown under different experimental treatments may result in changes to shape, so bright-field imaging is widely used to study observed morphological phenotypes. Nevertheless, recording and quantifying area or shape is a challenge for two reasons: 1) selection bias during image capture and 2) applying a two-dimensional parameter such as area to a three-dimensional sample. One strategy of image capture is to record a random field and measure a predetermined number of organoids in that field, but this can create bias during selection, and all organoids in the selected field may not be in focus. Whole-well imaging optimized for the 96-well microplate format eliminates this sampling bias by collecting the entire organoid population of interest. However, depending on the microscope objectives on hand, multiple fields may need to be collected and tiled in order to obtain a whole-well image. Some labs have described measuring area from a single z-plane¹², but to capture all organoids in focus, it is recommended to collect and stack multiple planes into a single EDF-image¹³. In some cases, a meniscus in the matrix gel may cause vignetting in the image, and background correction methods may need to be applied using image analysis software. Exact volume is ideally the most appropriate measurement for organoid size; however, this is difficult to precisely obtain, even with multiple z-stack images. Other useful morphological dimensions, such as circularity and maximum/minimum ratio, can easily be calculated from all the organoids in a whole-well EDF image. Together, these methods overcome sampling bias challenges and enable measurement of 2D parameters from 3D objects in a 3D space.

Formalin fixation and paraffin embedding of organoids is a common method to obtain hematoxylin and eosin (H&E), immunohistochemical (IHC), and immunofluorescent (IF) staining for visualization and analysis^{6,11,20}. However, publications that have described this technique lack comprehensive details on the embedding process. Additionally, locating organoids within the paraffin block can be challenging. Some labs pre-stain organoids with trypan blue prior to embedding to aid in location during sectioning¹¹. The method described here incorporates the use of a histological dye for orientation of the organoid/histology gel plug during embedding to promote efficiency of sectioning. H&E slides facilitate examination of necrosis, nuclear texture,

and proliferation, and thus it should be a mandatory endpoint for organoid culture to ensure that cells are healthy throughout the sphere. A strength of organoid culture is that samples are comprised of a heterogeneous mixture of cells that better resemble *in vivo* patient tissue. In the prostate, for example, both basal and luminal cells are present in prostate organoids⁵, while cells grown in 2D lack luminal differentiation⁸. To assess organoid differentiation, it is recommended that researchers assess basal markers such as CK5 and p63 and luminal markers such as androgen receptors and CK8⁸. Any other experimental proteins of interest can also be studied using these staining techniques.

Although FFPE sections allow visualization of cells residing in the inner compartment *via* histologic methods (H&E, IF, IHC), images are limited to cross sections, and organoid shape may be altered by the embedding process. Whole-mount staining enables an organoid to be stained and observed *in situ*, preserving morphological phenotypes and permitting images of protein localization. Whole-mounting is a tool utilized to stain whole-tissue or whole-animal specimens, such as the zebrafish or mouse embryo, and is easily adapted for organoids. The technique described here was modified from the procedure by Mahé *et al.*¹¹, which details the initial growth and culture of gastrointestinal organoids directly on a chamber slide for staining, and requires fixation of the entire parent culture. The method outlined here involves the transfer of a single organoid (or organoids) of interest onto a chamber slide at the time of staining. This enables the selection of individual organoids for whole-mount analysis in an ongoing experiment without fixation of the parent culture. When performing whole-mount staining, it is necessary to optimize permeabilization and the incubation time for primary and secondary antibodies to ensure penetration throughout the specimen (anywhere from one or more days is recommended). Once imaged *via* confocal microscopy, 3D renderings can be produced to enable visualization and localization of a specific protein and calculate the number of positively stained cells present in a sample. Flow cytometry is a useful endpoint to quantify populations of basal, luminal, or stem cells^{5,14}. To do this, cells are recovered from the matrix gel and gently dissociated for staining. Users should carefully select appropriate markers for separation based on the current literature. For human primary prostate epithelial cells, CD26 and CD49f are suitable luminal and basal markers, respectively^{5,21}.

In summary, this protocol details human primary prostate organoid growth, collection, and experimental endpoints. Of note, the mounting technique described can be applied to a variety of other assays that are normally employed for 2D cells, such as fluorescent probe-based experiments looking at proliferation¹⁹, apoptosis, and subcellular organelle stains. Additionally, the collection and dissociation method described here could be utilized in preparation for single-cell RNA sequencing¹⁸. Collectively, this demonstrates the possibility for a variety of novel, high-fidelity endpoints that researchers can optimize and explore in the future.

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DISCLOSURES:

The authors have nothing to disclose.

