Journal of Visualized Experiments

Prostate organoid cultures as tools to translate genotypes and mutational profiles to pharmacological responses

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60346R2
Full Title:	Prostate organoid cultures as tools to translate genotypes and mutational profiles to pharmacological responses
Section/Category:	JoVE Cancer Research
Keywords:	Prostate organoids, Prostate Cancer, Enzalutamide, Drug resistance
Corresponding Author:	Wouter Karthaus Memorial Sloan Kettering Cancer Center New York, New York UNITED STATES
Corresponding Author's Institution:	Memorial Sloan Kettering Cancer Center
Corresponding Author E-Mail:	karthauw@mskcc.org
Order of Authors:	Wouter Karthaus
	Kyrie J. Pappas
	Danielle Choi
	Charles L. Sawyers
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	New York

TITLE:

Prostate Organoid Cultures as Tools to Translate Genotypes and Mutational Profiles to Pharmacological Responses

AUTHORS AND AFFILIATIONS

Kyrie J. Pappas[1], Danielle Choi[1], Charles L. Sawyers[1,2], Wouter R. Karthaus[1]

[1]Human Oncology and Pathogenesis Program, Memorial Sloan Kettering Cancer Center, New York, NY, USA

[2]Howard Hughes Medical Institute, Chevy Chase, Maryland, USA

Corresponding Author:

Wouter R. Karthaus (Karthauw@mskcc.org)

Email Addresses of Co-Authors:

Kyrie Pappas (pappask@mskcc.org)

Danielle Choi (choid3@mskcc.org)

Charles L. Sawyers (sawyersc@mskcc.org)

KEYWORDS:

prostate organoids, prostate cancer, second generation anti-androgens, drug resistance, primary cell culture, prostate model systems

SUMMARY:

Presented here is a protocol to study pharmacological responses in prostate epithelial organoids. Organoids closely resemble in vivo biology and recapitulate patient genetics, making them attractive model systems. Prostate organoids can be established from wildtype prostates, genetically engineered mouse models, benign human tissue, and advanced prostate cancer.

ABSTRACT:

Presented here is a protocol to study pharmacodynamics, stem cell potential, and cancer differentiation in prostate epithelial organoids. Prostate organoids are androgen responsive, three-dimensional (3D) cultures grown in a defined medium that resembles the prostatic epithelium. Prostate organoids can be established from wild-type and genetically engineered mouse models, benign human tissue, and advanced prostate cancer. Importantly, patient derived organoids closely resemble tumors in genetics and in vivo tumor biology. Moreover, organoids can be genetically manipulated using CRISPR/Cas9 and shRNA systems. These controlled genetics make the organoid culture attractive as a platform for rapidly testing the effects of genotypes and mutational profiles on pharmacological responses. However, experimental protocols must be specifically adapted to the 3D nature of organoid cultures to obtain reproducible results. Described here are detailed protocols for performing seeding assays to determine organoid formation capacity. Subsequently, this report shows how to perform drug treatments and analyze pharmacological response via viability measurements, protein isolation, and RNA isolation. Finally, the protocol describes how to prepare organoids

for xenografting and subsequent in vivo growth assays using subcutaneous grafting. These protocols yield highly reproducible data and are widely applicable to 3D culture systems.

INTRODUCTION:

Drug resistance is one of the major clinical problems in cancer treatment. Metastatic prostate cancer (PCa) treatment is primarily directed at the androgen-signaling axis. Next-generation anti-androgen therapies (e.g., enzalutamide and abiraterone) have showed great clinical success, but virtually all PCa eventually progresses towards an androgen-independent state, or castration resistant prostate cancer (CRPC).

Recent genomic and transcriptomic profiling of CRPC revealed there are three general mechanisms of resistance in prostate cancer: 1) activating mutations resulting in the restoration of androgen receptor (AR) signaling[1]; 2) activation of bypass signaling, as exemplified in a pre-clinical model for next-generation anti-androgen therapy resistance in which activation of the glucocorticoid receptor (GR) can compensate for loss of AR signaling[2]; and 3) the recently identified process of lineage plasticity, in which tumor cells acquire resistance by switching lineages from a cell type dependent on the drug target to another cell type that is not dependent on this (which, in PCa, is represented as AR-negative and/or neuroendocrine disease [NEPC])[3,4]. However, the molecular mechanisms that cause drug resistance are not understood. Moreover, acquired anti-androgen resistance may lead to therapeutic vulnerabilities that can be exploited. Therefore, it is essential to evaluate drug responses in model systems that mimic patient phenotypes and genotypes.

Prostate organoids are organotypic cultures grown in a 3D protein matrix with a defined medium. Importantly, prostate organoids can be established from benign and cancerous tissue of murine or human origin, and they retain phenotypic and genotypic features found in vivo[5,6]. Importantly, both anti-androgen sensitive PCa and CRPC cells are represented in the current compendium of organoids. Moreover, prostate organoids are easily genetically manipulated using CRISPR/Cas9 and shRNA[5]. Thus, prostate organoids are a suitable model system for testing drug responses and elucidating resistance mechanisms. Here, a detailed protocol is described to perform drug testing and analyze pharmacological responses using prostate organoids.

PROTOCOL:

All work described in this protocol has been performed with previously established murine organoids and patient-derived organoids. All animal work was performed in compliance with the guidelines of Research Animal Resource Center of Memorial Sloan Kettering Cancer Center (IACUC: 06-07-012). All patient-derived tissues were collected in compliance with rules and regulations of Memorial Sloan Kettering Cancer Center (IRB: 12001).

1. Medium and buffer preparation

1.1. Thaw basement membrane matrix (e.g., Matrigel) at 4 °C overnight before starting the experiment. Keep it on ice during use.

1.2. Place culture plates at 37 ˚C for 24 h prior to experiments. This will help the basement membrane matrix dome (hereafter referred to as matrix dome) to polymerize. Plating organoids is described in step 2.1.2.

