jove

Establishment of Pancreatic Cancer-Derived Tumor Organoids and Fibroblasts From Fresh Tissue

Jesús Frutos Díaz-Alejo[*,1,2,3,4], **Simon April-Monn**[*,5], **Marina Cihova**[6], **Verona Buocikova**[6], **Jorge Villalón López**[1,3], **Maria Urbanova**[6], **Carmen G. Lechuga**[7], **Miroslav Tomas**[6,8], **Peter Dubovan**[6,8], **Bárbara Luna Sánchez**[3], **Sonia Camaño Páez**[3], **Alfonso Sanjuanbenito**[2,9], **Eduardo Lobo**[9], **Estefanía Romio de la Heras**[10], **Carmen Guerra**[2,7], **Carolina de la Pinta**[11], **Emma Barreto Melian**[1,2], **Mercedes Rodríguez Garrote**[1,2], **Alfredo Carrato**[1,2,4], **Laura Ruiz-Cañas**[3,12,13], **Bruno Sainz, Jr.**[2,3,12,13], **Ana Torres**[3], **Bozena Smolkova**[6], **Julie Earl**[1,2,3]

[1] Molecular Epidemiology and Predictive Tumor Markers Group, Area 3, Ramón y Cajal Health Research Institute (IRYCIS) [2] The Biomedical Research Network in Cancer (CIBERONC) [3] Biobank and Biomodels Platform (PT20/0045), ISCIII research and development platforms in biomedicine and health sciences, BioBank Hospital Ramón y Cajal-IRYCIS, Spanish National Biobanks Network (ISCIII Biobank Register No. B.0000678), Ramón y Cajal Health Research Institute (IRYCIS) [4] Faculty of Medicine, University of Alcalá de Henares [5] Institute of Tissue Medicine and Pathology, University of Bern [6] Department of Molecular Oncology, Cancer Research Institute, Biomedical Research Center of the Slovak Academy of Sciences [7] Experimental Oncology, Molecular Oncology Program, Centro Nacional de Investigaciones Oncológicas (CNIO) [8] Department of Surgical Oncology, National Cancer Institute, Slovak Medical University [9] Pancreatic and Biliopancreatic Surgery Unit, Hospital Universitario Ramón y Cajal [10] Department of Pathology, Hospital Universitario Ramón y Cajal [11] Department of Radiation Oncology, Hospital Universitario Ramón y Cajal [12] Department of Cancer, Instituto de Investigaciones Biomédicas “Alberto Sols” (IIBM) [13] Cancer Stem Cell and Fibroinflammatory Group, Chronic Diseases and Cancer, Area 3, IRYCIS

[*] These authors contributed equally

Corresponding Author

Julie Earl

julie.earl@live.co.uk

Citation

Díaz-Alejo, J.F., April-Monn, S., Cihova, M., Buocikova, V., Villalón López, J., Urbanova, M., Lechuga, C.G., Tomas, M., Dubovan, P., Sánchez, B.L., Páez, S.C., Sanjuanbenito, A., Lobo, E., Romio de la Heras, E., Guerra, C., de la Pinta, C., Barreto Melian, E., Rodríguez Garrote, M., Carrato, A., Ruiz-Cañas, L., Sainz, Jr., B., Torres, A., Smolkova, B., Earl, J. Establishment of Pancreatic Cancer-Derived Tumor Organoids and Fibroblasts From Fresh Tissue. *J. Vis. Exp.* (), e65229, doi:10.3791/65229 (2023).

Date Published

May 10, 2023

DOI

10.3791/65229

Abstract

Tumor organoids are three-dimensional (3D) *ex vivo* tumor models that recapitulate the biological key features of the original primary tumor tissues. Patient-derived tumor organoids have been used in translational cancer research and can be applied to assess treatment sensitivity and resistance, cell-cell interactions, and tumor cell interactions with the tumor microenvironment. Tumor organoids are complex culture systems that require advanced cell culture techniques and culture media with specific growth factor cocktails and a biological basement membrane that mimics the extracellular environment. The ability to establish primary tumor cultures highly depends on the tissue of origin, the cellularity, and the clinical features of the tumor, such as the tumor grade. Furthermore, tissue sample collection, material quality and quantity, as well as correct biobanking and storage are crucial elements of this procedure. The technical capabilities of the laboratory are also crucial factors to consider. Here, we report a validated SOP/protocol that is technically and economically feasible for the culture of *ex vivo* tumor organoids from fresh tissue samples of pancreatic adenocarcinoma origin, either from fresh primary resected patient donor tissue or patient-derived xenografts (PDX). The technique described

URL

jove.com/t/65229

herein can be performed in laboratories with basic tissue culture and mouse facilities and is tailored for wide application in the translational oncology field.

Introduction

Tumor organoids are *ex vivo* three-dimensional (3D) organized cultures that are derived from fresh tumor tissue and provide cancer models. Tumor organoids recapitulate the biological key features of the original primary tumor[1,2,3,4] and can be expanded for up to several months and cryopreserved, similar to conventional immortalized cell lines. Tumor organoids provide a biobank of patient-derived tumor models for translational/personalized medicine[5] and represent an important advance in cancer cell biology systems/models. Patient-derived tumor organoids can be used as *ex vivo* models to predict the efficacy of (neo)adjuvant oncological/pharmacological therapies, for which cultures are established from fresh tumor tissue and drug sensitivity assays or pharmacotyping are performed on a patient-specific basis to identify effective agents for subsequent lines of therapy[1,4]. Furthermore, tumor organoids overcome the limitation of the availability of primary tumor tissue and, more importantly, provide an excellent alternative or complementary system to *in vivo* mouse models, such as patient-derived xenografts (PDX)[2]. The complexity of tumor organoids is increased if the primary tumor cells are combined with stromal cells that are found in the tumor microenvironment (TME), such as cancer-associated fibroblasts (CAFs), endothelial cells, and immune cells, which mimic the functioning and complex cellularity of the primary tumor. Tumor organoids have been established for many tumor types using standardized protocols[6,7,8,9,10]. Organoid propagation from different solid tumors, including colorectal and breast cancer tissue, is well-established and technically affordable[11,12,13,14,15].