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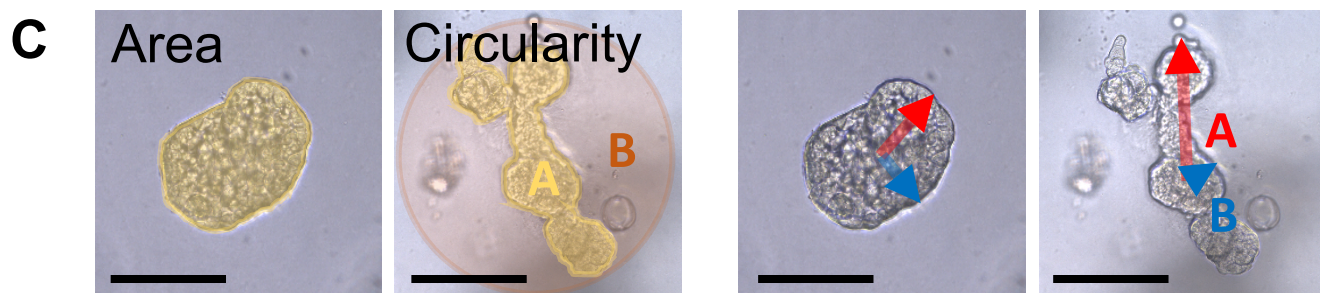
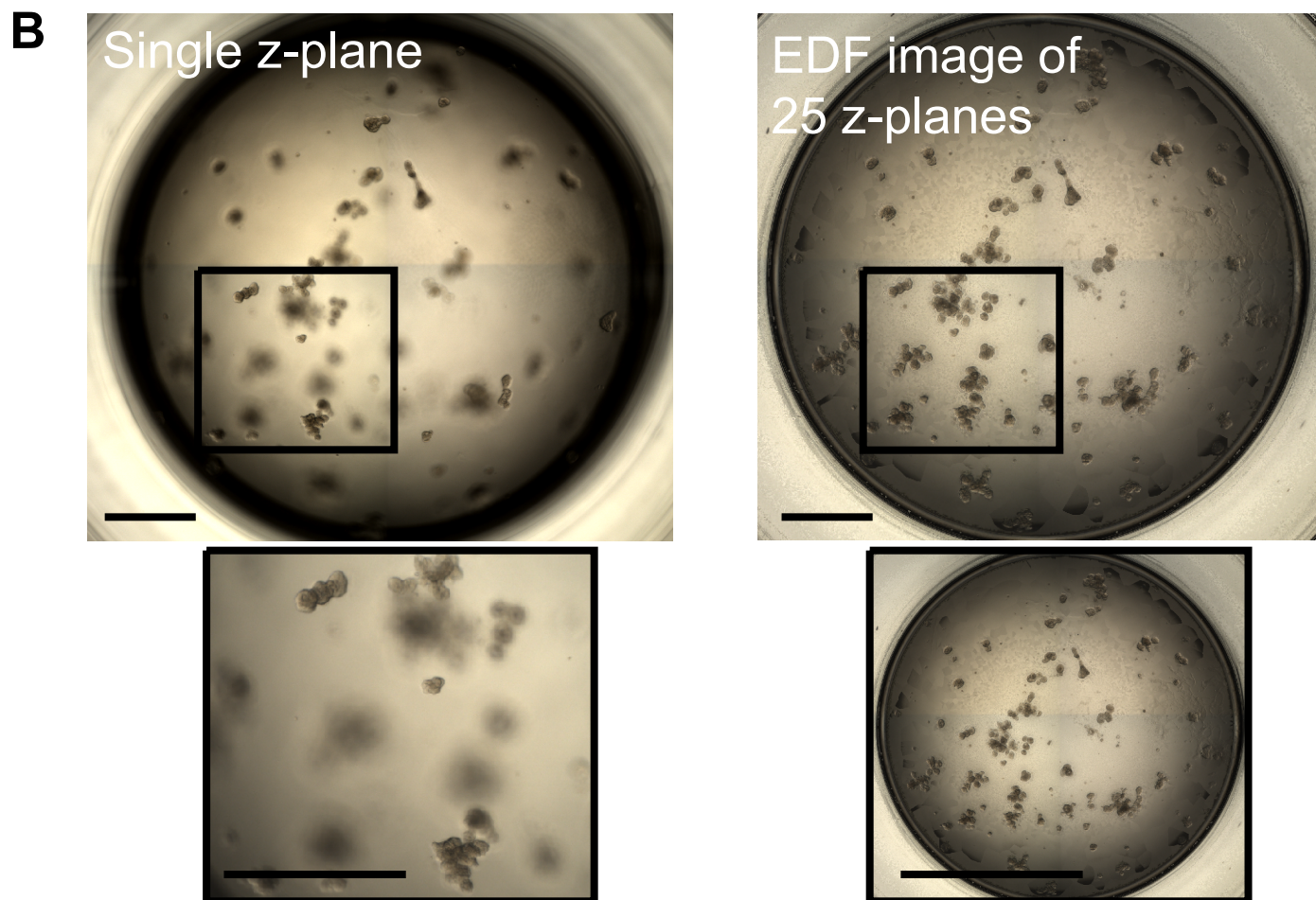
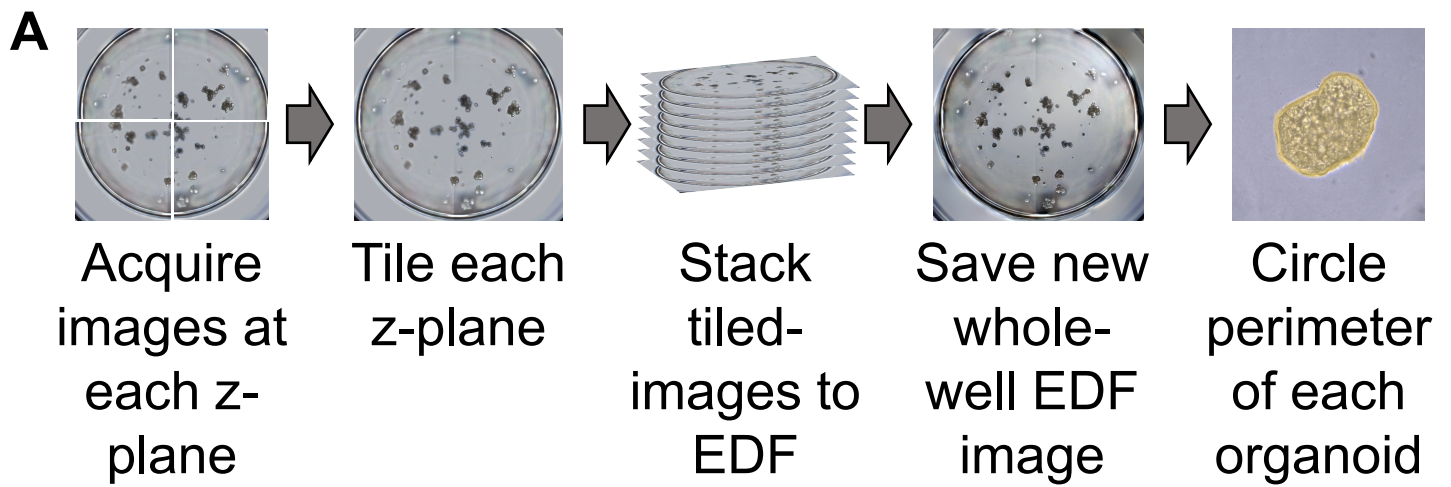
