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Budget-Friendly Generation, Biochemical Analyses, and Lentiviral Transduction of Patient-Derived Colon Organoids

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For the past decade, three-dimensional (3D) culture models have been emerging as powerful tools in translational research to overcome the limitations of two-dimensional cell culture models. Thanks to their ability to recapitulate the phenotypic and molecular heterogeneity found in numerous organs, organoids have been used to model a broad range of tumors, such as colorectal cancer. Several approaches to generate organoids exist, with protocols using either pluripotent stem cells, embryonic stem cells, or organ-restricted adult stem cells found in primary tissues, such as surgical resections as starting material. The latter, so-called patient-derived organoids (PDOs), have shown their robustness in predicting patient drug responses compared to other models. Because of their origin, PDOs are natural offspring of the patient tumor or healthy surrounding tissue, and therefore, have been increasingly used to develop targeted drugs and personalized therapies. Here, we present a new protocol to generate patient-derived colon organoids (PDCOs) from tumor and healthy tissue biopsies. We emphasize budget-friendly and reproducible techniques, which are often limiting factors in this line of research that restrict the development of this 3D-culture model to a small number of laboratories worldwide. Accordingly, we describe efficient and cost-effective techniques to achieve immunoblot and high-resolution microscopy on PDCOs. Finally, a novel strategy of lentiviral transduction of PDCOs, which could be applied to all organoid models, is detailed in this article. © 2023 The Authors. Current Protocols published by Wiley Periodicals LLC.

Basic Protocol 1: Establishment of PDCOs from biopsies
Basic Protocol 2: Long-term maintenance and expansion of PDCOs in BME domes
Basic Protocol 3: Cryopreservation and thawing of PDCOs
Basic Protocol 4: Lentiviral transduction of PDCOs
Basic Protocol 5: Immunoblot and evaluation of variability between donors
Basic Protocol 6: Immunofluorescence labeling and high-resolution microscopy of PDCOs
Basic Protocol 7: Transcriptomic analyses of PDCOs by RT-qPCR

Keywords: cancer • high-resolution microscopy • immunoblotting • lentiviral transduction • patient-derived organoids • RT-qPCR

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INTRODUCTION

This article describes protocols aiming at developing, routinely in the laboratory, the use of patient-derived colon organoids (PDCOs) under the scope of both a tight budget and reproducibility, which are very well-known limitations in the field. Indeed, the major goal of this article is to detail step-by-step robust protocols to isolate intestinal crypts from patient biopsies using both an enzyme-free reagent and mechanical disruptive approaches. Of note, these crypts are architectural subunits of the intestinal epithelium including adult stem cells enabling the self-renewal of the colon tissue previously fragmented, and consequently, the formation of organoids. Then the intestinal crypts are embedded in domes made of Cultrex, a basement membrane extract, and fed with IntestiCult, a commercially available conditioned medium. This ensures a potent establishment of the dome-organoid cultures, and their subsequent propagation passages after passages. On the contrary to most protocols in literature, it is crucial to notice that the PDCO cultures we developed originate not only from the patient tumor tissue but also from the patient healthy tissue. Growing both types of organoids is of high interest in the cancer research field to identify novel biomarkers. To reach this objective, this article also describes immunoblot, immunofluorescence, and RT-qPCR protocols. These three different downstream assays are relevant to study the levels of expression of proteins or mRNA, as well as the spatial localization of proteins within the organoids assessed by confocal microscopy. Finally, this work investigates an unresolved question in the organoid field, which is the efficiency of lentiviral transduction of organoids, to generate silenced tumor patient-derived organoids and identify target genes leading to translation in therapeutics.

This article presents comprehensive and detailed protocols for the production, culture, and analysis of patient-derived colon organoids (PDCOs). Firstly, we provide a protocol for the production of PDCOs (Basic Protocol 1) and their long-term maintenance and expansion in culture (Basic Protocol 2). We also describe all the steps for cryopreserving and thawing patient-derived organoids (Basic Protocol 3), facilitating the establishment of a biobank. Furthermore, we offer multiple protocols for the analysis of these PDCOs. Firstly, we propose a lentiviral transduction protocol for gene knockdown (Basic Protocol 4). Secondly, we describe two different protocols for the analysis of protein expression in PDCOs, including an immunoblot protocol (Basic Protocol 5) and an immunofluorescence protocol (Basic Protocol 6). Finally, we conclude with a protocol for the transcriptomic analysis of PDCOs using RT-qPCR (Basic Protocol 7). Importantly, we propose budget-friendly notes in each protocol. We provide a cost comparison of budget-friendly notes vs conventional procedures in Table 1.

STRATEGIC PLANNING

Before performing any of the Basic Protocols detailed hereafter, ensure to understand every single step, then make sure to have all the reagents ready and prepared in agreement with the safety data sheet (SDS). Aseptic culture conditions under an adequate biosafety cabinet (BSC) must be applied according to the protocol followed (Basic

Table 1 Cost Comparison With or Without Budget-Friendly Notes[a,b]

	Dome number		Budget-friendly notes		Conventional procedures	
	Tumor	Healthy	Solution/reagent	Price (€)	Solution/reagent	Price (€)
Basic Protocol 1	6	6	Cultrex	40.00	Geltrex	74.00
			DMEM/F-12	0.22	Intesticult	5.00
			Murine (M-) Wtn3a	2.60	Human (H-) Wtn3a	4.68
			(Refresh) IntestiCult	300.00	(Refresh) IntestiCult	360.00
			(Refresh) M-Wtn3a	20.40	(Refresh) H-Wtn3a	56.16
			Passage 1:2 Cultrex	79.90	Passage 1:2 Geltrex	148.00
			DMEM/F-12	0.22	Intesticult	5.00
Basic Protocol 2	12	12	M-Wtn3a	5.10	H-Wtn3a	9.36
			(Refresh) IntestiCult	600.00	(Refresh) IntestiCult	720.00
			(Refresh) M-Wtn3a	10.20	(Refresh) H-Wtn3a	18.72
Total cost (TC) (€)				**1058.60**		**1400.90**
		BP4	Plasmids to produce lentivirus particles	240.00	Commercial lentivirus	1700.00
			$CaCl_2$	<0.50	Transduction reagent	180.00
		TC(€)		**240.50**		**1880.00**
		BP5	Homemade gel	<1.00	(Bio-Rad) precast gel	15.00
			Wet-transfer buffer	<1.00	Trans-Blot Turbo kit	264.00
		TC(€)		**<1.00**		**279.00**

[a] In the first (top) part of this table, we compare the cost difference for generating 6 healthy and 6 tumor organoid-domes over 2 months, with or without the budget-friendly notes included in both Basic Protocol 1 (culturing) and Basic Protocol 2 (passaging and continued culture). Without the application of budget-friendly notes, these steps of both protocols would be 1.3 times more expensive.