Surgical tumor resections or tumor biopsies provide primary tumor tissue specimens. Ideally, tumor tissue specimens should come from the center of the tumor mass or the invading edge of the tumor, as well as normal-looking tissue adjacent to the tumor. Compared to conventional 2D cultures, tumor organoids require several "add-ons", including a biological basement membrane (such as Matrigel, hydrogel, or a collagen-based scaffold), which mimics the extracellular TME, and a liquid growth medium that supplies specific nutrients and growth factors and supports cell proliferation and viability in culture[16].

The most basic steps in primary cell culture are washing the tissue in saline solution to prevent contamination, mechanically cutting/digesting the tumor into small pieces of 1-3 mm^3, and treatment with collagenase for the enzymatic digestion of the tissue. The digested mix is then filtered to remove large tissue fragments, resuspended in a biological basement membrane such as Matrigel, and plated as domes in low-attachment culture plates to enhance non-attachment growth. The basement membrane matrix domes are covered with liquid culture medium and supplemented with glutamine and antibiotics to avoid contamination, as well as with specific growth factors depending on the tissue type[7,8,9,16,17]. Other relevant cells present within the bulk tumor and the TME may also be isolated, such as cancer-associated fibroblasts (CAFs) and immune cells. This technique, which has recently been reviewed[18], allows the establishment of co-cultures

with different cell types to study the response to therapy in a more "realistic" tumor environment. Furthermore, cell-cell interactions and the interaction between tumor cells and components of the surrounding biological matrix can be studied.

The reported success rate of tumor organoid establishment using fresh tissue from biopsies or resected gastrointestinal tumor tissue is around 50%[11], and the success rate from the latter is largely dependent on the tissue type and origin[4], particularly the tumor grade and overall tumor cellularity. Three-dimensional tumor models have varying complexity, from simple unicellular aggregates to highly complex engineered models consisting of various cell types. The terminology used to describe 3D cultures in the literature is highly inconsistent[19,20,21], as different terms such as spheroids, tumorspheres, and organoids are used, although the difference between them is unclear. As a clear consensus on the definition has not yet been reached, in this article, a tumor organoid is described as an organized tumor cell culture embedded into a biological basement membrane.

Herein, a validated protocol is reported for the establishment of tumor organoids from fresh tissue samples originating from fresh primary resected or PDX-derived pancreatic ductal adenocarcinoma (PDAC), and this protocol can be performed in most laboratories with basic tissue culture facilities. This protocol has been adapted from several state-of-the-art reported protocols that are currently used to establish tumor organoids or tumoroids from digestive tumor tissue from the groups of David Tuveson[9], Hans Clevers[8], and Aurel Perren[7].

This protocol does not discuss how the fresh tissue is harvested. To obtain high-quality fresh human tumor tissue, it is important to have efficient coordination between the surgeons that harvest the tissue and the pathology department that extracts the tissue sample for organoid culture. Likewise, when using PDX as a fresh tissue source, efficient coordination with the person that harvests the tissue sample is also important. It is critical to obtain the tissue sample as quickly as possible (within 30-60 min from harvesting time) in order to maintain a high quality.

Protocol

All procedures were performed in compliance with the institutional guidelines for the welfare of experimental animals approved by the Universidad Autónoma de Madrid Ethics Committee (CEI 103-1958-A337) and La Comunidad de Madrid (PROEX 294/19) and in accordance with the guidelines for Ethical Conduct in the Care and Use of Animals as stated in The International Guiding Principles for Biomedical Research Involving Animals, developed by the Council for International Organizations of Medical Sciences (CIOMS). The protocol followed the ethical principles for biomedical research with written informed consent. Prior ethical approval was obtained for the use of fresh tissue for the establishment of the tumor organoid cultures. The samples were provided by the BioBank Hospital Ramón y Cajal-IRYCIS (National Registry of Biobanks B.0000678), integrated into the Biobanks and Biomodels Platform of the ISCIII (PT20/00045), and processed following standard operating procedures with the appropriate ethical approval. The tumors were subcutaneously implanted, as previously described[22], into immunocompromised 6 week old female NU-Foxn1nu nude mice (see **Table of Materials**) and passaged *in vivo* to establish PDAC PDXs.

1. Experimental preparation

1. Handle human samples in a class II biosafety cabinet.

2. Wear a lab coat, protective gloves, and glasses throughout the procedure to avoid infection by tissue-borne pathogens.

   NOTE: A minimum of 3 h is required to process the fresh tissue sample and plate the organoids and fibroblasts.

3. Process the fresh tissue samples[22] within a maximum of 24 h from harvesting, and store at 4 °C in tissue culture medium (DMEM supplemented with 10% FBS and 1% penicillin-streptomycin) until processing.

4. Keep all the samples and reagents on the ice during the entire procedure, and ensure to pre-set a refrigerated centrifuge to 4 °C and use it at a low speed to avoid damaging the cell preparation.