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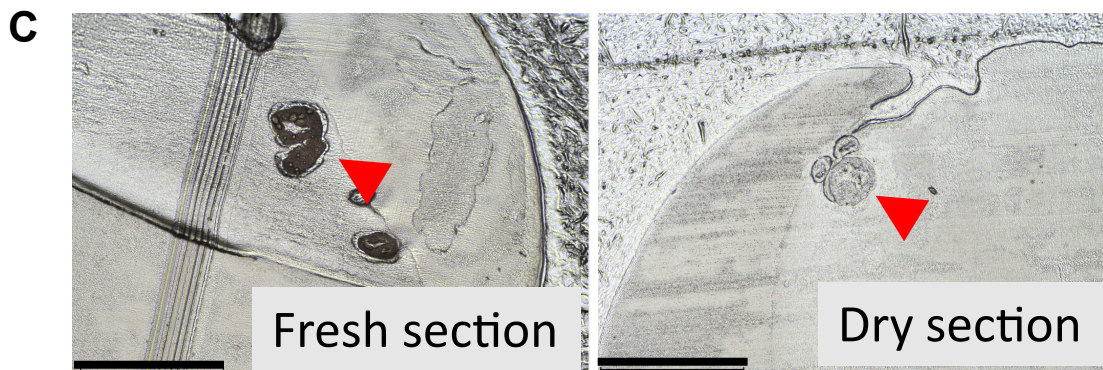
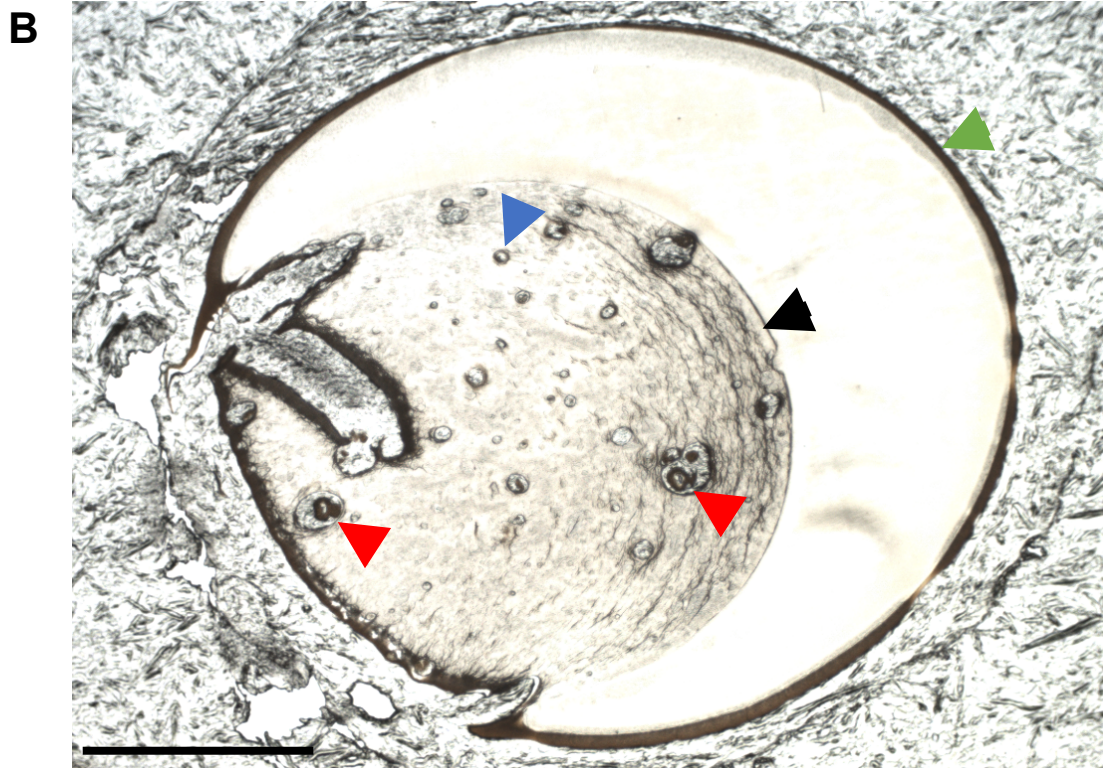
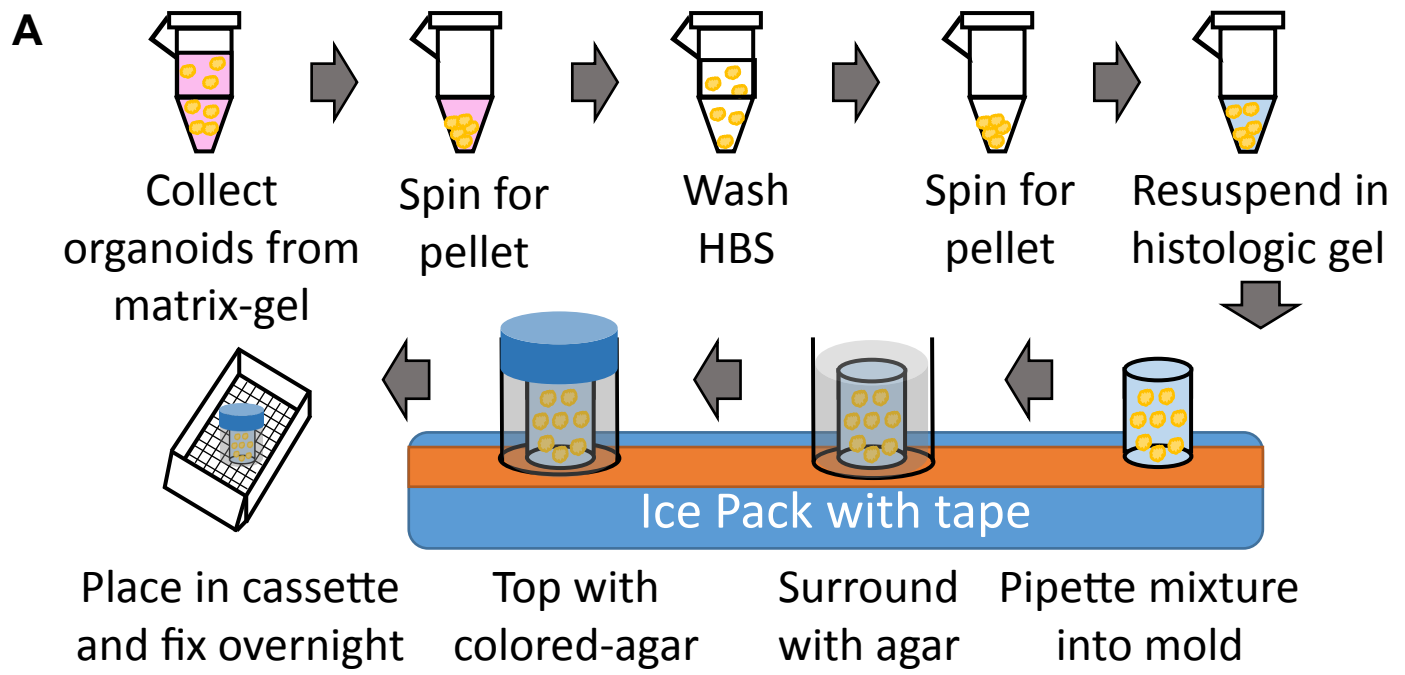