1.3. Prepare the organoid medium according to the established protocol[5].

1.4. Prepare the organoid medium without the addition of epidermal growth factor (EGF; see **Table of Materials** for components). EGF suppresses the AR transcriptional output and confers anti-androgen resistance[5].

1.5. Prepare Dulbecco's Modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS).

NOTE: This media is used to inhibit the enzymatic digestion with trypsin replacement in sections 2–4.

1.6. Prepare drug solutions according to the manufacturer's protocol. For enzalutamide/mdv3100 (hereafter referred to as second generation anti-androgen), prepare a stock solution of 100 µM in dimethyl sulfoxide (DMSO). Stock can be stored at -20 °C for up to 6 months and does not have to be made fresh.

2. Isolation, enzymatic digestion, and establishment of organoids

2.1. Isolate prostate organoids from mouse or human tissue according to the previously established protocols[5,7]. A brief description is provided below.

2.1.1. Mince and enzymatically digest prostate tissue to produce a single cell suspension. In this experiment, 1 mL of 5 mg/mL collagenase type II in ADMEM/F12 was used for the digestion of 50 mg of prostate tissue.

2.1.2 Collect cells by centrifugation at 300 *x g* for 5 min, count the cells, resuspend them in the basement membrane matrix, and plate at the appropriate density[5,7] in the matrix domes on pre-warmed organoid culture plates (plating method is shown in **Figure 1A**).

2.1.3. Allow the domes to solidify and add media onto the tops of the domes so that they are completely covered.

2.2. Grow organoids to the desired quantity for downstream applications. Cell number can be determined by standard counting methods. See the application-specific section of the protocol for additional details on density.

2.3. Using a P1000 pipette, draw up the medium and pipette up and down to disrupt. When basement membrane matrix is fully disrupted, transfer the suspension to a 15 mL conical tube. Do not place more than 10 domes per single 15 mL conical tube. Centrifuge at 300 *x g* for 5 min.

2.4. Draw off the supernatant and wash the cell pellet with 5 mL of PBS. Centrifuge at 300 *x g* for 5 min.

2.5. Draw off the supernatant and resuspend the pellet in 4 mL of trypsin replacement. Digest for 5–10 min with shaking at 37 ˚C. Add an equal volume of organoid medium + 10% FBS to inhibit the trypsin replacement. Centrifuge at 300 *x g* for 5 min.

2.6. Draw off the supernatant and resuspend in 1 mL of PBS.

2.7. Filter the suspension with a 40 µm filter to ensure a single cell suspension. Quantify the cell number using a hemocytometer or equivalent counting device.

NOTE: If obtaining viable single cells is difficult, use flow sorting to obtain a single cell solution.

3. Assessing organoid formation capacity

NOTE: To determine the percentage of cells that can generate an organoid, a seeding assay can be performed as a proxy for the stem/progenitor potential. The organoid formation capacity is also important for defining a cell seeding number for the viability assays.

3.1 Dilute the cell suspension obtained in step 2.7 to 100 cells per 10 µL of the suspension using organoid medium containing 10 µM Rho kinase inhibitor Y-27632.

3.2 Transfer 1,100 cells (110 µL of suspension) to a new conical tube.

3.3 Add 285 µL of basement membrane matrix and resuspend the cells. This will result in a ~70% matrix concentration.

NOTE: Dilution of the basement membrane matrix during seeding greatly reduces the variation in dome size, caused by the viscosity of the protein matrix.

3.4 Seed cells in 35 µL of matrix domes in a pre-warmed 24 well plate, resulting in 200 cells/well. Plate 3–5 replicates per sample (also see plating method in **Figure 1A** and step 2.1.2).

3.5 To ensure that the cells remain within the matrix dome, flip the plate and place it in a cell incubator to solidify the basement membrane matrix.

3.6 After 10 min, remove the plate from the incubator and add medium containing the Rho kinase inhibitor.

3.7 Refresh the medium every 2 days. After 7 days, quantify the number of organoids. Keep the Rho kinase inhibitor in media throughout the experiment.

3.8 Count the number of organoids established per dome and calculate the percent of organoids formed out of the total number of cells plated (200 cells).

NOTE: Organoid establishment ratios vary from 3%–60% depending on the cell type and genotype.

4: Determining pharmacological responses of organoids

4.1 Continuing from step 2.7, seed 1,000–10,000 cells in a matrix dome. Use the organoid formation efficiency and growth speed as a proxy for determining the final cell number.

NOTE: Recommended cell numbers are provided in **Table 1**. Use three to five replicates per condition per analysis. Use a final concentration of 70% basement membrane matrix to reduce pipetting errors induced by the viscosity.

4.2 Seed 35 µL of matrix domes in a 24 well plate and let the domes solidify as done in section 3 (also see plating method in **Figure 1A** and step 2.1.2). Add medium containing the Rho kinase inhibitor and drug of choice. This method can be applied to all drugs, but in this protocol, a second-generation anti-androgen is used at 10 µM for an example. To determine half maximal inhibitory concentration (IC50), perform a log10 incremental, and as a control, use the vehicle in which the drug was dissolved.

4.3 Refresh the medium every two or three days and analyze the organoids on day 7 to determine the pharmacological response of the drug. The timepoints may vary among the choice of experiment and drug.

NOTE: Organoids do not have to be trypsinized to perform these assays.

4.4 To keep organoids intact, using a P1000 pipette, draw up the medium and pipette up and down to disrupt the basement membrane matrix.

4.5 When the basement membrane matrix is fully disrupted, transfer the suspension to a 15 mL conical tube. Do not transfer more than 10 matrix domes per 15 mL conical tube.

4.6 Centrifuge at 300 *x g* for 5 min. Draw off the supernatant and wash with 5 mL of PBS.

4.7 Resuspend organoids in 1 mL of PBS and disrupt the organoids using trituration and a glass Pasteur pipette.

4.8. Quantify the number of organoid fragments. Seed 5 replicates containing 100 organoid fragments as described in step 2.1.2.

