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Isolation and Characterization of Patient Derived Pancreatic Ductal Adenocarcinoma Organoid Models

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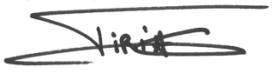
May 31, 2019

Dear Dr. Ronald Myers,

We are pleased to submit a manuscript for your consideration for publication in JoVE. Three-dimensional organoid models of disease have become a key resource for researchers. Yet, the implementation of this novel technology in the laboratory remains difficult. By submitting this manuscript to JoVE we intend to facilitate the adoption of this technology by taking advantage of JoVE's unique audio/visual and written format. Our manuscript entitled "**Isolation and Characterization of Patient Derived Pancreatic Ductal Adenocarcinoma Organoid Models**" contains our current protocols for the generation of organoid models from pancreatic tumors. In addition, we provide methods to characterize the organoids generated using our protocols. We highlighted text that we believe will be critical for your readers to be featured in the accompanying video. We look forward to your reviews and comments.

Sincerely,



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TITLE:

Isolation and Characterization of Patient-Derived Pancreatic Ductal Adenocarcinoma Organoid Models

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Organoid, pancreatic cancer, PDA, PDAC, patient-derived models, precision medicine

SUMMARY:

Patient-derived organoid cultures of pancreatic ductal adenocarcinoma are a rapidly established 3-dimensional model that represent epithelial tumor cell compartments with high fidelity, enabling translational research into this lethal malignancy. Here, we provide detailed methods to establish and propagate organoids as well as to perform relevant biological assays using these models.

ABSTRACT:

Pancreatic ductal adenocarcinoma (PDAC) is amongst the most lethal malignancies. Recently, next-generation organoid culture methods enabling the 3-dimensional (3D) modeling of this disease have been described. Patient-derived organoid (PDO) models can be isolated from both surgical specimens as well as small biopsies and form rapidly in culture. Importantly, organoid models preserve the pathogenic genetic alterations detected in the patient's tumor and are predictive of the patient's treatment response, thus enabling translational studies. Here, we provide comprehensive protocols for adapting tissue culture workflow to study 3D, matrix embedded, organoid models. We detail methods and considerations for isolating and propagating primary PDAC organoids. Furthermore, we describe how bespoke organoid media is prepared and quality controlled in the laboratory. Finally, we describe assays for downstream characterization of the organoid models such as isolation of nucleic acids (DNA and RNA), and drug testing. Importantly we provide critical considerations for implementing organoid methodology in a research laboratory.

INTRODUCTION:

Pancreatic ductal adenocarcinoma (PDAC) is a lethal disease characterized by late diagnosis in

most patients, a lack of effective therapies, and a resultant low 5-year overall survival rate that remains less than 10%[1]. Only 20% of patients are diagnosed with a localized disease suitable for curative surgical intervention[2,3]. The remaining patients are typically treated with a combination of chemotherapeutic agents that are effective in a minority of patients[4,5]. To address these pressing clinical needs, researchers are actively working on early detection strategies and the development of more effective therapies. To accelerate clinical translation of important discoveries, scientists are employing genetically engineered mouse models, patient derived xenografts, monolayer cells lines, and, most recently, organoid models[6].

Three-dimensional epithelial organoid culture using growth factor and Wnt-ligand rich conditions to stimulate proliferation of untransformed progenitor cells were first described for the mouse intestine[7] and were quickly adapted to normal human pancreatic tissue[8]. In addition to normal ductal tissue, organoid methodology allows for the isolation, expansion, and study of human PDAC[8]. Importantly, the method supports the establishment of organoids from surgical specimens, as well as fine and core needle biopsies, allowing researchers to study all stages of the disease[9,10]. Interestingly, patient-derived organoids recapitulate well-described tumor transcriptomic subtypes and may enable development of precision medicine platforms[9,11].

Current organoid protocols for PDAC enable the successful expansion of more than 70% of patient samples from chemo-naïve patients[9]. Here we present the standard methods employed by our laboratory to isolate, expand, and characterize patient-derived PDAC organoids. Other PDAC organoid methodologies have been described[12,13] but no comparison of these method has been thoroughly performed. As this technology is relatively new and advancing quickly, we expect that these protocols will continue to evolve and improve; however the principles of tissue handling and organoid culture will continue to be useful.

PROTOCOL:

All human tissue collection for research use was reviewed and approved by our Internal Review Board (IRB). All of the following protocols are performed under aseptic conditions in a mammalian tissue culture laboratory environment.

1. Media preparation

1.1. Conditioned media preparation.

NOTE: The human pancreatic organoid media requires abundant growth factors and nutrients as well as conditioned media supplementation to provide sufficient growth stimulation for organoid expansion. Both conditioned mediums described below are prepared from commercially available cell lines (see **Table of Materials**). For complete protocols and materials, please refer to the manufacturers' websites.