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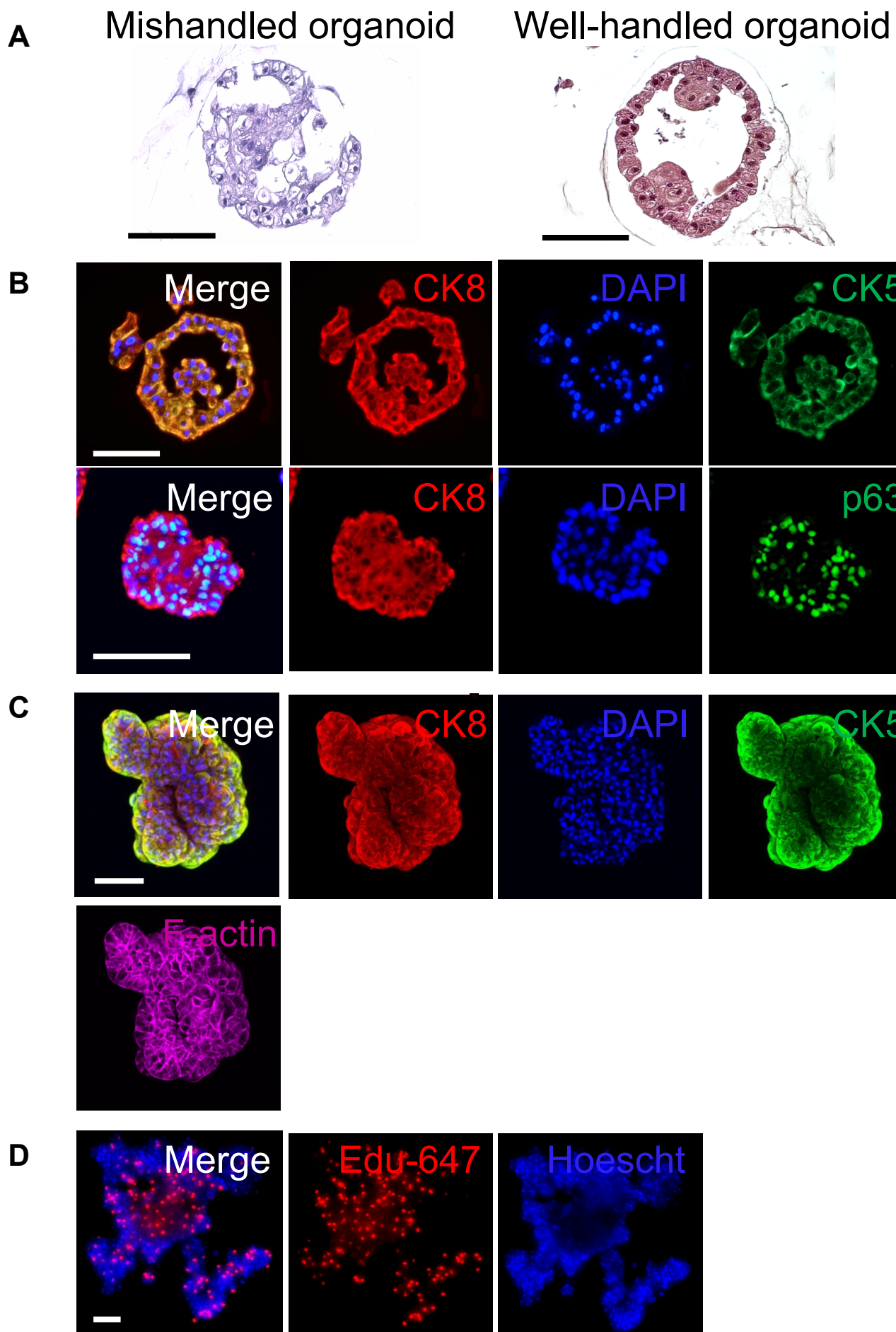
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$$\text{Circularity} = \frac{\text{Area A}}{\text{Area B}}$$