4.9. Perform the cell viability assay as described below in section 7.

5. RNA isolation from organoids

NOTE: Commercially available column-based methods yield good quantity and quality of RNA. To ensure good quantity RNA, use a minimum of one dome per sample; however, using three domes is recommended, which can be seeded in a single well of a 12 well plate.

5.1. Add ß-mercaptoethanol (1%) to the glutathione lysis buffer in the RNA isolation kit.

5.2. Draw off the medium from the basement membrane domes containing organoids and add 750 µL of this buffer. Pipette up and down using a P1000 pipette. Check that all the basement membrane matrix has been dissolved.

5.3. Add 750 µL of 70% ethanol and mix by pipetting. Subsequently transfer 700 µL of the mixture to the column, centrifuge at 12,000 *x g* for 1 min, and repeat with the remainder of the lysate.

5.4. Perform washes and on-column DNase treatment according to manufacturer's instructions. Elute RNA in 30–50 µL of RNAse-free water.

5.5. Measure the concentrations using a fluorometer at OD = 260 nm and 280 nm and store at -80 ˚C or continue with downstream applications.

6. Protein isolation from organoids

NOTE: For protein isolation, prepare standard RIPA buffer containing phosphatase and protease inhibitors (**Table of Materials**). Using at least three domes is recommended, which can be seeded in a single 12 well.

6.1. Using a P1000 pipette, draw up the medium from the cell with the basement membrane domes containing organoids and pipette up and down to disrupt the basement membrane matrix.

6.2. When fully disrupted, transfer the suspension to a 15 mL conical tube. Centrifuge at 300 *x g* for 5 min.

6.3. Draw off the supernatant and wash with 5 mL of ice-cold PBS. Centrifuge at 300 *x g* for 5 min.

6.4. Draw off the supernatant and resuspend the pellet in 4 mL of trypsin replacement. Digest for 5–10 min while shaking at 37 ˚C.

6.5. Add an equal volume of organoid medium + 10% FCS to inhibit the trypsin replacement. Centrifuge at 300 *x g* for 5 min.

NOTE: Post-centrifugation, no basement membrane matrix should be visible in the pellet.

6.6. Draw off the supernatant and wash with 5 mL of ice-cold PBS. Draw off the supernatant and resuspend the cell pellet in 300 µL of lysis buffer using a P1000 pipet, then transfer to a 1.5 mL microcentrifuge tube.

6.7. Incubate on ice for 10 min and subsequently sonicate 2x for 30 s each at cooled water with a temperature of 4 °C. Place the tube back on ice and perform protein quantification using standard methods.

6.8. Denature the protein by adding sodium dodecyl sulfate (SDS) containing loading dye and boil for 5 min at 95 ˚C. Store lysates at -80 °C or continue with downstream applications.

7. Cell viability assay with organoids

NOTE: Cell viability can be assessed using the commercially available cell viability assay kit and a luminometer. Prepare buffers according to the manufacturer's instructions. Five replicates per condition is recommended: one replicate consisting of one 35 µL basement membrane matrix dome in one well of a 24 well plate.

7.1. Draw off the medium of the organoid culture, being careful to leave the matrix domes intact.

7.2. Add 65 µL of PBS and pipette up and down to disrupt the matrix dome.

7.3. Add 100 µL of the cell viability assay kit buffer and resuspend by pipetting.

7.4. Incubate at room temperature (RT) for 10 min with shaking.

7.5. Transfer 100 µL of mixture to a non-translucent plate suitable for the luminometer and perform reading according to the manufacturer's instructions for the cell viability assay kit.

8. Preparation of organoids for xenografting

NOTE: Organoids are also amenable for subcutaneous grafting in both immune compromised animals, as well as, isogenic mice. To ensure injected organoids are distinguishable in vivo, label organoids with a constitutively expressing fluorophore[5]. It is recommended to perform a pilot experiment for grafting using 5 x 10^5 cells to 2 x 10^6 cells per injection, with increments of 5 x 10^6 cells, as grafting efficiency varies between organoid lines.

8.1. Using a P1000 pipette, draw up the medium and pipette up and down to disrupt the basement membrane matrix. When fully disrupted, transfer the suspension to a 15 mL conical tube. Centrifuge at 300 *x g* for 5 min.

NOTE: Do not transfer more than 10 matrix domes per 15 mL conical tube.

8.2. Draw off the supernatant and wash with 5 mL of PBS. Centrifuge at 300 *x g* for 5 min.

8.3. Draw off the supernatant and resuspend the pellet in 4 mL of trypsin replacement. Digest for 5–10 min while shaking at 37 ˚C.

8.4. Add equal volume of organoid medium + 10% FBS to inhibit trypsin replacement. Centrifuge at 300 *x g* for 5 min.

8.5. Draw off the supernatant and resuspend in 1 mL of PBS. Filter the suspension with a 40 µm filter to ensure a single cell suspension. Quantify cells using standard methods.

8.6. Spin down and resuspend the cells in PBS + Rho inhibitor to a concentration of 2 x 10^6 cells per 100 µL (see **Table 2** for cell concentrations and absolute cell number needed for varying concentrations). Use an equal volume of basement membrane matrix to generate a 1:1 suspension. Place the suspension on ice.

8.7. Inject cells according to standard protocols and monitor xenograft growth using standard methods[8].

REPRESENTATIVE RESULTS:

Seeding efficiency

Organoid formation capacity is determined by phenotype and genotype. Wild-type (WT) prostate basal cells showed superior organoid formation capacity (30%–40%) compared to luminal cells (3%) (**Figure 1A**). After organoid establishment, the formation capacity increased drastically. Typically, 25%–30% of cells derived from a WT organoid can form a new organoid (**Figure 1B**). CRISPR/Cas9-mediated loss of Pten ($Pten^{\Delta/\Delta}$) or p53 ($p53^{\Delta/\Delta}$) resulted in a minor increase in organoid formation capacity. Loss of both p53 and Pten further increased formation capacity (**Figure 1B**).

