REVISIONS TO JoVE MANUSCRIPT:

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

* 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 and Reagents. You may use the generic term followed by “(see table of materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Matrigel, Dispase, Eppendorf, TrypLE, EVOS FL, Photoshop, CELLESTE, HistoGel, STATMARK, triton-x100, Click-iT, Visikol HISTO-M, etc 🡪 Matrigel has been replaced with “matrix-gel”, dispase has been replaced with “neutral protease”, eppendorf replaced with “microcentrifuge tube”, TrypLE replaced with “cell-dissociation enzymes”, EVOS FL has been removed, HistoGel was replaced with “histology-gel”, STATMARK was removed, triton-x100 is a detergent that is sold from many companies and not a commercial product, visikol was removed, CELLESTE was removed and the image analysis portion was made more generic. Similarly, Click-IT was replaced with “fluorescently-labelled EdU proliferation kit” and “fluorescent reaction cocktail.”
* 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 dispase/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 concertation 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 whit 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.