$$\text{Maximum/minimum radius ratio} = \frac{A}{B}$$





Name of Material/ Equipment	Company
Cells of interest	
Keratinocyte-SFM (KSFM)	ThermoFisher Scientific
Fetal Bovine Serum, charcoal stripped, USDA-approved regions (FBS)	ThermoFisher Scientific
5 α -Dihydrotestosterone (DHT)	Sigma-Aldrich
Flat bottom, polystyrene 96-well cell culture treated plate	ThermoFisher Scientific
Matrigel (matrix gel)	Corning
Ice Bucket	
Cell Culture Hood	
1.5 mL micro-centrifuge tubes or 15 mL conical	ThermoFisher Scientific
Centrifuge	
Dispase (neutral protease)	STEMCELL Technologies
Hanks' balanced salt solution (HBSS)	ThermoFisher Scientific
TrypLE Express (cell dissociation enzymes)	ThermoFisher Scientific
TRizol	ThermoFisher Scientific
RIPA Lysis and Extraction Buffer	ThermoFisher Scientific
DNAzol	ThermoFisher Scientific
Organoids	
Flat bottom, polystyrene 96-well cell culture treated plate	ThermoFisher Scientific
Brightfield microscope with camera capabilities	
Photo analysis software	
Graphing software	
Organoids	
Ice pack	
Masking tape	
Pipette tips (1000 μ L)	
Razor blade	

Dispase Agarose HistoGel (histology-gel) Pencil "Plunger" from 1 cc insulin syringe Tissue Casette Container to hold fixative 10% neutral buffered formalin (NBF) Histology pen, xylene and EtOH-resistant Ethanol (histologic grade) Xylenes Deionized water Paraffin Tissue processor Embedding workstation Economy Tissue Float Bath Microtome Microtome blades Positively charged microscope slides Laboratory Oven Hematoxylin and Eosin Stain Kit ABC Peroxidase Standard Staining Kit Androgen Receptor Primary Antibody (AR)	STEMCELL Technologies Sigma-Aldrich ThermoFisher Scientific Thomas Scientific Sigma-Aldrich Sigma-Aldrich Fisher Scientific Sigma-Aldrich Leica Daigger Ted Pella Thomas Scientific ThermoFisher Scientific Vector Laboratories ThermoFisher Scientific Cell Signaling Technology
FFPE, sectioned organoid sample baked on a slide Ethanol (histologic grade) Xylenes Deionized water (DI H ₂ O) Antigen retrieval solution 1x Phosphate Buffered Saline - PBS Triton X-100 Normal Horse Serum Bovin Serum Albumin Counterstain (DAPI, Hoescht) Sodium azide Confocal microscope Coplin Staining rack Staining dish Decloaking chamber Humidity chamber Hydrophobic barrier pen Cytokeratin 8/18 primary antibody (CK8) p63-alpha antibody (p63) Keratin 5 Polyclonal Antibody (CK5) Goat anti-rabbit secondary	Fisher Scientific Sigma-Aldrich Sigma-Aldrich, Abcam ThermoFisher Scientific Sigma-Aldrich thermoFisher Scientific Sigma-Aldrich Sigma-Aldrich Sigma-Aldrich Fisher Scientific IHC World IHC World Biocare Medical Thomas Scientific Vector Laboratories ARP American Research Products Cell Signaling Technology Biolegend ThermoFisher Scientific

Goat anti-guinea pig secondary	ThermoFisher Scientific
Microscope cover glass	Globe Scientific
Anti-fade mounting media	ThermoFisher Scientific
Organoids	
Pipette tips (1000 µL)	
Pipette tips (200 µL)	
8-well chamber slide	ThermoFisher Scientific
Cell-Tak	Corning
1x Phosphate Buffered Saline (PBS)	ThermoFisher Scientific
4% paraformaldehyde (PFA)	Biotium
50mM NH ₄ Cl	sigma-Aldrich
Triton™ X-100 (non-ionic detergent)	sigma-Aldrich
Normal Horse Serum (NHS)	thermoFisher Scientific
Bovin Serum Albumin (BSA)	sigma-Aldrich
Counterstain (DAPI, Hoescht)	Sigma-Aldrich
Counterstain (phalloidin)	thermoFisher Scientific
Sodium azide	sigma-Aldrich
Confocal microscope	
Cytokeratin 8/18 primary antibody (CK8)	ARP American Research Products
p63-alpha antibody (p63)	Cell Signaling Technology
Keratin 5 Polyclonal Antibody (CK5)	Biolegend
Goat anti-rabbit secondary	ThermoFisher Scientific
Goat anti-guinea pig secondary	ThermoFisher Scientific
Visikol HISTO-M	Visikol
Organoids	
Pipette tips (1000 µL)	
Pipette tips (200 µL)	
8-well chamber slide	ThermoFisher Scientific
Cell-Tak	Corning
1x Phosphate Buffered Saline - PBS	ThermoFisher Scientific

Cell assay of interest

Various

Organoids
Ice bucket
Cell culture hood
1.5 mL eppendorf tubes or 15 mL conical
Microcentrifuge
Dispase

STEMCELL Technologies

TrypLE Express (cell dissociation enzymes)