[b] In the second (bottom) part of this table, we compare the cost difference between budget-friendly notes and conventional procedures for the lentiviral transduction of PDCOs (Basic Protocol 4) and immunobloting on PDCOs (Basic protocol 5). Without the application of budget-friendly notes, Basic Protocol 4 and 5 would be ~7.8 and 279 times more expensive, respectively.

Protocols 1 to 3), as well as wearing personal protective equipment (PPE). Good laboratory practices must be followed at any time. In addition, work with lentiviruses must be performed in a BSL2+ environment with adapted PPE (Basic Protocol 4). Finally, confocal fluorescent microscopy involves laser handling, so work according to health and safety rules and avoid exposing your eyes directly to the laser beam.

NOTE: We obtained colorectal biopsies of patients from the CHU UCL Namur Mont-Godinne hospital (Belgium), following ethical approval and upon informed consent of each patient. Informed consent must be obtained from all subjects before the collection of the primary human tissue material.

ESTABLISHMENT OF PDCOS FROM BIOPSIES

BASIC PROTOCOL 1

This protocol aims to establish colon organoid cultures from patient biopsies. The tumor and healthy tissues are processed in parallel, with a separate set of sterile material, and in a BSC to work under aseptic culture conditions. Once extracted from patients, the biopsies are put in ice-cold DMEM/F-12 supplemented with antibiotic-antimycotic and kept at 4°C for transport. Then, in a BSC, the biopsies are extensively washed and minced into small pieces in a solution of gentle cell dissociation reagent (GCDR). Biopsies are incubated at 37°C on a shaker to induce tissue dissociation resulting in a turbid solution containing small chunks and isolated intestinal crypts. This solution is filtered to mechanically fragment the chunks and obtain a higher yield of isolated intestinal crypts,

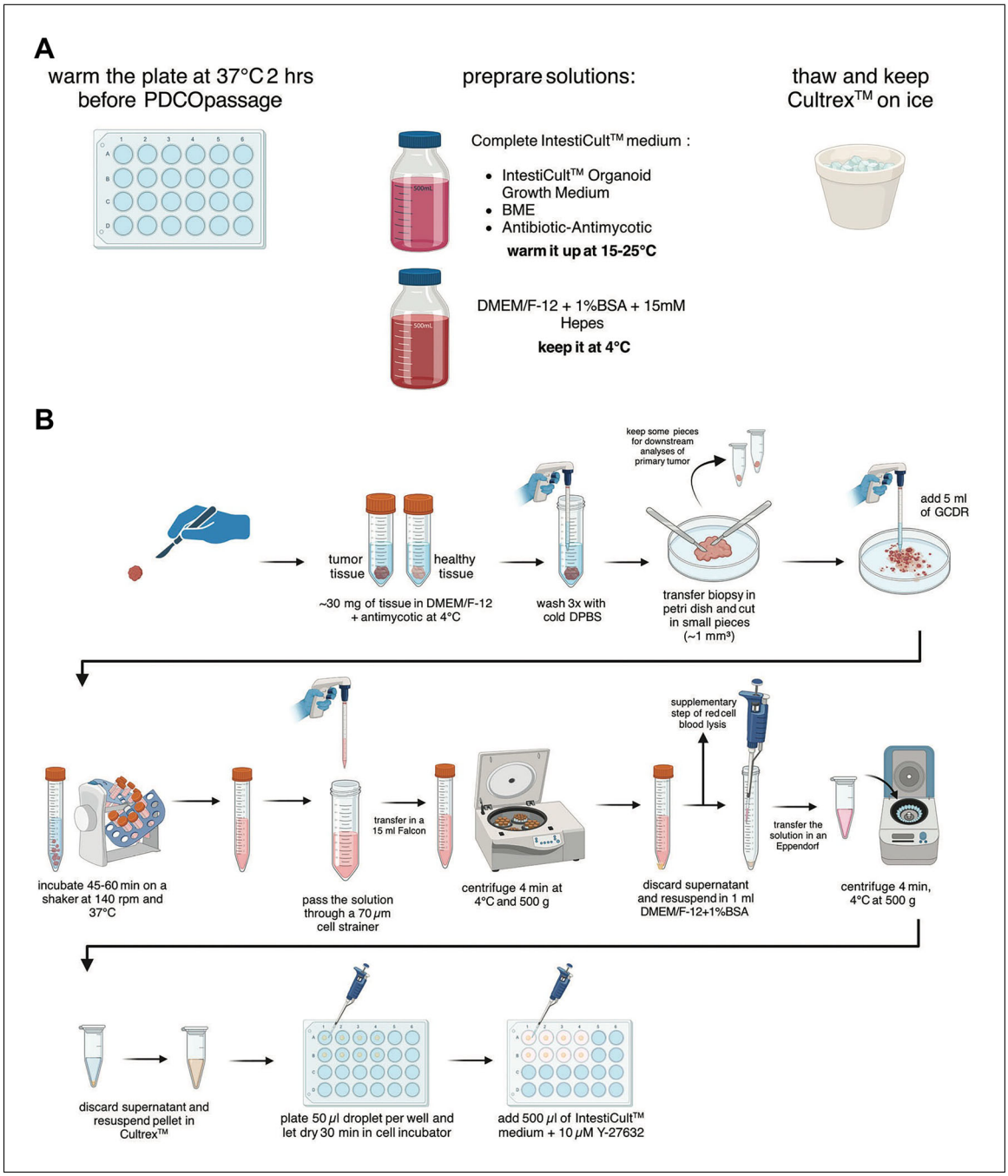

Figure 1 Workflow of PDCO generation. (**A**) Material preparation. (**B**) Workflow describing how biopsies (healthy and tumor tissues) are processed to be seeded into Cultrex-domes to generate PDCO cultures. In brief, once the biopsies are extracted from the patient, tumor and healthy tissues are weighed and processed separately. The biopsies are washed, minced successively into small pieces, and crypts are isolated by tissue dissociation. Finally, crypts are resuspended in Cultrex for subsequent dome formation. Intesticult medium is then added with the apoptosis inhibitor (Y-27632).

which will enable the growth and the maturation of colon organoids. The intestinal crypt solution is centrifuged, and the pellet is resuspended in Cultrex, which is basement membrane extract (BME). Then, domes containing Cultrex-intestinal crypts are seeded in a 24-well plate and dried before the incubation in IntestiCult to establish and propagate colon organoids derived from patients. An overview of this workflow is presented in Figure 1.