1.1.1. Produce R-spondin1 conditioned media according to the manufacturer's protocol.

1.1.1.1. First, expand the 293T cells expressing R-spondin1 using appropriate antibiotic (Zeocin) selection methods in the presence of abundant serum (10%). When producing conditioned media, withdraw and wash away the antibiotic selection and serum such that no antibiotic and serum are present in the final conditioned media. Importantly, the conditioned media must be filter sterilized after collection (0.2 µm) to prevent cross-contamination.

1.1.1.2. Store aliquoted conditioned media at 4 ˚C for short term use (within 6 months) or at -20 ˚C for long term storage (more than 6 months). Freeze-thaw cycles should be avoided.

1.1.2. Produce L-Wnt-3A conditioned media according to the manufacturer's protocol.

1.1.2.1. First, expand the L-M(TK-) cells expressing L-Wnt-3A using appropriate antibiotic (G-418) selection methods in the presence of abundant serum (10%). When producing conditioned media, withdraw and wash away the antibiotic selection such that no antibiotic is present in the final conditioned media; however, maintain the serum throughout the culturing and conditioning. Importantly the collected conditioned media must be filter sterilized (0.2 µm) to prevent cross contamination.

1.1.2.2. Store the aliquoted conditioned media at 4 ˚C for short term use, or -20 ˚C for long term storage. Freeze-thaw cycles should be avoided.

1.1.3. For quality control, perform a TOPFLASH assay to test the Wnt activity of R-spondin1 and the L-Wnt-3A conditioned media alone and in combination according to a previously published protocol[14].

1.2. Basal media preparation: Use basal media for preparation of complete organoid media as well as washing steps for organoid work. Supplement advanced DMEM/F-12 with Glutamine at a final concentration of 1X, HEPES (10 mM), and Penicillin/Streptomycin (100 U/mL). Store basal media at 4 ˚C.

1.3. Organoid Complete media preparation: Supplement the basal media with R-spondin1 conditioned media at a final concentration of 10% v/v, L-Wnt-3A conditioned media at a final concentration of 50% v/v, Human EGF (50 ng/mL), Human FGF (100 ng/mL), Human Gastrin I (10 nM), Mouse Noggin (100 ng/mL), A83-01 (500 nM), B27 supplement (1X), Nicotinamide (10 mM), N-acetylcysteine (1.25 mM), and Primocin (100 µg/mL).

1.3.1. For primary organoid isolations, thawed organoid cultures, and cultures starting with single cells, include Rho kinase inhibitor Y-27632 at a final concentration of 10.5 µM.

1.3.2. Prepare the human organoid complete media on ice and store at 4 ˚C for use within one month. For this reason, we recommend fresh weekly preparation of media.

2. Isolation of PDAC organoids

NOTE: Thaw the basement membrane extract (BME) solution (growth factor reduced; see **Table of Materials**) on ice in a 4 ˚C environment (fridge or cold room) for at least 12 h prior to use. Incubate the tissue culture plates for organoid culture in a 37 ˚C incubator for at least 12 h prior to use.

2.1. Collect and transport a surgically removed tumor specimen for research in a buffered storage solution to maintain viability of the tissue. Use basal media or a commercially available tissue storage solution.

2.2. Proceed to isolate organoids as soon as possible after receiving the specimen. Tissue can be stored in the storage solution up to 24 h post-surgery and still yield organoids.

2.3. Once in the laboratory, transfer tumor to a 10 cm tissue culture dish and remove storage solution.

2.4. While handling the tissue with metallic forceps, mince the tumor with a #10 scalpel into small fragments of 1 mm^3 or smaller. Larger fragments will take longer to digest; therefore, aim to generate homogenous tissue fragment sizes. If tissue is already disaggregated or in smaller than 1 mm^3 fragments, skip this step.

2.5. Transfer the tissue fragments to a 15 mL conical tube containing 10 mL of basal media. Mix by inverting the tube several times. Centrifuge the tube at 200 x *g* for 5 min. After spin, remove the basal media carefully to avoid losing tissue fragments.

2.6. To the tube containing the tissue fragments add 9 mL of basal media and 1 mL of 10x Gentle Collagenase/Hyaluronidase solution. To avoid clumping of cells during digestion, supplement this solution with 20 µL of 10 mg/mL DNAse I solution. Mix by inverting the tube several times.

2.7. Incubate the enzymatic digestion at 37 ˚C on a nutator or rotator. For adequate mixing during digestion, the tissue fragments must constantly be moving through the solution.

NOTE: Timing for this enzymatic dissociation of the tumor must be determined empirically for every specimen. A successful digestion of the tumor is characterized by the decrease in size of the tissue fragments until they are not clearly discernable to the naked eye. Concomitantly, the opacity of the solution will increase as microscopic tissue fragments are released.