ThermoFisher Scientific

Hanks' balanced salt solution (HBSS)
Flow Tube with Cell Strainer Snap Cap

ThermoFisher Scientific
Fisher Scientific

Cytokeratin 5 Antibody - FITC

Millipore Sigma

Cytokeratin 8 Antibody - Alexafluor 405

Abcam

CD49f - Alexafluor 647

BioLegend

CD26 - PE
Flow cytometer

BioLegend

Catalog Number	Comments/Description
17005042	Matrix-gel: growth Factor Reduced, Phenol-red free, etc. depending on application
12676029	
D-073-1ML	
161093	
Various	
05-408-130, 339650	
7923	neutral protease
14025076	
12605036	Cell dissociation enzymes (trypsin may also work, depending on cell type, but TrypLE is more gentle and recommended for primary cells)
15596018	suggested RNA extraction solution
89900	suggested protein extraction solution
10503027	suggested DNA extraction solution
161093	ex. EVOS FL Auto Imaging System ex. Photoshop, CELLESTE, ImageJ, MorphoLibJ, CellProfiler ex. Graphpad, Excel, etc

7923 A9045 HG-4000-012	
1202D72	
HT501128 Z648191-12EA A405P-4 214736-1L various EF4575E XH-1001	STATMARK pen For fixation and graded dilutions during processing
27243 1158B91 PR305225G H-3502 32020 5153S	Suggested H&E staining kit Suggested immunohistochemistry staining kit Suggested primary antibody for IHC
A405P-4 214736-1L	dilutions should be performed using deionized water
C9999, ab93684 10010023 X100-1L 31874 A2058 D9542 S2002	suggested for storage but not required
19-4 M905-12DGY M900-12B DC2012 1219D68 H-4000 03-GP11 4892S 905501 A21245	suggested primary antibody for IF suggested primary antibody for IF suggested primary antibody for IF suggested secondary antibody for p63 or CK5 detection (do not use at same time)

A-11075 1414-10 S36972	suggested secondary antibody for CK8 detection
154534PK CB40240 10010023 22023 254134 X100-1L 31874 A2058 D9542 A22287 S2002	It is also possible to use a confocal dish, depends on preference of user optional adherent reagent other fixatives such as methanol or formalin can be used suggested for storage but not required
03-GP11 4892S 905501 A21245 A-11075 various	suggested primary antibody for IF suggested primary antibody for IF suggested primary antibody for IF suggested secondary antibody for p63 or CK5 detection (do not use at same time) suggested secondary antibody for CK8 detection optional clearing agent
154534PK CB40240 10010023	It is also possible to use a confocal dish, depends on preference of user

Various

Click-iT EdU
Alexa Fluor
proliferation
assay
(fluorescentl
y-labelled
EdU
proliferation
kit), Image-iT
Lipid
Peroxidation
Kit, etc) and
fluorescent
probes/dyes
(ex. HCS
mitochondria
I Health Kit,
CellMask,
LIVE/DEAD
Viability
assays,
CellROX
reagents, etc)

7923

	12605036	Cell dissociation enzymes (trypsin may also work, depending on cell type, but TrypLE is more gentle and recommended for primary cells)
	14175079 08-771-23	
	FCMAB291F	Suggested flow antibody
	ab210139	Suggested flow antibody
	313609	Suggested flow antibody
	320576	Suggested flow antibody



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Article Title:			
Signature:			Date: August 29, 2018

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EDITOR COMMENTS: All comments have been addressed and are shown in blue.

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- Please define all abbreviations before use → all abbreviations have been defined
- Please use centrifugal force (x g) for centrifuge speeds. → rcf was changed to x g
- Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. 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. See examples below:
 - 1.2.3, 1.3, 2.7.1, 2.7.3, 5.20.1, etc.: Please specify the incubation temperature throughout. → added
 - 2.7.2: Please provide more specific details or add relevant references. → added to table of materials
 - 2.7.3: What volumes of TrypLE and HBS are used? → added
 - 3.2.1: Should “step 4.1.4” be 3.1.4? → corrected
 - 3.2.3: Please describe how to perform a background subtraction. → This is a correction that the software is able to complete for you by (‘Process’ > ‘Subtract’), an extra sentence was added to draw attention to this step.
 - 4.1.2: Please specify the volume of the tubes used. → ‘microcentrifuge tube’ was added to clarify this
 - 4.1.3: What volume of histologic marking dye is added? → 1-2 drops has been added
 - 4.2.2: What volume of HBS is used to wash? → added
 - 4.6: Please specify the baking temperature. → added
 - Line 344: Please update the step number. Please provide more specific details or add relevant references. → Added a suggested H&E and IHC kit to the materials list, added protocol section for FFPE IF.
- Line 209: step 4.2.2 is a washing step. Please check whether it is a typo. → This is not a typo
- Line 213: step 4.2.3 is not an imaging step. Please check. → the embedding process as a whole would be useful to be imaged
- 3.2.4: Please split into two steps. → this has been split up into sub-steps
- 4.2.1: Since this step is highlighted for filming, steps 2.1-2.7 should also be highlighted. → these are now highlighted
- Please include at least one paragraph of text to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. However for figures showing the experimental set-up, please reference them in the Protocol. Data from both successful and sub-optimal experiments can be included. → a representative results section has been added after the references
- JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations: --
 - Critical steps within the protocol → ice cold matrix-gel is a critical step, along with H&E staining to assure there is no necrosis

- Any modifications and troubleshooting of the technique → concentration of matrigel varies per lot number and plating efficiency varies between patients, and incubation time needs to be optimized for antibody of interest, background correction for vignetting of images during analysis
- Any limitations of the technique → embedding can lead to changes in organoid morphology
- The significance with respect to existing methods → the 96-well over a droplet improves seeding conditions, the EDF image is an improvement from the single z-plane method, and the whole mount of a selected organoid(s) is an improvement from staining the entire sample,
- Any future applications of the technique → a whole mounted sample can be analyzed with 3D software to create a 3D image or count positive cells in a sample, single cell collection could be applied to new techniques such as single-cell RNA sequencing.
- **References: Please do not abbreviate journal titles.** → revised
- Please remove the titles and Figure Legends from the uploaded figures. Please include all the Figure Legends together at the end of the Representative Results in the manuscript text. → a representative results section has been added after the references
- Table of Equipment and Materials: Please remove trademark (™) and registered (®) symbols. Please provide lot numbers and RRIDs of antibodies, if available. → these have been removed from table of materials

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The authors report methods to generate prostate organoids from fresh human primary prostate epithelial cells, to collect the organoids, to acquire whole-well images of the organoids, to fix and embed the organoids in paraffin, and to perform whole-mounting of organoids for immunofluorescent staining. The procedures described in the methods are easy to follow, which may be of significance in the field of prostate organoid culture.