Pharmacological response

Based on seeding efficiency, seeding of 1,000–10,000 cells in 35 µL of basement membrane matrix dome in a 24 well plate was performed. Recommended cell seeding numbers based on organoid formation efficiency is provided in **Table 1**. However, organoid proliferation speeds can differ greatly depending on genotype. Additional changes to the cell seeding number can be made based on proliferation.

In **Figure 2,** the effects of anti-androgenic molecules on growth were tested in murine organoids with different genotypes. A total of 2,500 cells were seeded from murine organoids with a WT genotype, p53 loss, Pten loss, or dual p53 and Pten loss. p53 and Pten loss was initiated by lentiviral introduction of a gRNA targeting the *p53* and/or *Pten* locus in organoids constitutively expressing Cas9 under the control of the Rosa26 promoter with a C57/Bl6 genetic background[9].

Loss of p53 did not cause resistance to the anti-androgenic molecules. Loss of Pten increased resistance to anti-androgenic compound, as shown previously[10]. Dual loss of p53 and Pten, however, resulted in complete resistance to the second-generation anti-androgen (**Figure 2A**). AR inhibition also altered organoid phenotypes. In control $Cas9^{+/+}$ organoids, as well as $P53^{\Delta/\Delta}$-deleted and $Pten^{\Delta/\Delta}$ organoids, a decrease in organoid lumen size was observed (**Figure 2B**). $p53^{\Delta/\Delta}$ $Pten^{\Delta/\Delta}$ organoids were phenotypically unaffected (**Figure 2B**). In line with these results, when 1 x 10^6 cells were grafted subcutaneously in the flank, only $p53^{\Delta/\Delta}$ $Pten^{\Delta/\Delta}$ organoids grew (**Figure 2C**). Overall, these results demonstrate that $p53^{\Delta/\Delta}$ $Pten^{\Delta/\Delta}$ co-deletion results in resistance to the second-generation anti-androgen in murine organoids.

Patient-derived PCa organoids are heterogeneous in phenotype and genotype[11,12]; therefore, responses to drugs can differ greatly between human PCa organoid lines. In **Figure 3**, the anti-androgenic molecules response of two distinct human PCa organoids, MSKPCA2 and MSKPCA3 are shown. Proliferation of MSKPCA2 organoids was strongly inhibited by anti-androgenic molecules, whereas MSKPCA3 organoids remained unaffected (**Figure 3A,B**). MSKCPCA2 organoids expressed high levels of AR and the AR-target FKBP5, and they expressed hallmark luminal proteins such as CK8 and CK18. In contrast, MSKPCA3 organoids also expressed basal (CK5) and mesenchymal (Vimentin) markers and showed no expression of FKBP5. These results suggest that these organoids model a non-luminal androgen-independent phenotype.

FIGURE LEGENDS:

Figure 1: Measuring organoid formation rates of human and mouse prostate cells. (**A**) Schematic overview of cell resuspension in basement membrane matrix (left) and organoid seeding in matrix domes (right). (**B**) Relative organoid formation of human (CD49f+)-derived basal and (CD26+)-derived luminal cells (%, y-axis; mean ± SD) in the presence of 1 nM DHT. A total of 200 cells were seeded and the number of organoids was quantified 7 days post-seeding (n = 3, ***p < 0.01, t-test). (**C**) Relative organoid formation of murine WT, $Pten^{\Delta/\Delta}$, $P53^{\Delta/\Delta}$, and $P53^{\Delta/\Delta}$ $Pten^{\Delta/\Delta}$ organoids (%, *y*-axis; mean ± SD) in the presence of 1 nM DHT. A total of 200 cells were seeded and the number of organoids was quantified 7 days post-seeding (n = 3). *p53* and *Pten* loss was mediated by the gRNA's targeting of the p53 and/or Pten locus in organoids expressing Cas9 constitutively under a Rosa26 promoter.

Figure 2: Assessing the pharmacological response of organoids derived from genetically engineered mice. (**A**) Relative cell proliferation of murine WT, $Pten^{\Delta/\Delta}$, $P53^{\Delta/\Delta}$, and $P53^{\Delta/\Delta}$ $Pten^{\Delta/\Delta}$ organoids (*y*-axis, mean ± SD, measured by cell viability assay kit) of 2,500 cells 7 days post-establishment of organoids; (n = 3, *p < 0.05, t-test) with 1 nM DHT or 10 µM of second-

generation antiandrogen as indicated. (**B**) Representative brightfield images of WT, Pten$^{\Delta/\Delta}$, P53$^{\Delta/\Delta}$, and P53$^{\Delta/\Delta}$ Pten$^{\Delta/\Delta}$ organoid cultures treated with 1 nM DHT or 10 µM second-generation anti-androgen as indicated. (**C**) Representative growth curve of WT, Pten$^{\Delta/\Delta}$, P53$^{\Delta/\Delta}$, and P53$^{\Delta/\Delta}$ Pten$^{\Delta/\Delta}$ organoids injected subcutaneously in the flank of isogenic C57/Bl6 mice. Only P53$^{\Delta/\Delta}$ Pten$^{\Delta/\Delta}$ organoids showed growth. A total of 1 x 10^6 cells were injected. Three independent curves of P53$^{\Delta/\Delta}$ Pten$^{\Delta/\Delta}$ organoids are shown to show heterogeneity in growth speed.