2.8. After 30 min, remove the conical tube from the 37 ˚C incubator and observe. If the tissue fragments are still clearly visible, continue the dissociation at 37 ˚C with constant mixing. Repeat this observation every 30 min until most or all tissue fragments have been dissociated. Depending on the stringency of mixing as well as the size and amount of tumor fragments, this step can take as little as 30 min for small specimens or as long as 12 h for larger tumor samples.

2.9. Once the enzymatic dissociation is complete, centrifuge the tube at 200 x *g* for 5 min. After spin, a cell pellet should be visible and the supernatant should be clear, indicating that all cells have been spun out of solution. If that is not the case, repeat the spin.

2.10. Carefully remove the supernatant to avoid losing the cell pellet. Immediately wash the cells with 10 mL basal media by gently mixing the tubes with inversion. Centrifuge the tube at 200 x *g* for 5 min. Carefully remove the basal media to avoid losing cells and repeat this washing step one more time for a total of two washes.

2.11. After the second wash, remove all of the basal media carefully and place the tube on ice for 5 min.

2.12. At this time, place an aliquot of organoid complete media in a 37 ˚C water bath to pre-warm.

2.13. Mix the cell pellet with ice cold BME while maintaining the tube on ice. Seeding density should be high for organoid isolation. For a small pellet (~ 50 µL volume) use 200 µL of BME, while for a large pellet (~ 200 µL volume) use 800 µL of BME. Mix the cells and BME on ice using a p200 pipette until the solution appears homogenous while avoiding creating bubbles in the solution.

2.14. Spot a 100 µL dome in the center of a well of a pre-warmed 12 well plate using a p200 pipette. Repeat this until all of the BME solution have been dispensed. Carefully transfer the plate to the 37 ˚C incubator to allow the BME gel to solidify.

2.15. After 10 min, retrieve the plate and add 1 mL of pre-warmed organoid complete media per well. Dispense the media on the side of the well to avoid disruption of the BME dome.

2.16. Observe the cell fragments using an inverted tissue culture microscope using a 4x lens in brightfield.

NOTE: Single cells and microscopic tissue fragments should be clearly visible. A successful isolation is characterized by the appearance of more than 10 organoids after 24–48 h; the culture should be confluent within one week. As tumor specimens are heterogenous, some organoid isolations are more challenging as only a few organoids grow per well, as shown in **Figure 1**. In this case, allow the culture to continue for up to two weeks to maximize the number of cells available for passaging. To account for media consumption and evaporation during culture, top up the cultures with 200 µL of pre-warmed organoid complete media every 5 days.

3. Passaging of PDAC organoids

3.1. Retrieve the plate from tissue culture incubator. With a sterile cell lifter, gently lift the BME dome such that it is floating in the complete media. Avoid scraping the bottom of the well so as to not remove any monolayer cells attached to the plastic, as these are typically fibroblasts.

3.2. With a p1000 pipette, carefully transfer the BME dome and media to a 15 mL conical tube. Repeat this step for every organoid-containing well.

3.3. Gently wash each well that was harvested with 1 mL of cold basal media, and transfer to the 15 mL tube containing the organoids.

3.4. Thoroughly mix the solution and organoids with a p1000 pipette and spin down at 200 x *g* for 5 min. After spinning, a BME layer containing organoids in suspension and an organoid pellet will appear at the bottom of the tube. Carefully remove the supernatant, avoiding loss of the BME/organoid layer. Place the tube on ice.

3.5. Add 10 mL of ice-cold cell recovery solution (CRS) to the tube and thoroughly mix by inversion. This will depolymerize the protein matrices of the gelled BME at 4 ˚C. Incubate on ice for 30 min while mixing every 3 min by inversion. If available, place the tube on a rotating mixer in a cold room or 4 ˚C fridge for 30 min.

3.6. After the 30 min incubation, spin down at 200 x *g* for 5 min. The organoid pellet should be apparent while the BME layer should be gone. If the BME layer is decreased in size but still visible, incubate for an additional 30 min at 4 ˚C with mixing and repeat spin.

3.7. Once the BME has been depolymerized, remove the CRS leaving behind the organoid pellet. Wash the organoids with 10 mL basal media by mixing with inversion. Spin down at 200 x *g* for 5 min and remove the supernatant. Place the tube with the organoid pellet on ice for at least 5 min.

3.8. Optionally, if a single cell preparation is desired, incubate the organoids with 3 mL trypsin supplemented with 30 µL of 10 mg/mL DNAse I solution to avoid clumping of cells during digestion.

3.8.1. Incubate the enzymatic reaction at 37 ˚C with gentle inversion mixing every 2 min for up to 10 min. Monitor and confirm successful single cell dissociation under brightfield microscope.