Major Concerns: None.

Minor Concerns:

- Authors should advise which culture medium and factors that they used are the best for prostate organoid culture. They mentioned several media with citations, but readers do not have to try each of the media to know which is the best. → This has been clarified in the text in 1.1 with a note.
- As a method paper, the authors should give sources and catalog numbers of key reagents. → This can be found the table of materials
- Each figure/panel should be called in the text description in the order of appearance, rather than, e.g., in the section title. → This has been corrected in the text.
- In section 4, it is not clear at which time the organoids are fixed. In section 5.6, organoids are first allowed to adhere to glass for 30-45 minutes, and then fixed at step 5.8. A previous method paper (Ma, et al., Am J Clin Exp Urol, 2017; 5(3):25-33) reported that the organoids were first fixed in the 24-well plates and then processed. Please discuss both methods. Particularly, how well the 3D morphology is kept after the organoids adhered to glass? Is it possible to fix the organoids before letting them adhere to glass? → In Section 4 fixation occurs at step 4.2.6 when the formed organoid plug is placed into a cassette in 10% NBF. An illustration of the process has been added to the figures for clarity and the previous paper has been added to the discussion. The second portion of the comment refers to section 6.5 handling of the organoid for whole mount staining. By transferring the live organoid the chamber slide it rapidly (x minutes) lightly adheres to the glass without any visible change in morphology. Ma et al show fixation prior to whole mounting, but we have not tried this method due to concerns with organoid loss during the wash steps. We have added this to the discussion.

Reviewer #2:

Manuscript Summary:

This manuscript describes an important set of methods to handle prostate organoids in culture

Minor Concerns:

- Section 1.1 the methods of primary cell culture used in the examples should be briefly described and referenced. → a note has been added to reference primary prostate epithelial cell methods and mentions that they are commercially available as well

- Briefly discuss the merits of the different media proposed. → A single media was selected based off of reviewer 1's comment and its merits have been addressed.
- 2.4. the sentence says dissociate dispace. Do they mean dissociate the dispace/matrigel mixture? → This has been corrected in the text.
- 2.7.1 and 2.7.3: how many HBS washes to remove the trypsin → This has been clarified in the text.
- 4.1.1 in "advance" not "advanced" → This has been corrected in the text.

Reviewer #3:

Manuscript Summary:

The authors describe a protocol for propagating human prostate organoids and performing endpoint assays to determine organoid morphology and marker expression. The protocol could be useful as a starting point for researchers that are inexperienced with any of these assays, however, the methods described could be simplified and optimized at various sections and technical details are missing throughout the text. The specific concerns are detailed below.

With complete respect to Reviewer #3's valid comments, the format for JoVE is distinct from other manuscripts. We are not permitted to cited vendors and brands. This is also a report of our protocol to guide others in analyzing organoids. We have added references and discussion about the alternatives brought up by this reviewer.

Major Concerns:

- The authors should include a paragraph in the introduction with some background on prostate (cancer) biology and a description of the biological relevance of the organoid assays they describe. For what purposes are endpoint assays useful? What biological issues can be addressed by measuring for instance the circularity or max/min radius ratio of organoids? Etc. → The introduction section has been expanded to include the biological relevance of prostate organoids as a model system. The significance of the endpoints and considerations are now included in the representative results and discussion. Importantly, interpretation of these various endpoints will vary by experimental design and antibodies used for analysis. For example, organoid circularity may reflect changes in activation of prostate branching morphogenesis in an experiment in which budding factors are added.
- The figure legends are incomplete and should be thoroughly revised. They lack information on organoid type (prostate; mouse / human), type of microscopy, type of imaging software, scale bars (Fig 1), abbreviations (CK?), etc. Also, the calculation of maximum/minimum radius ratio should be better explained. It is not clear for what applications this could be useful. Could the authors cite papers in which this method has been successfully used? → Figure legends have been revised for clarity as suggested but within the guideline of JoVE (no brands or company names). A representative results section has been added to paper after references. Citation for maximum/minimum radius is included in introduction in the representative results section.
- The writing poor and sloppy and should be thoroughly revised. → The paper has been thoroughly inspected for typos and revised where necessary.
- Line 66-73: The authors list previous protocols or methods related to organoid culturing and analyses. It should be better explained how their protocol relates to these studies and whether any gap in the field is being addressed with this paper. → The introduction has been revised to clearly state the purpose of this protocol. A limitation of the many prostate organoid papers is brevity in organoid handling and methods. The goal of this protocol is that researchers can set up an organoid experiment from start to finish from one protocol. Steps that are modified from published reports are referenced. This JoVE protocol will serve as a single tool kit and video guidance to aid new users in learning these techniques.
- Figure 2B. Why are these images provided? What should the reader pick up from comparison of the fresh and dry sections? Better explain in legend. Images of the molds they generate to embed the organoids would be more informative, as these procedures are unclear from only reading the text. → JoVE guidelines require images of examples when a step is done incorrectly. In this case the purpose is to discern organoids from bubbles to help a user who has never seen an FFPE organoid unstained under a microscope and is sectioning for the first time. This clarification is included in 4.5.10 and in the figure legend. A mold workflow for embedding has been added and the JoVE publication will involve filming the entire embedding process.
- Figure 2C. Provide an image of a well-processed organoid as a comparison. → All stained organoids are moved to figure 3 for comparison.