5. Store P1,000 and P200 pipette tips in a −20 °C freezer to use during the protocol.

6. Aliquot the basement membrane matrix (see **Table of Materials**) prior to use. For this protocol, 250 mL aliquots are recommended.

2. Processing fresh primary tumor tissue to establish tumor organoids and primary fibroblasts

NOTE: A minimum of 3 h is required to process the fresh tissue sample (either primary human resectable tumors or PDXs) and to plate the tumor organoids and fibroblasts. An outline of the organoid preparation process is shown in **Figure 1**, from tissue digestion to the plating of the tumor organoids. Before starting the protocol, take out an aliquot of the basement membrane matrix from the −20 °C freezer, and leave it on ice for approximately 30-60 min before use.

1. Put a 6-well culture plate in a 37 °C cell culture incubator 3 h before plating the organoids.

2. Measure, photograph, and wash the tissue (step 1.3) with 3 mL of Advanced DMEM-F12 (Dulbecco's Modified Eagle's Medium (DMEM)/Nutrient Mixture F-12 Ham (F12), supplemented with 5% fetal bovine serum (FBS), 15 mM Hepes, 1% L-Glutamine, 1% Penicillin-Streptomycin, 125 ng/mL Amphotericin B, 0.1 mg/mL Normocin) (see **Table of Materials**) by pipetting up and down and then wash with 3 mL of phosphate-buffered saline (PBS) by pipetting up and down.

3. Remove the media by aspiration from the tissue culture plate and cut the tissue into 1 mm^3 pieces in a sterile tissue culture plate using two sterile blades and 2 mL of Digestion Medium (Advanced DMEM-F12 + 10 mg Collagenase IV/mL, 0.000625% Trypsin-EDTA, and 10 µg/mL DNase). Collect the tissue in a 50 mL tube with 5 mL total of Digestion Media and incubate for 1 h at 37 °C with mechanical digestion with commercially available equipment (see **Table of Materials**) or with periodic mixing of the sample, by pipetting the sample up and down.

4. Add 5 mL of Advanced DMEM-F12 to inactivate the trypsin, and filter the sample through a 70 µm pore strainer to remove large debris.

5. Add 2 mL of DMEM with 10% FBS to the top of the filter, and collect the debris and tissue remnants to establish cultures of fibroblasts (see step 5).

6. Centrifuge the filtrate at 200-300 x *g* for 5 min at room temperature, and aspirate the supernatant, leaving only the cell pellet. Add 5 mL of Advanced DMEM-F12, and centrifuge for 5 min at 300 x *g*.

7. Resuspend the pellet in 4 mL of ammonium-chloride potassium (ACK, see **Table of Materials**) lysis buffer.

Pass the mixture to a 15 mL tube, and incubate at room temperature for 2 min to lyse the red blood cells.

NOTE: This red blood cell lysis step can be removed when there is no visible evidence of contamination.

8. Centrifuge at 300 x *g* for 5 min at room temperature, and aspirate the supernatant. Add 5 mL of Advanced DMEM-F12, and centrifuge for 5 min at 300 x *g*.
9. Add 1 mL of commercially available cell dissociation reagent (see **Table of Materials**) supplemented with DNase (1 µg/mL) to the cell pellet, and incubate for 2-3 min at room temperature.
10. Centrifuge at 300 x *g* for 5 min, and aspirate the supernatant. Add 5 mL of Advanced DMEM-F12, and resuspend the cell pellet.
11. Pipette up and down 30 times with a P1,000 pipette to dissociate the cells, centrifuge for 5 min at 300 x *g*, and remove the supernatant.
12. Take P1,000 and P200 pipette tips from the −20 °C freezer, and resuspend the cell pellet in the membrane matrix using a P1,000 pipette and cold tips (50 µL per drop, taking into account the pellet volume, six to seven domes per well). Take out the previously heated plate from the incubator. Set a P200 pipette to 48 µL, and use cold tips to create domes of the basement membrane matrix in the pre-heated 6-well plate.
13. Put the plate with the basement membrane matrix-cell domes into the 37 °C 5% CO_2 incubator for 15 min to solidify the basement membrane matrix.
14. Add 2-2.5 mL of Advanced DMEM-F12, supplemented with 20 ng/mL recombinant human epidermal growth factor (EGF), 100 ng/mL human placenta growth factor (PlGF), 10 ng/mL recombinant human basic fibroblast growth factor (bFGF), 769 ng/mL insulin-like growth factor-1 (IGF-1), and 10.5 µM ROCK Inhibitor (Advanced DMEM-F12 + growth factors) (see **Table of Materials**).

3. Monitoring the organoids

1. Monitor the tumor organoid culture visually for the first 7 days and then three times per week thereafter.
2. Change the medium twice per week with Advanced DMEM-F12 containing 20 ng/mL recombinant human epidermal growth factor (EGF), 100 ng/mL human placenta growth factor (PlGF), 10 ng/mL recombinant human basic fibroblast growth factor (bFGF), 769 ng/mL insulin-like growth factor-1 (IGF-1), and 10.5 µM ROCK inhibitor (Advanced DMEM-F12 + growth factors).
3. Capture images of the tumor organoid culture periodically on day 1, day 3, day 7, day 10, and day 15 after processing the sample and plating the culture in the matrix to observe the growth and viability.