3.8.2. To stop the enzymatic reaction, add 10 mL of room temperature basal media and spin down at 200 x g for 5 min.

3.8.3. Wash cells with 10 mL basal media by mixing with gentle inversion. Spin down at 200 x g for 5 min and remove the supernatant and repeat the wash one more time. Place the tube with the cell pellet on ice for at least 5 min.

NOTE: We recommend performing this step every 3-5 passages during regular maintenance of the organoids to generate homogeneous culture with a large number of organoids.

3.9. Add ice cold BME to the cell pellet and mix gently on ice until solution is homogenous using a p200 pipette. While avoiding generating bubbles in the mixture, pipette up and down at least

5–10x, placing the tip of the pipette close to the bottom of the tube to help mechanically break up the organoids. As a guide, use 4–6x the volume of the BME/cell pellet such that the splitting ratio is no more than 1:2 from one passage to the other.

3.10. Spot a 100 µL dome in the center of a well of a pre-warmed 12 well plate using a p200 pipette; repeat until all of the BME solution has been dispensed. Carefully transfer the plate to the 37 ˚C incubator to allow the BME gel to solidify. After 10 min, retrieve the plate and add 1 mL of pre-warmed organoid complete media per well. Dispense the media on the side of the well to avoid disruption of the BME dome.

3.11. Organoids should reform and start growing within 24 h. Organoid cultures are typically passaged ever 7-10 days depending on the culture density and cell proliferation. If necessary, top up the cultures with 200 µL of pre-warmed organoid complete media every 5 days to compensate for growth factor depletion and evaporation.

4. Freezing and thawing of PDAC organoids

4.1. To freeze down the organoids, proceed to harvest the organoids and depolymerize the BME as described in steps 3.1–3.7.

4.2. To the cell pellet, add 1 mL of freezing media, mix gently, and transfer the mixture to a pre-labeled cryovial.

4.3. Place the cryovial in a freezing container to maintain a safe constant temperature decrease and place in a -80 ˚C freezer. After 24 to 48 h, transfer the frozen vials to liquid nitrogen storage. Organoids can be cryopreserved for months to years using this method.

4.4. To thaw the organoids, retrieve the frozen cryovial from liquid nitrogen storage. Transport the frozen vial on dry ice to the tissue culture room.

4.5. Place the frozen cryovial in the 37 ˚C water bath to rapidly thaw the organoids and transfer the contents of the vial to a 15 mL conical tube containing 9 mL of basal media warmed to room temperature.

4.6. Spin down at 200 x *g* for 5 min and remove the supernatant. Place the tube with the organoid pellet on ice for at least 5 min.

4.7. Proceed to resuspend in BME and plate the organoids as described in steps 3.9–3.11.

NOTE: Organoid cultures recover slowly after a freeze/thaw cycle, therefore cultures may be grown for up to two weeks before passaging.

5. Characterization of PDAC organoids

NOTE: The characterization of the organoids should be performed on an established culture after several passages to diminish the risk of contamination from non-epithelial cell types such as fibroblasts and immune cells.

5.1. Nucleic acid extraction from PDAC organoids

5.1.1. Plate organoids on a 12 well plate dedicated to nucleic acid extraction. Plate no more than 100 µL of BME per well. For adequate DNA/RNA yield, plate at least 2–4 wells per culture. Grow organoids for up to 5 days until cultures are close to confluent but devoid of the excessive cell debris that accumulates in longer term cultures.

5.1.2. Retrieve the plate from the 37 ˚C incubator and completely remove all trace of organoid complete media, leaving only the BME dome containing the organoids on the plate.

5.1.3. Add 900 µL of acid phenol reagent (see **Table of Materials**) to each well and mix thoroughly using a p1000 pipette until solution becomes homogeneous. The BME will be completely dissolved in the acid phenol reagent and organoids will lyse after a short 5 min incubation.

CAUTION: Acid phenol is a hazardous chemical that can cause chemical burns. Handle with care using appropriate protections and technique.

5.1.4. Transfer the homogenous solution to a 2 mL tube and add 200 µL of chloroform. Incubate 5 min and centrifuge at 12,000 x *g* for 15 min at 4 ˚C.

5.1.5. Proceed to the RNA and DNA extraction according to manufacturer's protocol. RNA can be extracted from the aqueous phase while DNA can be extracted from the interphase and organic phase. Once purified and quantified, RNA and DNA isolated from different wells of the same culture can be pooled to increase yield.

NOTE: (1) While we strongly recommend using this nucleic acid extraction method for RNA, there are many good methods for isolating DNA from cultured cells. (2) To ascertain the presence of tumor cells within the organoid culture, we recommend sequencing the DNA using your preferred next generation sequencing method to identify mutations within PDAC hallmark genes (*KRAS, TP53, SMAD4, CDKN2A*).