Materials

IntestiCult organoid growth medium (human) (StemCell Technologies, cat. no. 06010)
Antibiotic-antimycotic (100×) (ThermoFisher Scientific, cat. no. 15240062)
DMEM/F-12 (ThermoFisher Scientific, cat. no. 11320033)
HEPES, 1 M (ThermoFisher Scientific, cat. no. 15630056)
Bovine serum albumin (BSA) fraction, protease-free, TSE-/BSE-free (Carl Roth, cat. no. T844.3)
Cultrex UltiMatrix reduced growth factor basement membrane extract (RGF BME) (Bio-techne R&D Systems, cat. no. BME001-05)
Geltrex LDEV-free reduced growth factor basement membrane matrix (ThermoFisher Scientific, cat. no. A1413201)
DPBS, no calcium, no magnesium (ThermoFisher Scientific, cat. no. 14190144)
Liquid nitrogen, or dry ice and methyl-2 butane (isopentane), ≥99%, GPR RECTAPUR (VWR, cat. no. 24872)
4% PFA (see recipe)
Sucrose (ThermoFisher Scientific, cat. no. S0389)
OCT embedding matrix (Carl Roth, cat. no. 6478.2)
Gentle cell dissociation reagent (GCDR) (StemCell Technologies, cat. no. 100-0485)
1× RBC lysis buffer, eBioscience (ThermoFisher Scientific, cat. no. 00-4333-57)
Y-27632, ROCK inhibitor (see recipe)
Recombinant human Wnt-3a protein (Bio-techne R&D Systems, cat. no. 5036-WN)
Wnt-3a, murine (see recipe)

24-well clear multiple well plates, flat bottom with lid, sterile, Corning CellBIND (Corning, cat. no. 3337)
Incubator for cell culture, CellXpert C170 (Eppendorf, cat. no. 6734000011)
Sterile disposable scalpels, shape 10 (Laborimpex, cat. no. AGAR_T5217-10)
10-cm Petri dish, glass (Merck, cat. no. BR455742-10EA)
Cryogenic vials (VWR, cat. no. 479-1220)
15-ml centrifuge tube (Corning, cat. no. 430791)
Tube revolver rotator (ThermoFisher Scientific, cat. no. 88881001)
10-ml pipette, Stripette (Corning, cat. no. 4488)
70-µm reversible strainers, large (StemCell Technologies, cat. no. 27260)
50-ml centrifuge tube (Corning, cat. no. 430829)
Stripettor ultra pipet controller (Corning)
Assorted pipettor and tips
Centrifuges, 5702 and 5425 (Eppendorf, cat. nos. 5702000010 and 5405000310)
1.5-ml Eppendorf microtubes (Merck, cat. no. Z606340)

Preparation

1. Warm an empty tissue-culture treated 24-well plate at 37°C, at least 2 hr before the beginning of the experiment.

2. Prepare complete IntestiCult organoid growth medium (human) following the supplier guidelines, and warm it up to room temperature (15° to 25°C).

   To prevent any bacterial and fungi contamination, due to the extraction of the biopsies in non-aseptic conditions once removed from the patient, an antibiotic-antimycotic (1×) is added to complete IntestiCult Organoid Growth Medium.

   For the remainder of this article, this complete medium with antibiotics will be referred to as "IntestiCult" for ease of reading.

3. Prepare 12 ml of sterile DMEM/F-12 with 15 mM HEPES and 1% (w/v) BSA (or a larger volume for storage at 4°C for up to 3 months) and keep it on ice.

   BSA is crucial to prevent the biopsy pieces to stick to the plastics and consequently lose material.

4. Thaw Cultrex UltiMatrix RGF BME on ice and keep it on ice during all the following steps of the protocol.

   For each well of a 24-well plate, 50 µl will be required. A ∼300 mg biopsy allows the seeding of 4 to 6 wells.

   The yield of intestinal crypts per tissue (and subsequently number of domes) is dependent on the patient and the nature of the tissue.

   In the rest of this article, Cultrex UltiMatrix RGF BME will be referred to as "Cultrex" for ease of reading.

   Cultrex is stored at −20°C and can be aliquoted to be stored at 4°C for up to 3 weeks.

   Budget-friendly note: Alternatively, Cultrex can be replaced by Geltrex LDEV-free reduced growth factor basement membrane matrix.

Biopsy mechanical processing

5. Weigh the biopsies that are in 10 ml DMEM/F-12 supplemented with antimycotic. Keep the biopsies on ice. Process the two biopsies (tumor tissue and healthy tissue) side-by-side and at the same time. Make sure to either rinse or change the scalpel set between each tissue to prevent cross-contamination.

   Biopsies are collected on the same day as the tumor resection, ideally within a few hours after resection. Between resection and collection, biopsies are stored in DMEM/F-12 supplemented with antimycotic at 4°C.

6. Wash the biopsy 3 times with 5 ml of cold DPBS. Between each washing step, aspirate the supernatant carefully and discard it.

   Ensure to leave a minimal volume of supernatant to prevent accidental aspiration and subsequent loss of material during washing steps.

7. Add 3 ml of fresh DPBS and transfer the biopsy into a 10-cm Petri dish.

8. Using 2 sterile scalpels to create a neat scission, split the biopsy into 2 pieces accounting for 1/3 and 2/3 in size of the original biopsy, respectively. The smallest part can be cut into 2 pieces, placed in cryogenic vials, snap-frozen in liquid nitrogen, and kept at −80°C for future downstream experiments (protein or RNA extraction for example). The larger piece is processed as follows.

   As mentioned earlier, please make sure to use separate pairs of scalpels for the tumor tissue and healthy tissue. You may use the same scalpels throughout the entire protocol.