- 1.2.2. and 1.2.4: The authors should note that the percentage of matrigel used for optimal performance can differ per matrigel batch as they vary in protein concentration and consistency. → [Matrigel protein concentration considerations have been added to discussion.](#)
- What is the advantage of propagating organoids as described in section 1.1-1.4 as compared to culturing in for instance 5 ul drops of matrigel per well in 96-wells format? This would simplify the procedure and allow for a proper medium exchange (instead of refreshing only 50% of the medium each time). → [This has been added to the discussion.](#)
- The dispase treatment (2.3-2.7) to recover intact organoids from the matrigel is time-consuming and unnecessary when organoids are processed into single cells; TrypLE can be applied directly to the well for disruption of matrigel and processing into single cells. → [A note about this has been added to the text.](#)
- The authors claim that their 2D projected image of a 3D organoid by compression of Z-stacks is more informative than measuring the area of the most representative plane (line 493-507). The authors should compare both methods in a figure to show better performance of their compression method. The limitations of presenting 3D organoids in 2D images should be better emphasized. → [A single z-plane has been added to Figure 2 to show that at a single plane only a few organoids are in focus for detection.](#)
- Throughout the text, volumes for washings or antibody incubations are not indicated. → [This has been corrected in the text.](#)
- Section 5: The authors transfer a drop of 25-50 ul matrigel with organoids from their culture to a chamber slide. Is the matrigel solid at this stage? Why would the organoids sink and stick to the bottom as claimed in 5.6? What happens with the matrigel during fixation & washings? → [This step has been revised for clarity. Details on how to remove the matrigel have been added \(step 6.4\) leaving whole organoids able to tether to the slide during wash steps.](#)
- 5.8.: 4% PFA fixation at 4C is commonly used for fixating cultures and tissue. Why do the authors perform their method at RT? → [We prefer room temperature because the matrix is solid rather than liquid at 4C, but both temperature will work.](#)
- Abbreviations are not explained throughout the text (e.g. 'NHS' line 401) → [This has been corrected in the text.](#)
- Section 5: It is unnecessary to perform separate steps for DAPI and Phalloidin, it can be added to secondary antibody mix. → [This has been corrected in the text.](#)
- Line 524-525. The authors state that their method involves transferring of a single organoid into a chamber slide for staining, which is inconsistent with methods described in section 5. → [This has been clarified for consistency in the text, a single organoid or many organoids can be transferred as needed.](#)

Minor Concerns:

- Line 414: The washing step appears to be short for washing 3D organoids. → [This length works for our culture conditions, a note regarding longer wash steps has been added.](#)
- Line 301: explain 'the biopsy protocol' → [Biopsy protocol is a setting on a processor, the processing steps were previously included but this section has been reworded for clarity.](#)
- Line 304: define 'different' → [This has been reworded for clarity.](#)
- Line 356: Pre-wet what? → [This has been clarified in the text.](#)
- Line 359: Define 'large' → [This has been reworded in the text.](#)

Reviewer #4:

Manuscript Summary:

The manuscript by McCray et al describes a detailed methodology for determining the phenotypes generated from prostate organoids. The advantage of this method is that the authors show a 3D-assessment/based on image-based expression in real-time over a soft-agar 3D colony (which is hard to image for expression of various phenotype markers in real-time). Each step of method is written succinct. The method is written in a very simplistic way which is understandable by researchers as well as undergraduate students. Following are the some minor concerns which should be addressed:

- Although the manuscript is written very well, there are several typos in the manuscript. Words such as "reaction" appear numerous times either in lower case or upper case. It should be uniform throughout the manuscript → [This has been corrected in the text.](#)

- Pages 8-9: the yellow color-highlighting of texts on pages 8-9 should be removed. → Highlights are required by JoVE and denote which portions of the protocol are to be filmed
- Line 480: section 6.12; how soon the imaging should be done after mounting? → This has been clarified in the text.
- To make it reader friendly (for the benefit of undergraduate and graduate students), authors should write an appendix for abbreviations used and their full names. → Abbreviations are now included in the materials table for reference.

Reviewer #5:

Manuscript Summary:

In the manuscript "Assessment of Prostate Organoid Phenotypes by Imaging" McCray et.al. provide detailed methods for culturing prostate organoids, assessment of organoid morphology and histological phenotypes. The protocols may be also useful for analysis of prostate cancer organoids from patients. The authors listed all the materials and equipment in the table, highlighted critical steps, and clearly presented most steps, except one (see below).

Major Concerns: On page 10: the methods need to be described in greater detail because it is not clear how to draw up a drop of solid organoids-containing Matrigel and dispense it onto a chamber slide. → This method uses 33% matrigel which is less viscous and can be pipetted using a trimmed tip similar to a biopsy punch, this has been clarified in the text.

Minor Concerns:

1. On page 2: The concentration of Matrigel may be too low. Reference 5 (Nature Protocol) used 75% Matrigel for 3D prostate organoid culture. However, the current protocol uses 33%-50%. Is the Matrigel concentration high enough to remain solid for 2-3 weeks? → Yes, this has been clarified in the discussion.
2. On page 7: 7.1 should be changed to 5.1 → This has been corrected in the text.
3. On page 4: 1.1: It would be useful to mention "keep the media cold or on ice", since cold growth media needs to be used to make the 50% Matrigel mixture. → Thank you for catching this important detail. This has been added to the text.
4. This manuscript has 13 pages so the footnote should be changed to reflect that. → This has been corrected in the text.