5.2. Pharmacotyping of PDAC organoids

5.2.1. Isolate single cells from organoids as described above in steps 3.1–3.8. To maximize cell number, harvest at least 10 confluent wells of a 12 well plate

5.2.2. Resuspend single cells in 1 mL human organoid complete media. The presence of small cell clumps (~2–10 cells) is acceptable, however larger cell clumps will negatively affect the downstream analysis.

5.2.3. Count cells using an automated cell counter and record cell viability. Calculate the total number of cells and viable cells present in the 1 mL suspension.

5.2.4. For therapeutic testing, plate 1000 viable cells per well of a 384 well plate. For a 384 well plate we estimate we will use 400,000 viable cells (calculated for 400 wells).

5.2.5. Prepare cells for plating by mixing on ice 800 µL of BME with 7.2 mL of organoid complete media containing the cells. Keep mixture on ice and plate 20 µL per well of a 384 well plate. Use a 12-channel pipette and reservoir kept on ice to efficiently plate the cells. To avoid excessive evaporation from the plate, the outer wells can be used as a reservoir (60 µL PBS or water per well), but this will lead to a loss of 76 experimental wells.

5.2.6. Spin the plate down at 100 x *g* for 1 min in a swing bucket. This allows the small 20 µL volume and organoids to settle at the bottom of the plate.

5.2.7. Place plate in the 37 ˚C tissue culture incubator to allow organoids to form. After 24 h ,check for presence of organoids using a brightfield microscope.

5.2.8. Proceed to dosing therapeutic compounds dissolved in DMSO onto the plate using a drug printer or equivalent instrument. Test each drug dose in at least triplicate wells. To perform an effective dose-response analysis, determine dose range for each drug empirically, starting with a low, ineffective, dose and ending with a high dose where maximum effect is observed. Examples of dose ranges for chemotherapeutic compounds have been recently published[9].

5.2.9. Expose the cells to the therapeutic compounds for 3–5 days.

5.2.10. Optionally, at the end of the assay, image the plate to evaluate the therapeutic effect on organoid size, number, and morphology.

5.2.11. Assess viability using 20 µL per well of luminescence cell viability reagent (see **Table of Materials**) according to manufacturer's protocol. A luminometer will be necessary for data acquisition and a graphing software for data analysis.

REPRESENTATIVE RESULTS:

To illustrate the challenges associated with isolating organoids from PDAC, we show the establishment of a patient derived organoid culture from a small hypocellular tumor sample. After initial plating, only a few organoids were visible per well, as shown in **Figure 1**. Organoids were allowed to grow larger over the span of 2 week and were passaged according to our protocol to establish a more robust culture, as shown in the early and late passage 1 representative pictures (**Figure 1**). It is important to note that the larger cystic organoids observed in the late primary isolate were easily broken down into smaller fragments during the mixing of organoids with ice-cold BME, as described in step 2.13.

To demonstrate the outcome of the pharmacotyping protocol, we prepared single cells from an

established and fast growing representative PDAC organoid as described in this protocol. 1000 viable cells were plated per well and allowed to recover over 24 h before cytotoxic chemotherapeutic agents, Gemcitabine and Paclitaxel, were dosed. We performed a 9-point dose response assay in triplicate starting with a low dose of 100 pM and ending with a high dose of 2 µM. After 5-day treatment, representative pictures were taken for vehicle (DMSO), 2 µM Gemcitabine, and 2 µM Paclitaxel treated wells (**Figure 2**). Immediately after taking the pictures, cell viability was assessed using luminescence cell viability reagent and plotted using graphing software (**Figure 2**).

FIGURE AND TABLE LEGENDS:

Figure 1: Representative pictures are shown for a PDAC organoid isolation as well as after the first passage. Both early (1–3 days) and late (7–10 days) time points are shown to illustrate organoid growth over time. Scale bar indicates 200 µm.

Figure 2: Dose response analysis. (Left) Representative dose response analysis obtained using an established PDAC organoid culture, with standard deviation of triplicates shown as error bars. (Right) Pictures illustrating the effect at the end of the assay of vehicle (DMSO) treatment as well as 2 µM Gemcitabine and 2 µM Paclitaxel. Scale bar indicates 100 µm.

DISCUSSION:

Here, we present current protocols for isolating, expanding and characterizing patient-derived PDAC organoids. Our current success rate of establishing organoid culture is over 70%; therefore, these methods have not yet been perfected and are expected to improve and evolve over time. Important consideration should be given to sample size, as PDAC has a low neoplastic cellularity. Consequently, small specimens will contain few tumor cells, and will only generate a handful of organoids. Additionally, many patients receive chemotherapy and/or chemoradiation-based neoadjuvant treatment prior to surgical intervention[15]. If the treatment is effective for a particular patient, the tumor tissue may be devoid of viable cells. Acquisition of chemo-naive patient samples is preferred for initial optimization of these methods, but this is not always possible. Interestingly we have found that ischemia time following surgical removal of the tumor tissue is not a major criterion for successful organoid isolation, as long as the sample is processed within 24 h.