   If liquid nitrogen is unavailable, you can snap-freeze the smaller pieces by immersing them in a bath of dry ice and isopentane at −80°C. Be cautious, as isopentane is highly volatile.

   Additionally, for immunohistochemistry purposes, a third piece can be cut and fixed in 4% PFA for 30 min at room temperature and then transferred into a 15-ml centrifuge tube with a 30% sucrose solution for 48 hr. Then the piece of tissue can be embedded in optimal cutting temperature (OCT) compound, snap frozen, and kept at –80°C for long-term storage.

9. Use the scalpels to mince tissues into the smallest pieces you can generate. Add 5 ml of GCDR to start the dissociation of the tissue pieces and transfer the solution into a 15-ml centrifuge tube.

10. Rinse the 10-cm dish with 5 ml of fresh GCDR and transfer the solution with the remaining tissue chunks to the 15-ml tube.

Biopsy disruption to isolate intestinal crypts

11. Incubate the GCDR solution with the tissue pieces for 45 to 60 min at 37°C, on a tube rotator at 140 rpm.

    Every biopsy is different from one patient to another, and from healthy tissue to tumor tissue. Consequently, the time required for tissue dissociation will vary. It is crucial to troubleshoot the incubation time of the biopsy since over-incubation, as well as under-incubation, will lead to the loss of intestinal crypts. Therefore, double-check the turbidity of the solution every 15 min. Once the tissue is dissociated, chunks of small sizes will appear leading to a very turbid solution.

12. Transfer the GCDR solution, containing chunks and isolated intestinal crypts, with a 10-ml pipette onto a 70-μm cell strainer placed on a 50-ml centrifuge tube. In the event of a hindered filtration, pipette-mediated scratching of the top of the strainer should help to resume the process.

    Pre-coat the 10-ml pipet with GCDR to prevent the tissue pieces to stick onto the plastic.

13. To maximize material collection, rinse the strainer with 3 ml of DMEM/F-12 with 15 mM HEPES and 1% BSA. The collected medium is then transferred to the 50-ml tube containing the rest of the filtered solution (total of ∼13 ml).

14. Transfer the filtrated solution of intestinal crypts into a 15-ml tube.

    Replacing the 50-ml tube with a 15-ml tube is crucial to achieve a well-defined pellet after centrifugation and avoid losing material.

15. Centrifuge once for 4 min at 500 × *g*, 4°C. Carefully remove the supernatant. If a red coloration of the pellet is observed due to the presence of blood cells, proceed to step 16, otherwise go to step 17.

16. Resuspend the pellet in 3 ml of red blood cell lysis buffer and incubate for 5 min at room temperature. Then, add 5 ml of DMEM/F-12 with 15 mM HEPES and 1% BSA medium to filtrated solution. Centrifuge 4 min at 500 × *g*, 4°C, and aspirate the supernatant.

17. Wash the pellet 2 times with 3 ml of DMEM/F-12 with 15 mM HEPES and 1% BSA. Each time, spin 4 min at 500 × *g*, 4°C, and carefully remove the supernatant.

Dome-organoid seeding in 24-well plate

18. Resuspend the pellet in 1 ml DMEM/F-12 with 15 mM HEPES and 1% BSA and transfer the solution containing intestinal crypts into a 1.5-ml Eppendorf tube. Spin for 4 min at 500 × *g*, room temperature.

    Budget-friendly note: For economic reasons, DMEM/F-12 with 15 mM HEPES and 1% BSA has been extensively tested to replace IntestiCult in this step.

    Do not aspirate the totality of the supernatant. Leave a thin layer of DMEM/F-12 with 15 mM HEPES and 1% BSA (∼30 μl). Then, resuspend carefully the pellet in it, avoiding bubbles.

19. Resuspend the final pellet (+ thin layer of DMEM/F-12 with 15 mM HEPES and 1% BSA) in undiluted Cultrex. Avoiding bubble formation. A dome of 50 μl of Cultrex-intestinal crypt solution is plated per well of a warmed 24-well plate.

    The volume of Cultrex depends on the pellet size obtained from the biopsy. Usually, a pellet coming from ∼300 mg of biopsy is plated into 4 to 6 domes. Consequently, a volume of 4 × 50 μl = 200 μl Cultrex or 6 × 50 μl = 300 μl Cultrex can be used.

With this density, a notable growth of the organoid cultures is achieved. Of important note, when seeded at a very high density, cells in the center of the dome will die. Conversely, seeding cells too sparsely can hinder cell growth due to the lack of neighboring cell signals. The standard growth timeline is shown in Figure 2A.

Budget-friendly note: Alternatively, Cultrex can be replaced by Geltrex LDEV-free reduced growth factor basement membrane matrix.

20. Incubate the plate for 30 min at 37°C under 5% CO_2 to allow dome solidification.

21. Once dome solidification is achieved, remove the 24-well plate from the incubator and add 500 µl of IntestiCult medium supplemented with 10 µM of the ROCK inhibitor Y-27632 to each single well, as well as 50 ng/ml of Wnt3a for healthy tissue exclusively.

    Freshly prepare a mix of IntestiCult + Y-27632 for the total amount of wells containing domes.

    ROCK inhibitor improves the survival rates of cells by preventing dissociation-induced apoptosis. Therefore, it is exclusively added when domes are seeded after biopsy processing or after a passage, but not for routine maintenance of the dome-organoid cultures.

    Wnt3a must be supplemented in the medium of healthy colon organoids, because IntestiCult has been developed to propagate and maintain tumor colon organoids, in which Wnt/β-catenin pathway is over-activated. This pathway is known to be crucial for intestinal organoid growth. Hence the necessity to add Wnt3a in healthy colon organoids to activate Wnt/β-catenin pathway, but not in the tumor ones.

    Budget-friendly note: Murine Wnt3a (muWnt3a) is preferred to the human Wnt3a (huWnt3a) from the same supplier, because in our hands (1) muWnt3a led to a higher yield of organoid propagation than the huWnt3a, and (2) muWnt3a is cheaper than the huWnt3a.

22. Incubate the plate at 37°C under 5% CO_2 for 2 weeks until the first passage. Refresh the medium every two days with 500 µl of IntestiCult only for tumor organoids and of IntestiCult supplemented with Wtn3a for healthy organoids.