4. Passage and cryostorage of the organoids

1. Remove the culture medium from the 6-well plate.
2. Add 1 mL of an appropriate cell recovery reagent (see **Table of Materials**) to disassociate the basement membrane matrix and obtain a cell suspension, and recover the supernatant in a 15 mL tube. If the matrix does not dissociate immediately, incubate the sample at 4 °C for 15-20 min until completely dissolved.
3. Add 1 mL of the same cell recovery reagent used in step 4.2 to the well of the plate to recover additional dissociated cells, and add the supernatant to the same 15 mL tube as in step 4.2.
4. Add 5 mL of cold Advanced DMEM-F12, and centrifuge at 200 x *g* for 5 min.

5. Remove the supernatant, and dry the pellet carefully by removing any remaining liquid with a 5 mL pipette; avoid moving the tube as much as possible. Resuspend the cell pellet in twice the original volume of basement membrane matrix in order to obtain a passage of 1:2.
6. For the cryostorage of the organoid cultures, resuspend the pellet obtained in step 4.5 in 1 mL of freezing medium (10% DMSO, 20% FBS, 50% DMEM-F12, 10.5 µM ROCK inhibitor), and store at −80 °C.
   NOTE: The volume of basement membrane matrix can be adjusted to perform different passages, such as 1:1 (using the same volume of basement membrane matrix as the original) or 1:3 (adding three times the original volume of basement membrane matrix).

5. Establishment of fibroblasts

1. After recovering the debris and tissue remnants in 2 mL of DMEM with 10% FBS, add this to a 6-well plate, and incubate at 37 °C with 5% CO_2.
2. Remove the tissue remnants from the fibroblast cultures from step 2.5 by the aspiration of the medium 2 days or 3 days after plating, and replace with fresh DMEM with 10% FBS.
3. Monitor the fibroblast culture visually three times per week, and change the culture medium at least twice per week using DMEM supplemented with 10% FBS.

Representative Results

It is important to document how the tumor organoid culture progresses over time, particularly in the first few weeks, in order to estimate how the culture will behave in downstream assays. **Figure 2** shows an example of optimal tumor cell isolation and tumor organoid establishment from fresh tissue over a 15 day period. Sometimes, there is a large volume of cell debris in the sample, and it is difficult to see the developing tumor organoids, as shown in **Figure 3**. Furthermore, the phenotype of the developing organoids can vary from isolated, rounded organoids (**Figure 4A**) to spheroid/aggregate-like cultures (**Figure 4B-D**), and this depends on the origin of the tumor. Fibroblasts are a common by-product of primary tumor cell culture establishment from solid tumors, especially those with a high stroma content, and can often contaminate the organoid culture. **Figure 5** shows how these cells migrated from the basement membrane matrix domes and adhered to the culture plates after approximately 7 days. These cells consume the nutrients from the culture medium, thus compromising the optimal growth of organoids.

Primary fibroblasts can be obtained as an isolated culture when they migrate out of the tissue sections, adhere to the culture plates, and grow as a mono-layer culture, as shown in **Figure 6**. This protocol recommends using standard DMEM medium with 10% FBS for the culture of the fibroblasts. However, a specialized medium for fibroblasts can also be used. Adherent fibroblasts are visible approximately 7 days post-plating (**Figure 6**).

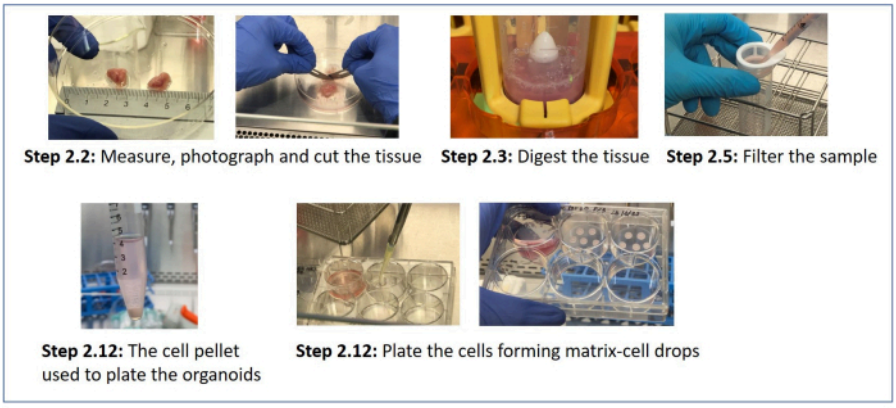

Figure 1: Processing fresh tissue samples to establish tumor organoids. An outline of the tumor organoid preparation process is shown, from tissue digestion to the plating of the organoids. Please click here to view a larger version of this figure.

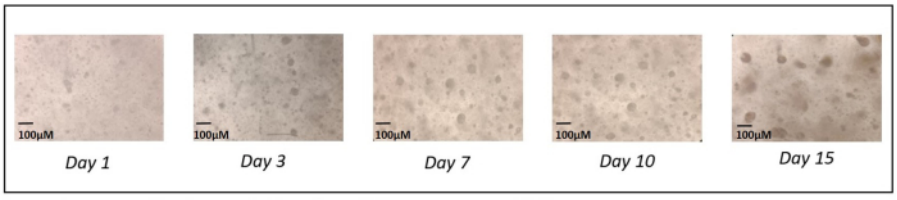

Figure 2: A tumor organoid culture established from a PDX originating from a fresh PDAC tumor. The progression of the tumor organoid culture over several days is shown, with images of the organoids in one microscopic plane on day 1, day 3, day 7, day 10, and day 15. Scale bar: 100 µm. Please click here to view a larger version of this figure.