Pancreatic ductal adenocarcinoma is a disease characterized by a strong desmoplastic reaction and deposition of a dense stromal matrix. While organoids are an excellent tool for the rapid isolation and expansion of the epithelial compartment, the model does not recapitulate the complex stroma of PDAC. Other methodologies such as patient derived xenografts[16] or air liquid interface culture[17] allow for a stromal compartment, however they may be challenging to expand quickly. When choosing a model system, the researcher should carefully consider the strengths and weaknesses of each[6].

The heterogeneous biology of this disease impacts organoid establishment as some patient-derived organoids grow extremely well in our conditions while other are much slower by comparison. The protocols above describe a Wnt ligand rich condition to isolate and expand all

patient-derived organoids, yet others have shown that some patient's tumors are able to grow in the absence of Wnt conditioned media[11,12]. Further testing will be required to determine if using a range of media conditions enhances the successful establishment of organoids, as was recently demonstrated for ovarian cancer organoids[18]. This multiplex approach is however limited by the low number of tumor cells that can be isolated from small patient samples. Additionally, normal untransformed ductal organoids can arise from an organoid isolation, particularly if the tumor tissue is adjacent to normal tissue[9]. To reduce the risk of normal organoid contamination, larger tissue samples can be subdivided into smaller independent fragments using morphological differences such as well vascularized (blood is visible) versus hypovascular regions, and hard nodules versus soft tissue.

The methods and protocols described here are the current standard approaches used in our laboratory for organoid isolation and they should be tested and adapted for each laboratory environment. For instance, the enzymatic dissociation (steps 2.6 to 2.9) of the tumor tissue is particularly important to optimize. Small equipment differences (nutator vs rotator mixer) can lead to significantly different timing for this step. Furthermore, the tissue dissociation can be fine-tuned by increasing or reducing the concentration of the Collagenase/Hyaluronidase mixture. Care must be taken to not treat all samples in the same manner. For example, in some cases organoids can be isolated from ascites fluid from advanced PDAC patients without mechanical or enzymatic dissociation.

DNA sequencing is the current gold standard to determine the presence or absence of tumor organoids as PDAC is driven by frequent mutations in KRAS, TP53, SMAD4 and CDKN2A. Transcriptomic analysis can reveal different tumor subtypes while pharmacotyping can uncover patient-specific therapeutic vulnerabilities[9]. These protocols enable PDAC researchers to develop their own library of patient-derived organoids and to profile the biology of these models.

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DISCLOSURES:

None.

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Figure 1

Click here to access/download;Figure;Jove_figure1_revised.pdf

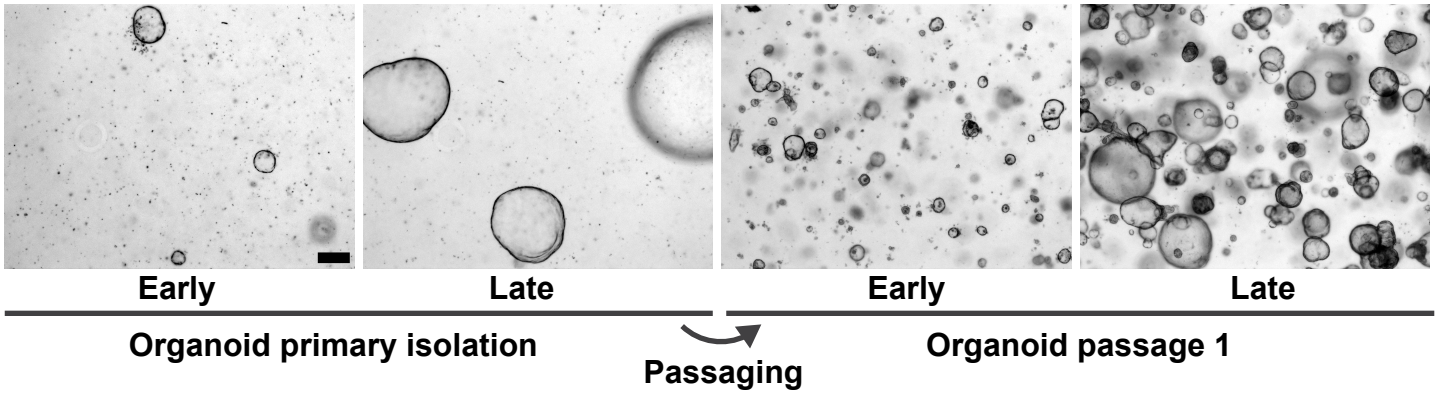
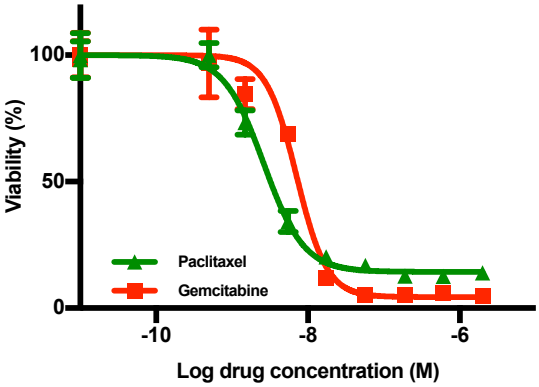
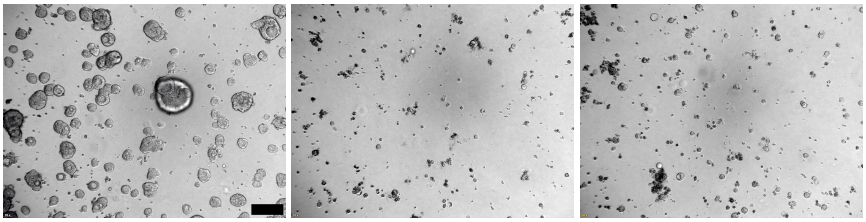