    Budget-friendly note: Alternatively, when dealing with a newly established organoid culture (<2 weeks), IntestiCult solution can be replaced as follows: 750 µl for 5 days, and 500 µl for the following three days from Friday to Sunday. This workflow will allow notable savings on the budget without impairing organoid growth. Days and cognate volumes have been extensively tested to allow both optimal growth of the organoid cultures and to deal with a tight budget.

BASIC PROTOCOL 2

LONG-TERM MAINTENANCE AND EXPANSION OF PDCOS IN BME DOMES

This protocol aims to maintain and expand PDCO cultures in BME domes, here Cultrex-domes. The goal is to perform different downstream assays, such as lentiviral transduction, immunoblotting, immunofluorescence, or RT-qPCR. Cultrex-organoid dome cultures are passaged every 1 to 2 weeks depending on the confluency and the morphology of the organoids. Indeed, large doughnut-like organoids and budding ones have been observed to produce robust and fertile fragments leading to a sustained expansion of PDCOs (Fig. 2A and B). On the contrary, small organoid (<50 µm) structures or mature ones (>250 µm) are hardly expandable due to their lack of intestinal crypts containing Lgr5+ stem cells. Briefly, domes to be passaged are mechanically disrupted by flushing them with GCDR and then filtered to obtain a solution rich in small fragments containing intestinal crypts that will self-assemble to generate novel organoids. IntestiCult is used for the maintenance and expansion of PDCOs. An overview of this workflow is presented in Figure 2C.

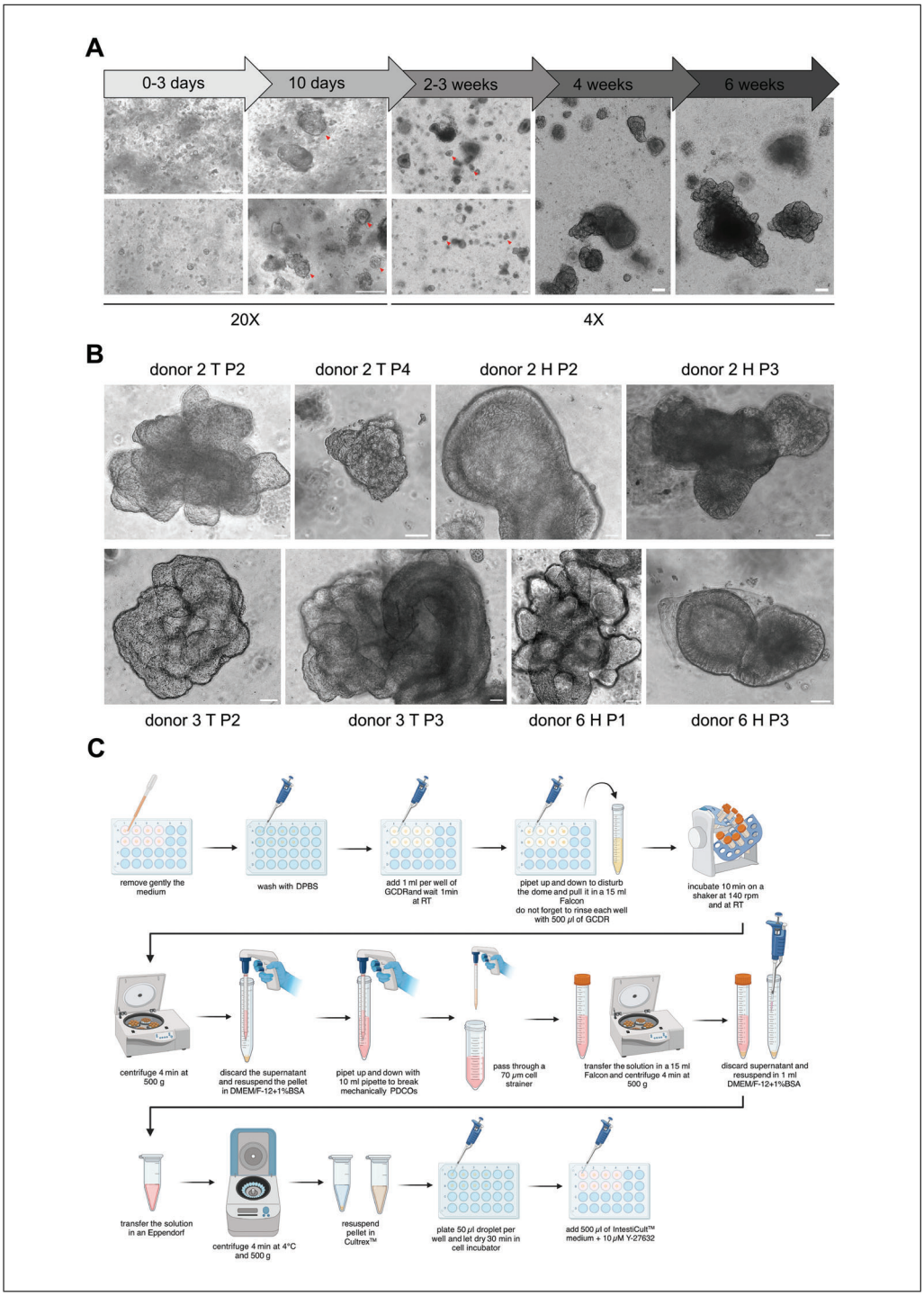

Figure 2 Long-term culture of PDCOs. (**A**) Representative brightfield images of the evolution of PDCO cultures over time. Red arrows show doughnut-like structures, which correspond to proliferative PDCOs. Scale bars, 100 µm. (**B**) Representative brightfield images of mature PDCOs from different donors (1, 2 and 3), different passages (P1 to 4) and generated from both tumor (T) or healthy (H) biopsy tissues. Scale bars, 150 µm. (**C**) Experimental workflow of PDCO passage. In brief, PDCO-domes are disrupted to fragment organoids. Fragments are seeded back in BME domes to achieve PDCO expansion.