Figure 3: Assessing the pharmacological response of organoids derived human prostate cancer biopsies. (A) Relative cell proliferation of patient-derived MSKPCA2 and MSKPCA2 organoids (*y*-axis, mean ± SD, measured by cell viability assay kit) of 5,000 cells 7 days post-establishment of organoids (n = 4, *p < 0.05, t-test) with 1 nM DHT or 10 µM second-generation anti-androgen as indicated. (**B**) Representative brightfield images of MSKPCA2 and MSKPCA3 organoids cultures treated with 1 nM DHT or 10 µM second-generation anti-androgen as indicated. (**C**) Western blot analysis of AR, FKBP5 (AR-target gene), CK8 and CK18 (luminal markers), CK5 (basal marker), and Vimentin (mesenchymal marker) in MSKPCA2 and MSKPCA3 organoids. GAPDH was used as a loading control.

Table 1: Cell seeding numbers used to assess pharmacological response based on organoid formation capacity. Table by column (left to right) includes organoid formation capacity ranges and the corresponding number of cells to seed per matrix dome.

Table 2: Recommended cell numbers for organoid xenografting experiments. Columns from left to right include absolute total cell number for 10 injections, total volume of PBS + Y-27632 for 10 injections, basement membrane matrix volume, and cell number per injection.

DISCUSSION:

Understanding the molecular mechanisms underlying anti-androgen resistance and discovering potential therapeutic vulnerabilities requires testing of pharmacological responses in model systems mimicking prostate cancer. Described here is a detailed protocol for the reliable analysis of pharmacological responses in patient-derived and genetically engineered prostate organoids and preparation of these organoid samples for downstream applications.

There are two critical steps in this protocol. The first is determining the seeding efficiency and growth rate of organoids. Organoid growth speed varies greatly. This is dependent on species, as murine derived organoids grow about two-fold faster than human-derived organoids. Apart from species, growth speed is dependent on genotype and phenotype. However, when seeding efficiency and growth speed are determined, this protocol can be adapted to all prostate organoid types.

The second critical step is working with the protein-matrix-based 3D culture to prepare for subsequent downstream applications. The introduction of seeding variation by viscosity of the basement membrane matrix during plating can be avoided by using diluted (70%) basement

membrane matrix, as described. Properly breaking up the polymerized matrix without excessively disturbing the organoids is also described in detail. This protocol enables disruption of the matrix without introducing variation in the organoid readout, which can be adapted for the screening of drug libraries for different genetic backgrounds in PCa. Moreover, by performing CRISPR/Cas9- or shRNA-based expression interference, genes conferring drug resistance can be queried.

A point of consideration is that the medium composition of prostate organoid culture can influence the pharmacological response. For example, EGF, a component of both murine and human prostate organoid culture, greatly reduces sensitivity to anti-androgen. Hence, EGF is omitted in this protocol from the medium, and sensitivity to anti-androgen is restored. It is advised to determine if any organoid ingredients influence sensitivity for the drug being tested. This holds especially true for the complex human prostate organoid culture medium, which (apart from EGF, Noggin, R-spondin1, DHT, and A83-001 [the composition of murine organoid medium]) contains fibroblast growth factor 10 (FGF10), FGF2, prostaglandin E2, nicotinamide, and the p38i inhibitor SB202190.

The organoid medium composition favors the growth of benign prostate epithelium over cancer tissue, thus no primary hormone sensitive PCa organoid lines have been established. Currently, all human PCa organoids are derived from patients with advanced metastatic anti-androgen resistant PCa; hence, most of these lines are anti-androgen resistant and suitable for identifying new treatments. As proof-of-concept, delta-like 3 (DLL3) has been identified as a therapeutic target using patient-derived NEPC organoids that are targetable with rovalpituzumab tesirine[13]. This method is suitable for these types of experiments and is also suitable for prostate organoids from normal benign tissue, primary prostate cancer, and CTCs.

One shortcoming of prostate organoid culture is the absence of a cellular niche. Thus, contributions to drug resistance by non-tumor cells cannot be studied using the current platform. However, co-cultures of colorectal cancer and lung cancer organoids with autologous T-cells have recently been established, enabling studies of interactions between the tumor and immune system[14]. Other co-culture systems may be established to further study non-cell-autonomous interactions.

In conclusion, this report provides a detailed protocol for the reproducible assessment of pharmacological responses in prostate organoids and subsequent downstream applications. Importantly, this protocol is broadly applicable and can be used for organoid cultures of other organs, including the colon[15], small intestine[16] , stomach[17,18], liver[19], pancreas[20], kidney[21], and mammary gland[22].

ACKNOWLEDGEMENTS:

K.P. is supported by NIH 1F32CA236126-01. C.L.S. is supported by HHMI; CA193837; CA092629; CA224079; CA155169; CA008748; and Starr Cancer Consortium. W.R.K. is supported by Dutch Cancer Foundation/KWF Buit 2015-7545 and Prostate Cancer Foundation PCF 17YOUN10.

DISCLOSURES:

C.L.S serves on the board of directors of Novartis; is a cofounder of ORIC Pharmaceuticals and coinventor of enzalutamide and apalutamide; is a science advisor to Agios, Beigene, Blueprint, Column Group, Foghorn, Housey Pharma, Nextech, KSQ, Petra, and PMV; and is a cofounder of Seragon, purchased by Genentech/Roche in 2014. W.R.K. is a coinventor and patent holder of organoid technology.