Figure 2





Name of Material/ Equipment	Company	Catalog Number
12 channel pipette (p20, p100, or p200) with tips		
12 well plates	Olympus	25-106
15 ml LoBind conical tubes	Eppendorf	EP0030122208
15 ml tube Rotator and/or nutator		
37 ˚C CO2 incubator		
37 ˚C water bath		
384 well plates	Corning	4588
A 83-01	TOCRIS	2939
ADV DMEM	ThermoFisher	12634010
Animal-Free Recombinant Human I	Peprotech	AF-100-15
Automated cell counter		
B27 supplement	ThermoFisher	17504044
Cell Recovery Solution	Corning	354253
CellTiterGlow	Promega	G7570
Chloroform	Sigma	C2432
Computer		
CryoStor CS10	StemCELL Tech	07930
Cultrex R-spondin1 (Rspo1) Cells	Trevigen	3710-001-K
DMEM	ATCC	30-2002
DNase I	Sigma	D5025
Drug printer	Tecan	D300e
Excel		
Extra Fine Graefe Forceps	Fine Science Tools	11150-10
FBS	ThermoFisher	16000044
G-418	ThermoFisher	10131035
Gastrin I (human)	TOCRIS	3006
Gentle Collagenase/hyaluronidase	STEMCELL Tech	7919
GlutaMAX	ThermoFisher	35050061
GraphPad Prism		
HEPES	ThermoFisher	15140122
Laminar flow tissue culture hood		
Luminometer		
L-Wnt-3A expressing cells	ATCC	CRL-2647
MACS Tissue Storage Solution	Miltenyi biotec	130-100-008
Matrigel Matrix	Corning	356230
Mr. Frosty Freezing Container	ThermoFisher	5100-0001

N-Acetylcysteine	Sigma	A9165
Nicotinamide	Sigma	N0636
p1000 pipette with tips		
p200 pipette with tips		
PBS	ThermoFisher	10010049
Penicillin/Streptomycin	ThermoFisher	15630080
primocin	InvivoGen	ant-pm-2
Rapid-Flow Filter Units (0.2 µm)	ThermoFisher	121-0020
Recombinant Human FGF-10	Peprotech	100-26
Recombinant Murine Noggin	Peprotech	250-38
Sterile Disposable Scalpels, #10 Bla	VWR	89176-380
Tissue culture centrifuge		
Tissue Culture Dishes 10 cm	Olympus	25-202
TRIZol	ThermoFisher	15596018
TrypLE Express	ThermoFisher	12605010
Y-27632	Sigma	Y0503
Zeocin	ThermoFisher	R25001

Comments/Description

Ultra low attachment, black and optically clear

Reagent that depolymerizes the Basement Membrane Extract at 4 ˚C
Luminescence cell viability reagent

Cell Freezing Solution

This is the drug printer we use in our laboratory
For data analysis

Glutamine solution
For data analysis

Basement Membrane Extract (BME), growth factor reduced

Acid Phenol solution

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Author(s):	Hervé Tiriac, Randall French and Andrew M. Lowy

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Institution:	University of California, San Diego
Title:	Associate Project Scientist

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Reviewers' comments:
Reviewer #1:

Manuscript Summary:
Tiriac et al describe updated methodology for the generation of organoid culture models derived from Pancreatic Ductal Adenocarcinoma patient samples. The organoid models preserve the pathogenic and genetic phenotype of the patient tumor and therefore provide an optimal model for translational studies. The authors having provided highly detailed methodology including the sourcing of all materials to ensure anyone could reproduce the generation of these organoid models as well as to use them in propagation and pharmacotyping.