Materials

IntestiCult organoid growth medium (human) (StemCell Technologies, cat. no. 06010)
Antibiotic-antimycotic (100×) (ThermoFisher Scientific, cat. no. 15240062)
DMEM/F-12 (ThermoFisher Scientific, cat. no. 11320033)
HEPES, 1 M (ThermoFisher Scientific, cat. no. 15630056)
Bovine serum albumin (BSA) fraction, protease-free, TSE-/BSE-free (Carl Roth, cat. no. T844.3)
Cultrex UltiMatrix reduced growth factor basement membrane extract (RGF BME) (Bio-techne R&D Systems, cat. no. BME001-05)
Geltrex LDEV-free reduced growth factor basement membrane matrix (ThermoFisher Scientific, cat. no. A1413201)
DPBS, no calcium, no magnesium (ThermoFisher Scientific, cat. no. 14190144)
Gentle cell dissociation reagent (GCDR) (StemCell Technologies, cat. no. 100-0485)
Y-27632, ROCK inhibitor (see recipe)
Recombinant human Wnt-3a protein (Bio-techne R&D Systems, cat. no. 5036-WN)
Wnt-3a, murine (see recipe)

24-well clear multiple well plates, flat bottom with lid, sterile, Corning CellBIND (Corning, cat. no. 3337)
Incubator for cell culture, CellXpert C170 (Eppendorf, cat. no. 6734000011)
Assorted pipettor and tips
15-ml centrifuge tube (Corning, cat. no. 430791)
Tube revolver rotator (ThermoFisher Scientific, cat. no. 88881001)
50-ml centrifuge tube (Corning, cat. no. 430829)
Centrifuges, 5702 and 5425 (Eppendorf, cat. nos. 5702000010 and 5405000310)
10-ml pipette, Stripette (Corning, cat. no. 4488)
70-µm reversible strainers, large (StemCell Technologies, cat. no. 27260)
Stripettor ultra pipet controller (Corning)
1.5-ml Eppendorf microtubes (Merck, cat. no. Z606340)

Preparation

1. Warm an empty tissue-culture treated 24-well plate at 37°C, at least 2 hr before the beginning of the experiment.

2. Prepare complete IntestiCult organoid growth medium (human) following the supplier guidelines, and warm it up to room temperature (15° to 25°C).

   To prevent any bacterial and fungi contamination, due to the extraction of the biopsies in non-aseptic conditions once removed from the patient, an antibiotic-antimycotic (1×) is added to complete IntestiCult Organoid Growth Medium.

3. Prepare sterile DMEM/F-12 with 15 mM HEPES and 1% (w/v) BSA and keep it on ice. The final volume depends on the number of domes that need to be passaged.

   BSA is crucial to prevent the biopsy pieces to stick to the plastics and consequently lose material.

4. Thaw Cultrex on ice and keep it on ice during all the following steps of the protocol.

   For each well of a 24-well plate, 50 µl will be required.

   Budget-friendly note: Alternatively, Cultrex can be replaced by Geltrex LDEV-free reduced growth factor basement membrane matrix.

Mechanical disruption of domes

5. Identify the domes to be passaged and remove gently the medium in each well, without disturbing the integrity of the Cultrex-dome.

   To identify which domes need to be passaged, it is important to examine the size and shape of the organoids in the dome using phase-contrast optical microscopy. The ideal organoid shape and size for passage is illustrated in Figure 2A between day 10 and weeks 2 to 3.

6. Gently wash the exposed domes with DPBS at room temperature and then discard the DPBS.

7. Add 1 ml of GCDR on top of each dome and incubate for 1 min at room temperature.

8. Mechanically break down each dome using a P1000 pipette tip, and pipet GCDR up and down above the dome to create a disruptive flow.

   Avoid touching the side and the bottom of the well with the tip.

9. Transfer the resulting solution of dome clumps in a 15-ml centrifuge tube.

   Pool all the domes that need to be passaged altogether (i.e., domes from a same donor and same tissue subtype normal versus tumor). Use a 50-ml tube if you have >6 domes to pool together.

10. Add 500 µl per dome of GCDR to the newly emptied well to rinse it. Then, transfer the 500 µl solution in the 15-ml tube from step 8. Incubate it for 10 min on a tube rotator at ∼160 rpm, at room temperature.

11. Centrifuge the tube 4 min at 500 × *g*, room temperature. Discard the supernatant.

12. Add 1 ml per passaged dome of ice-cold DMEM/F-12 with 15 mM HEPES and 1% BSA in the 15-ml centrifuge tube. Proceed to a mechanical disruption of the dome clumps to get organoid chunks, by performing vigorous up and down 15 times using a 10-ml pipette.

    Pre-coat the 10 ml pipette with DMEM/F-12 with 15 mM HEPES and 1% BSA solution to prevent the organoids to stick to the plastic.

13. Using the same 10-ml pipet, transfer the solution onto a 70-µm cell strainer placed on a 50-ml centrifuge tube. In the event of a hindered filtration, pipette-mediated scratching of the top of the strainer should help to resume the process.

    This step mechanically sub-fragments the chunks of organoids, thereby increasing the number of small structures in the culture containing intestinal crypts. These new organoid fragments will self-assemble to form novel organoids, which is crucial for organoid expansion.

14. To collect a maximum of material, rinse the strainer with 3 ml of DMEM/F-12 with 15 mM HEPES and 1% BSA. The collected medium is then transferred to the 50-ml centrifuge tube containing the rest of the filtered solution.

15. Transfer the filtrated solution into a 15-ml centrifuge tube.

    Replacing the 50-ml centrifuge tube with a 15-ml centrifuge is crucial to achieve a well-defined pellet after centrifugation and avoid losing material.

16. Centrifuge once for 4 min at 500 × *g*, 4°C. Aspirate the supernatant.

Dome-organoid cultures seeding in 24-well plate

17. Resuspend the pellet in 1 ml DMEM/F-12 with 15 mM HEPES and 1% BSA and transfer the solution into a 1.5-ml Eppendorf tube. Spin 4 min at 500 × *g*, room temperature.

Budget-friendly note: DMEM/F-12 with 15 mM HEPES and 1% BSA has been extensively tested to replace IntestiCult in this step, to save on budget.

18. Partially remove the supernatant. Leave a thin layer of medium (~30 µl) in which the pellet will be resuspended carefully. Then, add Cultrex and resuspend the residual 30 µl carefully, avoiding bubbles. The final volume of Cultrex depends on the number of passaged domes and the final dilution.

    The final dilution is dependent on the organoid growth speed. If the organoids are growing fast, a dilution of 1:4 is recommended. Otherwise, the standard dilution is 1:2. For instance, if four domes need to be passaged according to a standard dilution of 1:2, the final number of domes will be 8. As 50 µl of Cultrex per dome are needed, the total necessary volume of Cultrex will be 400 µl. The dilution ratio will proportionally impact the number of seeded wells.