REFERENCES:

1. Robinson, D. et al. Integrative Clinical Genomics of Advanced Prostate Cancer. *Cell.* **162** (2), 454 (2015).
2. Arora, V. K. et al. Glucocorticoid Receptor Confers Resistance to Antiandrogens by Bypassing Androgen Receptor Blockade. *Cell.* **155** (6), 1309–1322 (2013).
3. Ku, S. Y. et al. Rb1 and Trp53 cooperate to suppress prostate cancer lineage plasticity, metastasis, and antiandrogen resistance. *Science.* **355** (6320), 78–83 (2017).
4. Mu, P. et al. SOX2 promotes lineage plasticity and antiandrogen resistance in TP53- and RB1-deficient prostate cancer. *Science.* **355** (6320), 84–88 (2017).
5. Karthaus, W. R. et al. Identification of Multipotent Luminal Progenitor Cells in Human Prostate Organoid Cultures. *Cell.* **159** (1), 163–175 (2014).
6. Gao, D. et al. Organoid Cultures Derived from Patients with Advanced Prostate Cancer. *Cell.* **159** (1), 176–187 (2014).
7. Drost, J. et al. Organoid culture systems for prostate epithelial and cancer tissue. *Nature Protocols.* **11** (2), 347–358 (2016).
8. Bose, R. et al. ERF mutations reveal a balance of ETS factors controlling prostate oncogenesis. *Nature.* **546** (7660), 671–675 (2017).
9. Platt, R. J. et al. CRISPR-Cas9 Knockin Mice for Genome Editing and Cancer Modeling. *Cell*. **159** (2), 440–455 (2014).
10. Carver, B. S. et al. Reciprocal feedback regulation of PI3K and androgen receptor signaling in PTEN-deficient prostate cancer. *Cancer Cell.* **19** (5), 575–586 (2011).
11. Puca, L. et al. Patient derived organoids to model rare prostate cancer phenotypes. *Nature Communications.* **9** (1), 2404 (2018).
12. Gao, D. et al. Organoid Cultures Derived from Patients with Advanced Prostate Cancer. *Cell.* **159** (1), 176–187 (2014).
13. Puca, L. et al. Delta-like protein 3 expression and therapeutic targeting in neuroendocrine prostate cancer. *Science Translational Medicine.* **11** (484), eaav0891 (2019).
14. Dijkstra, K. K. et al. Generation of Tumor-Reactive T Cells by Co-culture of Peripheral Blood Lymphocytes and Tumor Organoids. *Cell.* **174** (6), 1586–1598.e12 (2018).
15. van de Wetering, M. et al. Prospective derivation of a living organoid biobank of colorectal cancer patients. *Cell.* **161** (4), 933–945 (2015).
16. Sato, T. et al. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature.* **459** (7244), 262–265 (2009).
17. Barker, N. et al. Lgr5(+ve) stem cells drive self-renewal in the stomach and build long-lived gastric units in vitro. *Cell stem cell.* **6** (1), 25–36 (2010).
18. Bartfeld, S. et al. In vitro expansion of human gastric epithelial stem cells and their

responses to bacterial infection. *Gastroenterology.* **148** (1), 126–136.e6 (2015).
19. Huch, M. et al. Long-term culture of genome-stable bipotent stem cells from adult human liver. *Cell.* **160** (1-2), 299–312 (2015).
20. Boj, S. F. et al. Organoid models of human and mouse ductal pancreatic cancer. *Cell.* **160** (1-2), 324–338 (2015).
21. Schutgens, F. et al. Tubuloids derived from human adult kidney and urine for personalized disease modeling. *Nature Biotechnology.* **37** (3), 303–313 2019).
22. Sachs, N. et al. A Living Biobank of Breast Cancer Organoids Captures Disease Heterogeneity. *Cell*. 1–25 (2017).