We thank the reviewer for their evaluation of this protocol and thoughtful comments. To address these issues, we have added clarification to the text. Furthermore, we have responded to the questions bellow. If there are any further concerns we are happy to provide addition clarification.

Major Concerns:
No major concerns.

Minor Concerns:

Overall a very well written and documented methodology. Some corrections/additions will add clarifications.
1. (5.1.3) Please clarify why you lyse from the plate, since previously you stated that it has lots of adherent fibroblasts that attach to the plastic (section 3.1). Would these not contaminate the analysis? Would it be better to lift the BME dome then add the trizol? Maybe this is an alternate method to prevent fibroblastic contamination? Have you performed any tests that would show it does/doesn't effect qPCR analysis.

We clarified in the protocol that this procedure should be performed on organoid cultures that have been well established and passaged several times. This will dramatically reduce the contamination of non-epithelial cell types. Additionally, the nucleic acid extraction should represent all the cells in the culture as we use the DNA/RNA to determine what we are growing. We prefer not to disturb the BME as it is technically challenging. Finally, the trizol helps quickly lyses the cells and liquifies the BME allowing the user to collect good quality RNA for downstream analysis.

2. (5.2.5) Why do you dilute the BME 800:7200 in this analysis when previously you used the BME at 100%? Do the organoids form the same in both conditions? It would be prudent to show they do, otherwise you might not be treating true organoids and just treating cells in a suspension.

Organoids do from in both conditions as we have described in our Cancer Discovery article last year. The presence of organoids is required before the compounds are added to the plate and in this protocol we specify that the reader check for organoids formation 24 hrs post plating. Indeed treating single cells would defeat the purpose of the assay.

3. (lines 218-221) Please clarify whether after spinning there are one or two distinct layers (a BME layer and a separate pellet layer) or whether this is a combined (BME/organoid layer).

This is now clarified in the text. We are indeed expecting a BME/organoid layer.

4. (line 83, 94) Please clarify that filtering is after CM collection.

This is now clarified in the text.

5. (line 85) Please provide an estimate for short term and long term as these are subjective.

This is now clarified in the text.

6. (figure 1, line 379) Please provide an estimate for early (xx-xx days) and late (xx-xx days) time points.

This is now clarified in the figure legend text.

Reviewer #2:

Manuscript Summary:
Tiriac et al. describe here protocols for isolation, culture, nucleic acid isolation, and drug testing of human pancreatic cancer organoids. While the culture of patient-derived organoids, including that from pancreas tumors, is becoming more common, and variations of these protocols are described in primary literature, e.g. PMID: 29853643, there are nuances in the successful culture and use of primary pancreatic tumor organoids that are not readily transferred to other laboratories via written methods. Therefore, a visual guide to these methodologies would be of some value to the scientific community.

We thank this reviewer for their evaluation of this protocol and thoughtful comments. To address these issues, we have added clarification to the text. Furthermore, we have responded to the questions bellow. If there are any further concerns we can provide addition clarification.

Major Concerns:

While some of the caveats and limitation of PDAC organoid culture are discussed, the manuscript should include an unbiased discussion of where other methodologies, such as tumor explant or PDX models may be better suited to address specific questions, for example, the inclusion of stromal tissues.

We have now included a brief paragraph in the discussion section to highlight this point. A more detailed review comparing model systems is referenced in the text.

It would be helpful to review methods for confirming malignant origin of primary organoid cultures.

Additional text has been added to 5.1.5 to provide clarification. This new text reflects the last paragraph of the discussion thus making the point clearly for the reader.

Please compare and contrast specific differences from published protocols, e.g. those including a step to break up organoids using a needle and syringe during passage.

There are a few differences in this protocol compare to published protocols. We do not recommend using syringes for human organoids during passaging because it leads to loss of precious organoid material. Additionally, by breaking up the organoid enzymatically every few passages the cultures never accumulate very large organoids. We have added text to steps 3.8 and 3.9 to emphasize the importance of enzymatic and mechanical passaging during regular culture. Another difference that is not discussed here is the use of 12 well plates instead of the published 24 well plates. This is simply personal preference in the scaling of the culture and should not affect the use of this protocol.

Minor Concerns:
Some reagents are poorly defined early in the protocol, making it necessary for the reader to search for reagent descriptions or make assumptions, e.g. "BME" is used in the protocol and is not described, but referred to only by the specific brand name "Matrigel" in the Materials section; it is not clear if the BME is growth factor-reduced; it is not clear in the protocol without searching the Media and Materials sections that Cell Recovery Solution is a commercial reagent for depolymerizing protein matrices.

This is now clarified in the text and in the materials table.