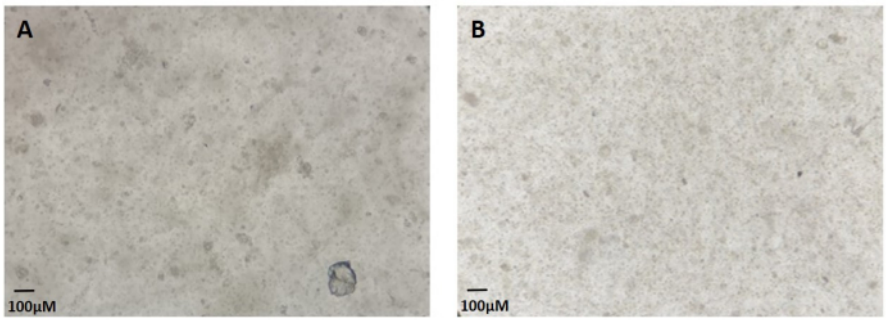

Figure 3: Examples of a non-optimal tumor organoid culture on day 2 with excess cell debris. Scale bar: 100 µm. Please click here to view a larger version of this figure.

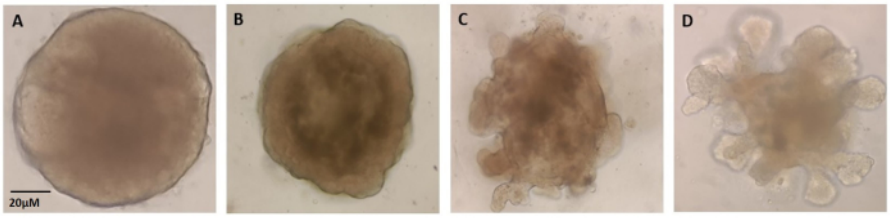

Figure 4: Established tumor organoids from different PDAC PDX tissues showing differences in size and morphology. Tumor organoids can have a (**A**) classical rounded phenotype or (**B-D**) a more spheroid/aggregate-type appearance. The average size of the organoids ranges from 80 uM to 100 uM, although some organoids may be as large as 200 uM. Scale bar: 20 µm. Please click here to view a larger version of this figure.

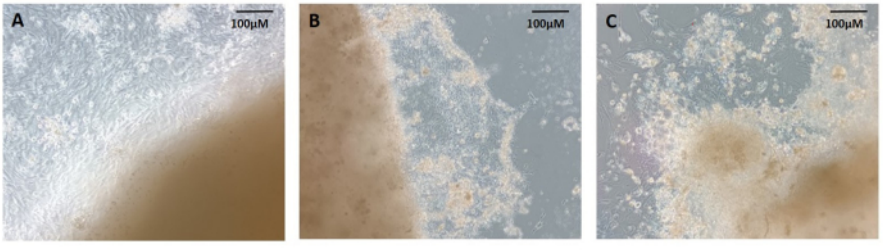

Figure 5: Examples of a non-optimal tumor organoid culture (A-C) contaminated with CAFs/fibroblasts after 30 days in culture. Scale bar: 100 µm. Please click here to view a larger version of this figure.

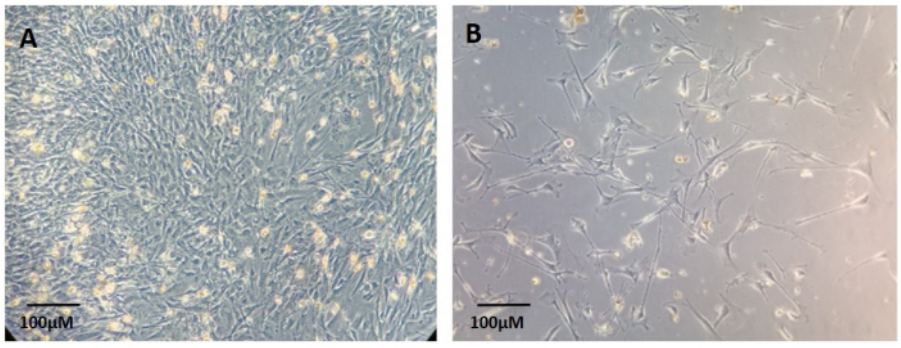

Figure 6: Examples of primary fibroblast cultures established from the remnants of the digested primary tumor tissue. (**A**) Confluent culture. (**B**) Non-confluent culture. Scale bar: 100 µm. Please click here to view a larger version of this figure.

Supplementary File 1: Troubleshooting of tumor organoid culture establishment. Please click here to download this File.

Discussion

Major advances in pharmacological cancer therapies are challenging, as the likelihood of the approval of drugs in phase I oncology clinical trials is 5.1%, which is the lowest of all disease types[23]. The main reason is that cancer is very heterogenous, and therefore, patient cohorts do not uniformly respond as expected to the given treatment, which highlights that a more personalized approach is needed. Two-dimensional (2D) cultures have been used in translational cancer research for many years but lack the structural 3D organization found in primary tumors. Thus, they do not accurately reflect patient therapy responses and tumor cell communication with one another or with their microenvironment[3,23]. The underlying core principle of all 3D culture systems is to promote cellular interaction with the surroundings and to organize cells in a spatially relevant manner, similar to in the primary tumor (*in situ*). The morphology of tumor organoids varies from round, mass, and grape-like to stellate depending on the inherent nature of cultured cells and the culture conditions used, as shown in **Figure 4**. Established patient-derived tumor organoid cultures can be treated with the same first-line treatment used in the patient in order to confirm that the tumor organoid recapitulates the clinical situation (i.e., the clinical therapy response)[1,13,24,25].