Figure 1

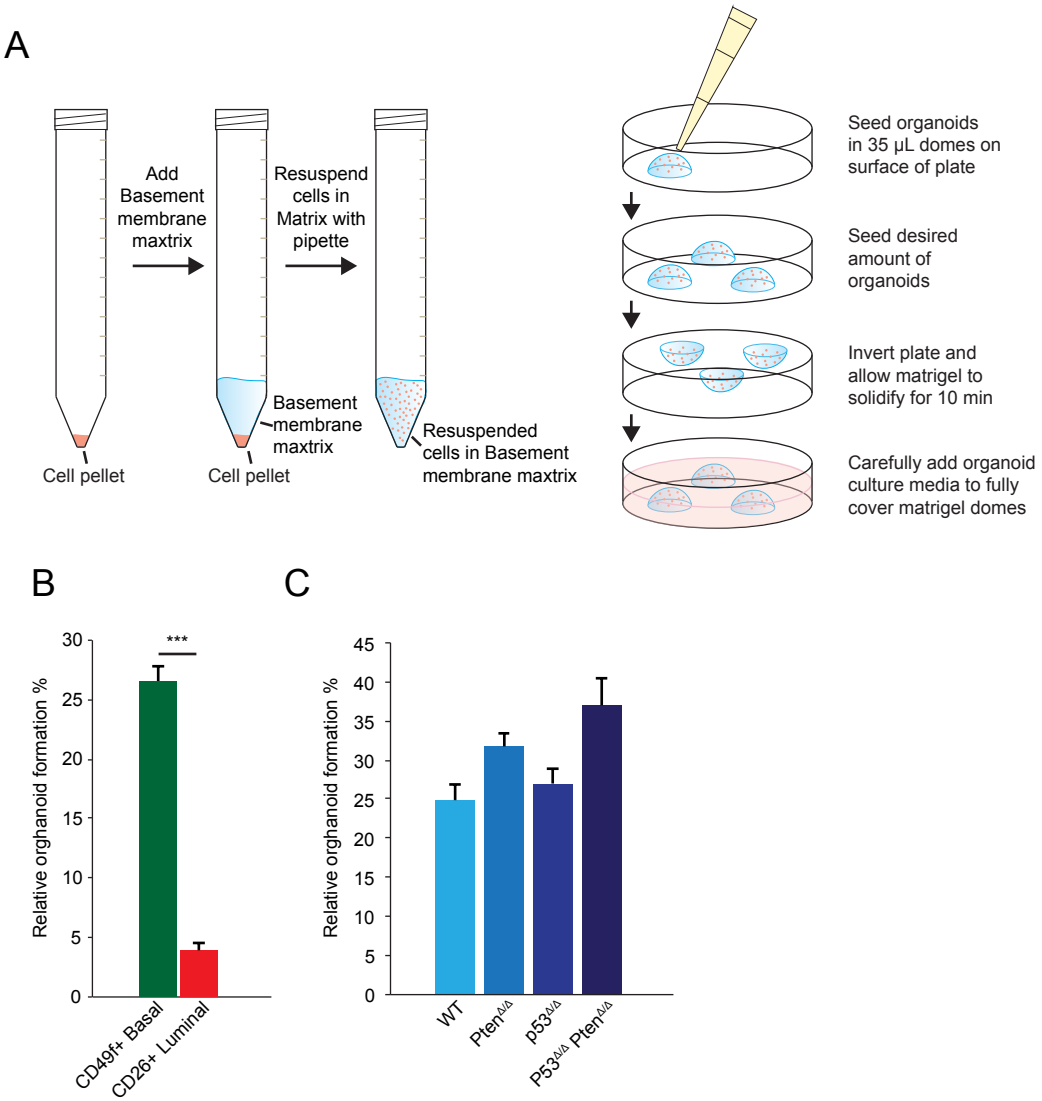


Figure 2

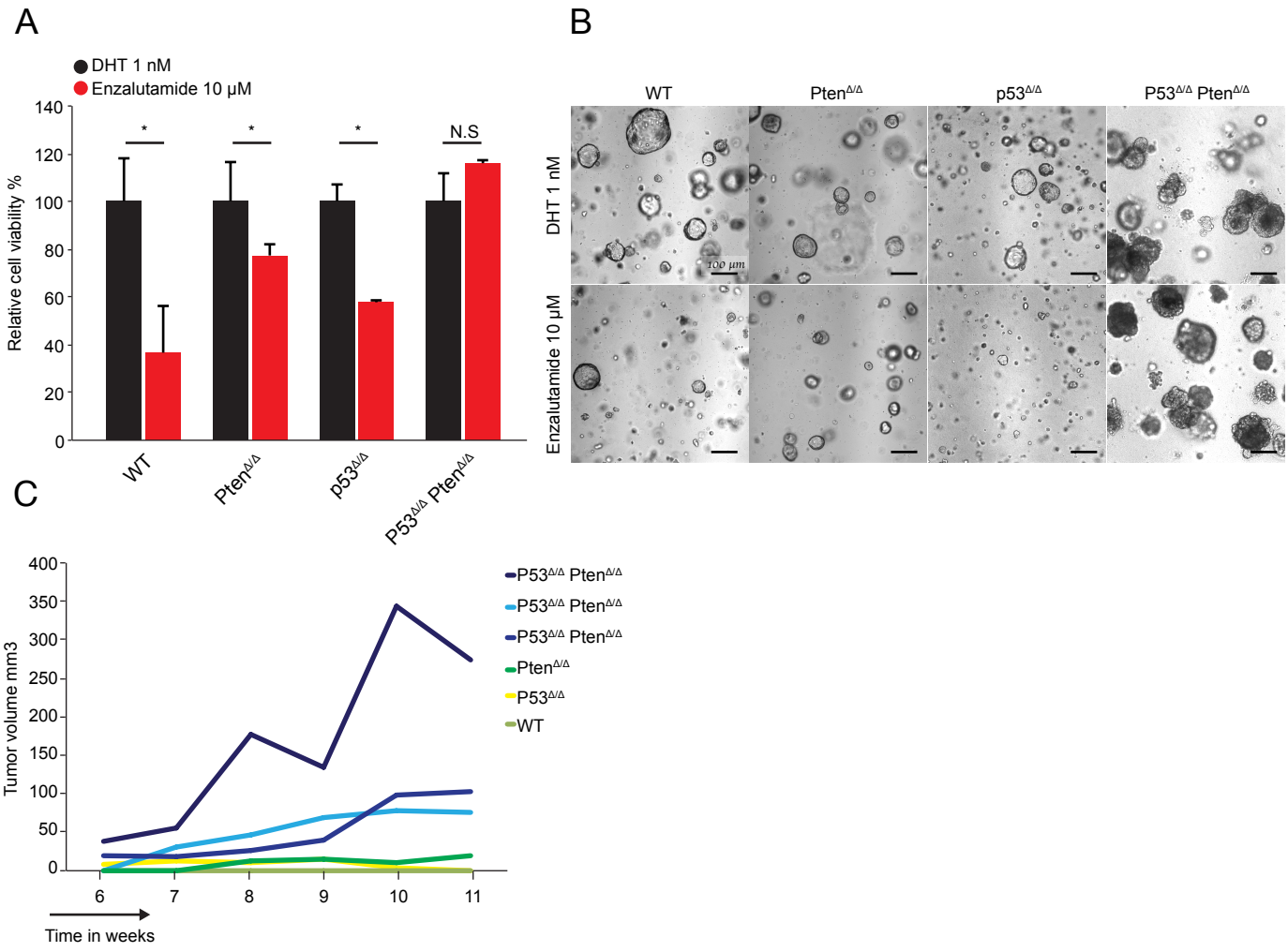


Figure 3

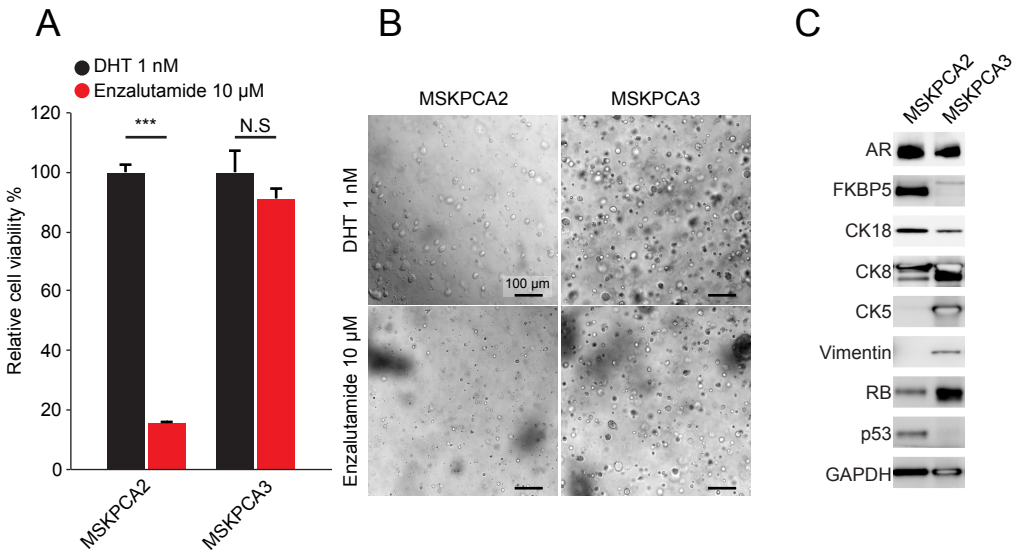


Table 1

Organoid seeding efficiency (%)	**Cell-seeding number (per dome)**
1-10%	100000
10-20%	5000
20-60%	2500
60-100%	1000

Table 2: Cell numbers needed for 10 injections

Total Cell number	PBS0+ Y-27632	Matrigel volume	cell concentration / Injection
5×10^6	500 µl	500 µl	5×10^5
10×10^6	500 µl	500 µl	1×10^6
15×10^6	500 µl	500 µl	1.5×10^6
20×10^6	500 µl	500 µl	2×10^6

Reagent	Source
A83-01	Tocris
ADMEM/F12	Gibco/Life technologies
B27	Gibco/Life technologies
Cell culture plates	Fisher
Cell Titer Glo	Promega
DHT	Sigma-Aldrich
DMSO	Fisher
EGF	Peprotech
FGF10	Peprotech
FGF2	Peprotech
Glutamax	Gibco/Life technologies
HEPES	MADE IN-HOUSE
Matrigel (Growthfactor reduced & Phenol Red free)	Corning
N-Acetylcysteine	Sigma-Aldrich
Nicotinamide	Sigma-Aldrich

NOGGIN	Peprotech or stable transfected 293t cells with Noggin construct (Karthaus et al. 2014)
Penicillin/Streptavidin	Gemini Bio-Products
Phospatase inhibitors	Merck Millipore
Prostaglandin E2	Tocris
Protease Inhibitors	Merck Millipore
R-SPONDIN	Peprotech or stable transfected 293t cells with R-Spondin1 construct (Karthaus et al. 2014)
RIPA buffer	Merck
RNA-easy minikit	Qiagen
SB202190	Sigma-Aldrich
TryplE	ThermoFisher
Y-27632	Selleckchem

Catalog Number	Comments
2939	Organoid medium component: Final concentration 200 nM
12634028	Organoid medium component
17504-044	Organoid medium component
657185	
G7571	
D-073	Organoid medium component: Final Concentration 1 nM
BP231-100	
315-09	Organoid medium component: Final concentration 50 ng/ml for mouse, 5 ng/nl for Human
100-26	Human specific organoid medium component: Final concentration 10 ng/ml
100-18B	Human specific organoid medium component: Final concentration 5 ng/ml
35050079	Organoid medium component
N/A	Organoid medium component: Final concentration 10 mM
CB-40230C	Organoid medium component
A9165	Organoid medium component: Final concentration 1.25 mM
N0636	Human specific organoid medium component: Final concentration 10 mM

120-10C	Organoid medium component: Final Concentration 10% conditioned medium or 100 ng/ml
400-109	Organoid medium component
524629	
3632464	
539131	
120-38	Organoid medium component: Final Concentration 10% conditioned medium or 500 ng/ml
20-188	
74104	
152121-30-7	Human specific organoid medium component: Final concentration 10 µM
12605036	
S1049	Organoid medium component: Final Concentration 10 µM

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article:	Prostate organoid cultures as tools to translate genotypes and mutational profiles to pharmacological responses.
Author(s):	Kyrie J. Pappas, Danielle Choi, Charles L. Sawyers, Wouter R. Karthaus

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

☑ Standard Access ☐ Open Access

Item 2: Please select one of the following items:

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

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