19. Plate 50 µl of Cultrex-intestinal crypt solution in drop shape into each well in a pre-warmed 24-well plate.

20. Incubate the plate for 30 min at 37°C under 5% CO_2 to allow dome solidification.

21. Once dome solidification is achieved, remove the 24-well plate from the incubator and add 500 µl of IntestiCult medium supplemented with 10 µM of the ROCK inhibitor Y-27632 to each single well, as well as 50 ng/ml of Wnt3a for healthy tissue exclusively.

    ROCK inhibitor notably improves the survival rates of cells by preventing the dissociation-induced apoptosis. Therefore, it is exclusively added when domes are seeded after biopsy processing or after a passage.

    Wnt3a must be supplemented in the medium of healthy colon organoids because IntestiCult has been developed to propagate and maintain tumor colon organoids, in which Wnt/β-catenin pathway is over-activated. This pathway is known to be crucial for intestinal organoid growth. Hence the necessity to add Wnt3a in healthy colon organoids to activate Wnt/β-catenin pathway, but not in the tumor ones.

    Budget-friendly note: Murine Wnt3a (muWnt3a) is preferred to the human Wnt3a (huWnt3a) from the same supplier, because in our hands (1) muWnt3a led to a higher yield of organoid propagation than the huWnt3a, and (2) muWnt3a is cheaper than the huWnt3a.

    Freshly prepare a mix of IntestiCult + 10 µM Y-27632 for the total amount of your wells containing domes.

22. Incubate the plate at 37°C under 5% CO_2 for 1 to 2 weeks until the next passage. Refresh the medium every two days with 500 µl of IntestiCult only for tumor organoids and of IntestiCult supplemented with Wtn3a for healthy organoids.

    There is no defined maximum number of passages. We recommend users to regularly check the stability of proliferation markers (e.g., SOX2) either via immunoblotting or RT-qPCR.

    Budget-friendly note: Alternatively, when dealing with a newly established organoid culture (<2 weeks), IntestiCult solution can be replaced as follows: 750 µl for 5 days, and 500 µl for the following 3 days from Friday to Sunday. This workflow will allow notable saving on budget without impairing organoid growth. Days and cognate volumes have been extensively optimized to allow both an optimal growth of the organoid cultures and to deal with a tight budget.

BASIC PROTOCOL 3

CRYOPRESERVATION AND THAWING OF PDCOS

Cryopreservation of PDCOs is essential for biobanking, which is crucial in any preclinical study. The first step of this protocol is to select domes featuring most of the organoids in a doughnut-like shape (shown in Fig. 2A). Then, these PDCOs are

passaged. Of note, it is essential to cryopreserve the organoids 4 days after the passaging step to ensure that PDCOs are in an exponential growth phase, resulting in an optimal viability yield after thawing. Then, newly passaged Cultrex-dome organoid cultures are mechanically disrupted by flushing and incubating them with GCDR. The solution containing organoid fragments from 2 domes pooled together is then washed at 4°C, centrifuged, and the corresponding pellet is resuspended in 1 ml of ice-cold CryoStor CS10 and transferred into a cryovial. The cryovials are stored overnight at −80°C and the next morning transferred into a liquid nitrogen tank. Finally, a fast-thawing protocol at 37°C involving a water-bath is described.

Materials

DMEM/F-12 (ThermoFisher Scientific, cat. no. 11320033)
HEPES, 1 M (ThermoFisher Scientific, cat. no. 15630056)
Bovine serum albumin (BSA) fraction, protease-free, TSE-/BSE-free (Carl Roth, cat. no. T844.3)
CryoStor CS10 (StemCell Technologies, cat. no. 100-1061)
DPBS, no calcium, no magnesium (ThermoFisher Scientific, cat. no. 14190144)
Gentle cell dissociation reagent (GCDR) (StemCell Technologies, cat. no. 100-0485)
IntestiCult organoid growth medium (human) (StemCell Technologies, cat. no. 06010) with antibiotic-antimycotic (1×) (ThermoFisher Scientific, cat. no. 15240062)
Ethanol, absolute, molecular biology grade (ThermoFisher Scientific, cat. no. 16685992)

Assorted pipettor and tips
15-ml centrifuge tube (Corning, cat. no. 430791)
Tube revolver rotator (ThermoFisher Scientific, cat. no. 88881001)
50-ml centrifuge tube (Corning, cat. no. 430829)
1.5-ml Eppendorf microtubes (Merck, cat. no. Z606340)
Cryogenic vials (VWR, cat. no. 479-1220)
Cell freezing container, Corning CoolCell LX (Merck, cat. no. CLS432002)
Incubator for cell culture, CellXpert C170 (Eppendorf, cat. no. 6734000011)
Centrifuges, 5702 and 5425 (Eppendorf, cat. nos. 5702000010 and 5405000310)
37°C water bath
Class II biological safety cabinet (BSC)
10-ml pipette, Stripette (Corning, cat. no. 4488)

Cryopreservation

1. Place DMEM/F-12 with 15 mM HEPES and 1% (w/v) BSA(1 ml needed) and CryoStor CS10 on ice.
2. Organoid domes selected for freezing are passaged 4 days prior to this step (see Basic Protocol 2) to maximize post-thaw recovery. This ensures that the organoids are in the logarithmic growth phase at the time of cryopreservation. The shape and size of the organoids at this stage are illustrated in Figure 2A.
3. Gently wash the domes once with DPBS at room temperature and then discard the DPBS.
4. Add 1 ml of GCDR on top of each dome and incubate for 1 min at room temperature.
5. Mechanically break down each dome using a P1000 pipette tip and pipet GCDR up and down above the dome to create a disruptive flow.

   Avoid touching the side and the bottom of the well with the tip.

6. Transfer the resulting solution containing dome chunks in a 15-ml centrifuge tube. Pool all the domes that need to be passaged altogether (i.e., domes from a same donor and same tissue subtype normal versus tumor).

   Use a 50-ml centrifuge tube if you have >6 domes to pool together.

7. Add 500 µl per dome of GCDR to the newly emptied well to rinse it. Then, transfer the 500 µl solution in the 15-ml centrifuge tube of step 6. Incubate the 15-ml tube for 10 min on a tube rotator at ∼160 rpm, at room temperature.