The tumor organoids can also be treated with novel pharmacological agents, which provides an exploratory approach for identifying novel therapies that could be used in subsequent treatment lines in the clinic when standard therapy options have been exhausted. The culture is continuously monitored to assess the response of the tumor organoid cultures to the agent in real-time. A feasibility study of the molecular and immunohistochemical characterization of patient-derived xenograft tumor (PDX) and PDX-derived organoids showed that the morphology, protein expression, and genomic alterations were comparable between the two models. Furthermore, a pharmacotyping proof-of-concept

study showed that the *in vivo* and *ex vivo* predictions were also comparable and, thus, concluded that using tumor organoids as a cancer/disease model is a feasible and robust approach that can be applied in the clinical setting[2].

A range of challenges related to the establishment of tumor organoid cultures originating from different tumor types have been highlighted herein and recently reviewed[26,27,28]. Key issues include "contamination/overgrowth" by healthy cells, which form organoids with a higher growth rate than cancer cells, the high cost of culture maintenance compared to 2D cultures, the low establishment rate, inconsistencies in the somatic mutation profile and histology between the *ex vivo* culture and the tumor of origin, primary tumor heterogeneity, the use of a murine-based extracellular matrix, which may not be optimal for human cells and may impede drug delivery, the absence of the TME and associated cells, the interference of growth factors or molecular inhibitors with the drug response, and the lack of standardized and validated protocols for the establishment and maintenance of tumor organoids. However, over the years, the use of primary tumor models in cancer research has increased, and techniques to culture and maintain primary cultures have improved. These delicate *ex vivo* cultures are established and maintained by adding supplements to promote cell growth and stability. The Rho kinase inhibitor (ROCK) is now routinely added to primary cultures to avoid apoptosis and enhance cell-cell adhesion, thus promoting the long-term expansion of stable primary *in vitro* cultures[29]. Furthermore, it also increases the survival of thawed cryopreserved stem cells, which is important when generating frozen stocks of organoid cultures[29]. Supplementation with heparin may also be used to enhance the expansion of stem cells by increasing WNT and FGF signaling and, thus, cell proliferation[30]. Other important reagents used in this validated protocol to optimize tumor organoid establishment include Accutase and DNase. Accutase is a gentle digestion reagent recommended to segregate individual stem cells or detach them from the culture surfaces, and it helps to maintain cell viability better than using other reagents designed for this purpose, such as trypsin[31]. However, collagenases may also be used to specifically target the collagen type incorporated into the biological basement membrane. DNase has been used in cell isolation methods for many years[32] to eliminate DNA remnants from dead and necrotic cells during the extraction protocol and, thus, prevent the clumping/aggregation of cells due to the increased viscosity of the sample preparation. Cell pellets are often treated with lysis buffer, but this step can be excluded when there is no visible evidence of red blood cell contamination of the tissue sample[8] (**Supplementary File 1**).

The successful establishment of tumor organoids does not come without inherent biological complications. Ideally, tissues for tumor organoid cultures should be processed on the same day as the harvesting to optimize cell viability. The freezing of tissues is not always recommended as it may significantly reduce cell viability. However, tumor organoids can be established using samples that have been flash-frozen or slowly cryopreserved in DMSO-containing freezing medium[33,34], which allows sample shipment and biobanking. In some cases, it is possible to obtain 2D cultures from tumor organoid cultures that have been passaged several times by the recovery of the tumor cells attached to the plates after removing the basement membrane matrix domes. In our experience, the success rate of tumor organoid establishment is much higher with PDX PDAC tissue than with fresh PDAC tissue derived from a resected specimen[4]. This is due to the fact that larger pieces of fresh PDX PDAC tissue are usually available, and this tissue has higher cellularity, likely due to the more hospitable *in vivo* environment provided by

the PDX model compared with *ex vivo*-based approaches. Non-toxic agents can be added to the culture medium to monitor the cell viability and the proliferation of the culture. Furthermore, the overgrowth of non-neoplastic organoids must be avoided using selective conditions[35,36]. Pancreas tissue may contain digestive enzymes that could affect the viability of the isolated tumor cells; thus, trypsin may be added to the digestion step to overcome this problem[4]. Many other factors, such as the status of the patient/tissue (i.e., treated or untreated with oncological therapy), the type of tumor, the contamination of the sample from the operating room, and the original grade of the tumor, can also significantly influence the ability to establish a tumor organoid. Likewise, not every tissue has enough viable tumor cells to establish primary cultures. This is particularly true for PDAC tumors, which are notoriously stroma-rich (approximately 90%-95%) and contain a small percentage of epithelial tumor cells. In addition, tissues with a high stroma content may be difficult to cut and dissociate to obtain single tumor cells for culture. Specialized and commercially available digestion media can be added, or mechanical dissociation equipment can also be used if the tissue is not fully digested. Alternatively, a thorough tissue dissociation can be achieved by continuous cutting using two scalpel blades until the tissue has a semi-liquid appearance (see video demonstration).

There are many technical pitfalls during the procedure that must also be highlighted. For example, tissue remnants may block the filter, and the filter must then be changed, as the remnants impede the passage of the liquid supernatant. The filtration step can be repeated several times to recover the maximum amount of dissociated tumor cells. Furthermore, it is important to check the cell pellet after each centrifugation step as sometimes it is difficult to see when there is a low cell number. The pipetting of the cell pellets should be performed gently and slowly, as aggressive pipetting may result in a loss of cells or a reduction in the cell viability. Furthermore, when preparing the matrix-cell mix, media precooled to 4 °C and refrigerated tips stored at −20 °C should be used, as the matrix solidifies quickly. Finally, and as mentioned above, DNase helps prevent the clumping of cells during sample preparation. Aliquoting the reagents used in the protocol and preparing growth factors mixes are also recommended to avoid the repetitive freeze-thawing of the reagents.