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

ARTICLE AND VIDEO LICENSE AGREEMENT

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

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

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

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

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

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

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

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

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

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

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

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

ARTICLE AND VIDEO LICENSE AGREEMENT

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

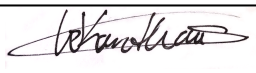
12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify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

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

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

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

CORRESPONDING AUTHOR

Name:	Wouter R. Karthaus
Department:	Human Oncology and Pathogenesis Program, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA
Institution:	Memorial Sloan Kettering Cancer Center
Title:	Prostate organoid cultures as tools to translate genotypes and mutational profiles to pharmacological responses.
Signature:	
Date:	05/30/2019

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

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

Ronald Myers, PhD.
Editor JoVE.

July 26th 2019,

Dear Dr Myers,

We are pleased to submit our revised manuscript our invited manuscript titled "Prostate organoid cultures as tools to translate genotypes and mutational profiles to pharmacological responses" for consideration at Journal of Visualized Experiments. We have addressed all concerns raised by the editor. We have added an extra illustration to figure 1A to clarify the organoid seeding method. Also We would like to stress that this method is generalizable for all drugs. Hence we have used in some cases a general term, but we have added that as an example we use antiandrogens in this protocol.

Regards

Wouter Karthaus

1. The editor has formatted the manuscript to match the journal's style. Please retain the same.
    a. We have done so
2. Please address specific comments marked in the manuscript.
    a. We have addressed all comments. We have also uploaded a Word document with comments for reference if anything is unclear.
3. We cannot have any commercial terms in our manuscript. This includes all trademarked, registered, patented terms, etc. I have changed to generic terms instead. Please check.
    a. We have removed all commercial terms
4. The table of materials in our case contains columns for name, company, catalog, and comments. Please move the details of the human and mouse organoid's details to the comment column.
    a. We have done so