The isolation of primary fibroblasts is of interest, as they are an important cell type in PDAC and can be used for subsequent co-culture experiments to determine the effects of the TME on tumor cell growth. After around 1 week, the fibroblasts have migrated out of the tissue sections and can usually be seen attached to the culture plates and growing as a monolayer culture. However, the presence of fibroblasts in the culture also has disadvantages, as they consume the nutrients from the culture medium and, thus, compromise the optimal growth of the tumor organoids. Ultra-low attachment (ULA) plates are often recommended for culturing tumor organoids to limit fibroblast growth, as they easily attach to standard cell culture-treated plates. This validated protocol recommends the use of standard DMEM media with 10% FBS for the culture of primary fibroblasts, although specialized media for fibroblasts may also be used.

It is important to document the state of the tumor organoid culture over time, especially during the first week, as the culture behaves differently depending on the tissue quality, quantity, and source (primary human resectable tumors versus PDXs). Non-toxic agents can be added to the culture medium to monitor the cell viability and the proliferation of the culture. The cultures must be visually monitored daily during the first 7 days and then two to three times per

week. Furthermore, the medium must be changed every 4-5 days or when it appears acidic (appears yellow), taking care not to damage the basement membrane matrix domes. At first, large brown clumps may appear due to a high cell density. However, this should disappear after the first passage, thus allowing clearly defined tumor organoids to be obtained on a clear background of the basement membrane matrix. Taking photographs of the tumor organoids is recommended, especially during the first 3 weeks, in order to determine the visual phenotype and growth rate of the tumor organoids. The passage of the tumor organoid culture is also a technical challenge. Generally, the culture medium is removed, and the matrix domes are disassociated with trypsin-based or specialized reagents at 4 °C until the basement membrane matrix completely dissolves. The cell pellet is then resuspended in fresh matrix, and the volume is adjusted to perform the desired passage, such as 1:1 (using the same matrix volume as the original) or 1:3 (adding three times the original volume of the approach). The group of David Tuveson, comprising experts in pancreatic organoids, provides online resources with protocols for establishing, maintaining, and staining pancreatic organoids[37].

Patient-derived *ex vivo* tumor organoids provide a valuable preclinical model for assessing patient-specific treatment sensitivity for application in personalized medicine. Furthermore, these models are more physiologically relevant tumor models compared to traditional 2D adherent cell monolayers. A clear and consistent nomenclature of 3D tumor models with periodic photographic and descriptive follow-up of the culture is needed in the tumor model field. Moreover, working with standardized protocols is of the utmost importance for the success of this technology in the clinic in determining drug sensitivity on a patient-specific basis. Researchers using this technology should be aware of the obstacles and pitfalls of these sensitive and complex models. However, working with a validated protocol and clearly defined conditions and troubleshooting options will aid in avoiding technical issues and optimize the establishment and maintenance of the culture. Here, a validated protocol is provided for establishing tumor organoids and isolating fibroblasts that can be readily implemented in many translational oncology laboratories with standard equipment and tissue culture experience.

Disclosures

None.

Acknowledgments

This study was supported by funding from the Plataforma biobancos y biomodelos - Unidades de las Plataformas ISCIII de apoyo ala I+D+i en Biomedicina y Ciencias de la Salud (PT20/00045), The European Union's Horizon 2020 Research and Innovation Programme under grant agreement No. 857381, project VISION (Strategies to strengthen scientific excellence and innovation capacity for early diagnosis of gastrointestinal cancers), Intramural call for new research projects for clinical researchers and emerging research groups IRYCIS (2021/0446) and the TRANSCAN II project JTC 2017 call "Establishing an algorithm for the early diagnosis and follow-up of patients with pancreatic neuroendocrine tumours (NExT)", grant number 1.1.1.5/ ERANET/20/03. The biological samples used in this protocol were provided by the BioBank Hospital Ramón y Cajal-IRYCIS (B.0000678) and integrated into the Biobanks and Biomodels Platform of the ISCIII (PT20/00045). We would also like to thank Yvonne Kohl, Agapi Kataki Vita Rovita, and Thorsten Knoll for their invaluable support to develop this protocol as part of the NExT and VISION projects.

References

1. April-Monn, S. L. et al. Patient-derived tumoroids of advanced high-grade neuroendocrine neoplasms mimic patient chemotherapy responses and guide the design of personalized combination therapies. *bioRxiv.* (2022).
2. Frappart, P. O. et al. Pancreatic cancer-derived organoids - A disease modeling tool to predict drug response. *United European Gastroenterology Journal.* **8** (5), 594-606 (2020).
3. Tiriac, H. et al. Organoid profiling identifies common responders to chemotherapy in pancreatic cancer. *Cancer Discovery.* **8** (9), 1112-1129 (2018).
4. Aberle, M. R. et al. Patient-derived organoid models help define personalized management of gastrointestinal cancer. *The British Journal of Surgery.* **105** (2), e48-e60 (2018).
5. Yoshida, G. J. Applications of patient-derived tumor xenograft models and tumor organoids. *Journal of Hematology & Oncology.* **13** (1), 4 (2020).
6. Hong, H. K. et al. Efficient primary culture model of patient-derived tumor cells from colorectal cancer using a Rho-associated protein kinase inhibitor and feeder cells. *Oncology Reports.* **42** (5), 2029-2038 (2019).
7. April-Monn, S. L. et al. 3D primary cell culture: A novel preclinical model for pancreatic neuroendocrine tumors (PanNETs). *Neuroendocrinology.* **111** (3), 273-287 (2020).
8. Pleguezuelos-Manzano, C. et al. Establishment and culture of human intestinal organoids derived from adult stem cells. *Current Protocols in Immunology.* **130** (1), e106 (2020).
9. Boj, S. F. et al. Organoid models of human and mouse ductal pancreatic cancer. *Cell.* **160** (1-2), 324-338 (2015).
10. Driehuis, E., Kretzschmar, K., Clevers, H. Establishment of patient-derived cancer organoids for drug-screening applications. *Nature Protocols.* **15** (10), 5739 (2020).
11. Yu, J., Huang, W. The progress and clinical application of breast cancer organoids. *International Journal of Stem Cells.* **13** (3), 295 (2020).
12. Barbáchano, A. et al. Organoids and colorectal cancer. *Cancers.* **13** (11), 2657 (2021).
13. Michels, B. E. et al. Human colon organoids reveal distinct physiologic and oncogenic Wnt responses. *Journal of Experimental Medicine.* **216** (3), 704-720 (2019).
14. Van De Wetering, M. et al. Prospective derivation of a living organoid biobank of colorectal cancer patients. *Cell.* **161** (4), 933-945 (2015).
15. Sato, T. et al. Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology.* **141** (5), 1762-1772 (2011).
16. Porter, R. J., Murray, G. I., McLean, M. H. Current concepts in tumour-derived organoids. *British Journal of Cancer.* **123** (8), 1209-1218 (2020).
17. Wang, Q., Guo, F., Jin, Y., Ma, Y. Applications of human organoids in the personalized treatment for digestive diseases. *Signal Transduction and Targeted Therapy.* **7** (1), 336 (2022).
18. Wang, J. et al. Patient-derived tumor organoids: New progress and opportunities to facilitate precision cancer immunotherapy. *Frontiers in Oncology.* **12**, 1382 (2022).

19. Hirschhaeuser, F. et al. Multicellular tumor spheroids: An underestimated tool is catching up again. *Journal of Biotechnology.* **148** (1), 3-15 (2010).
20. Weiswald, L. B., Bellet, D., Dangles-Marie, V. Spherical cancer models in tumor biology. *Neoplasia.* **17** (1), 1-15 (2015).
21. Marsee, A. et al. Building consensus on definition and nomenclature of hepatic, pancreatic, and biliary organoids. *Cell Stem Cell.* **28** (5), 816-832 (2021).
22. Mueller, M. T. et al. Combined targeted treatment to eliminate tumorigenic cancer stem cells in human pancreatic cancer. *Gastroenterology.* **137** (3), 1102-1113 (2009).
23. Wong, C. H., Siah, K. W., Lo, A. W. Estimation of clinical trial success rates and related parameters. *Biostatistics.* **20** (2), 273-286 (2019).
24. Ooft, S. N. et al. Patient-derived organoids can predict response to chemotherapy in metastatic colorectal cancer patients. *Science Translational Medicine.* **11** (513), eaay2574 (2019).
25. Chen, P. et al. Patient-derived organoids can guide personalized-therapies for patients with advanced breast cancer. *Advanced Science.* **8** (22), 2101176 (2021).
26. Furbo, S. et al. Use of patient-derived organoids as a treatment selection model for colorectal cancer: A narrative review. *Cancers.* **14** (4), 1069 (2022).
27. Xu, H., Jiao, D., Liu, A., Wu, K. Tumor organoids: Applications in cancer modeling and potentials in precision medicine. *Journal of Hematology & Oncology.* **15** (1), 58 (2022).
28. Xu, H. et al. Organoid technology in disease modelling, drug development, personalized treatment and regeneration medicine. *Experimental Hematology & Oncology.* **7** (1), 30 (2018).
29. Watanabe, K. et al. A ROCK inhibitor permits survival of dissociated human embryonic stem cells. *Nature Biotechnology.* **25** (6), 681-686 (2007).
30. Ling, L. et al. Effect of heparin on the biological properties and molecular signature of human mesenchymal stem cells. *Gene.* **576** (1 Pt 2), 292-303 (2016).
31. Bajpai, R., Lesperance, J., Kim, M., Terskikh, A. V. Efficient propagation of single cells Accutase-dissociated human embryonic stem cells. *Molecular Reproduction and Development.* **75** (5), 818-827 (2008).
32. Gonzalez, R. F., Dobbs, L. G. Isolation and culture of alveolar epithelial Type I and Type II cells from rat lungs. *Methods in Molecular Biology.* **945**, 145-159 (2013).
33. Walsh, A. J. et al. Drug response in organoids generated from frozen primary tumor tissues. *Scientific Reports.* **6**, 18889 (2016).
34. Bui, B. N. et al. Organoids can be established reliably from cryopreserved biopsy catheter-derived endometrial tissue of infertile women. *Reproductive BioMedicine Online.* **41** (3), 465-473 (2020).
35. Verissimo, C. S. et al. Targeting mutant RAS in patient-derived colorectal cancer organoids by combinatorial drug screening. *eLife.* **5**, e18489 (2016).
36. Drost, J. et al. Sequential cancer mutations in cultured human intestinal stem cells. *Nature.* **521** (7550), 43-47 (2015).
37. Cold Spring Harbor Laboratory. Tuveson Lab. Protocols for Generating, Manipulating, and Analyzing Pancreatic Organoid Cultures. <https://tuvesonlab.labsites.cshl.edu/protocolsreagents/>.