8. Centrifuge the tube 4 min 500 × *g*, room temperature. Discard the supernatant.

9. Resuspend the pellet in 1 ml of ice-cold DMEM/F-12 with 15 mM HEPES and 1% BSA and transfer it in a 1.5-ml Eppendorf tube.

   For the following steps, manipulations are performed on ice.

10. Centrifuge the tube 4 min at 500 × *g*, 4°C, and discard the supernatant.

11. Resuspend the pellet in 1 ml of ice-cold CryoStor CS10 per 2 domes and transfer each 1 ml of the solution into a well-labeled cryovial kept on ice.

    In each cryovial will be cryopreserved a pool of 2 domes.

    CryoStor CS10 is preferred to the standard cryopreservation mix composed of FBS + 10% DMSO, since it has been specifically developed for stem cell cryopreservation and showed in our hands a robust recovery of the frozen organoids once thawed. Moreover, CryoStor CS10 being commercially available strengthens the repeatability of this protocol, which would have been harder to pursue with FBS known for its variability from batch to batch.

12. Transfer the cryovials overnight in a freezing container at −80°C for controlled temperature decrease.

13. The next morning, transfer the cryovials to a liquid nitrogen container for long-term storage.

Thawing

14. Prepare a 15-ml centrifuge tube with 5 ml of pre-warmed IntestiCult.

15. Remove a cryovial from the liquid nitrogen tank.

16. Plunge the cryovial below the cap in a 37°C water bath to enable fast thawing.

    Ensure that this step does not last >2 min.

17. Remove the cryovial from the water bath and spray it extensively with ethanol, before opening it in a BSC and transferring the cells into the centrifuge tube containing 5 ml pre-warmed IntestiCult.

    To help the medium with crypts thawing, gently flush the cells with 500 ml of pre-warmed IntestiCult.

18. Perform Basic Protocol 2 beginning at step 16.

BASIC PROTOCOL 4 LENTIVIRAL TRANSDUCTION OF PDCOS

This protocol aims to generate tumor or healthy PDCOs in which genes are silenced using lentiviral transduction to investigate their function. It has been established with a GFP lentiviral vector but can be applicable with any shRNA plasmid. Lentivirus manipulation requires to work under aseptic culture conditions with adapted BSC (BSL2+) and PPE. This protocol is divided into several major steps, as depicted in the workflow presented in Figure 3. The first step is the production of lentiviral particles containing the plasmid

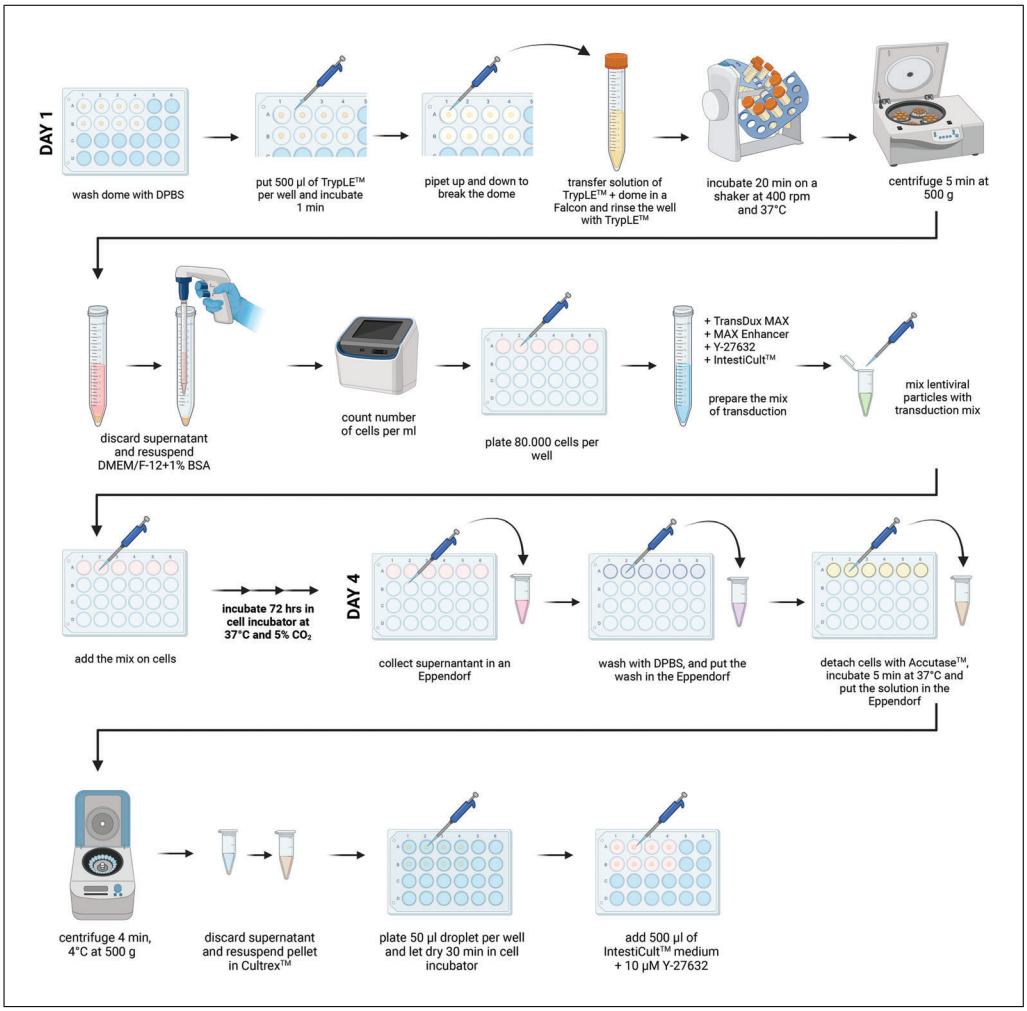

Figure 3 Workflow of lentiviral transduction of PDCOs. In brief, lentiviral particles containing the vector of interest, here a GFP expression plasmid, are first produced by transduction of HEK293T. Then, PDCOs dissociated into a single-cell suspension are transduced with a given number of lentiviral particles to achieve an efficient transduction yield. After 72 hr of incubation, cells are seeded back in domes to enable the formation of organoids.

of interest by HEK293T cells. The concentration of lentiviral particles is quantified using the Lenti-X RT-qPCR Titration kit from Takara, which is a crucial step to determine the multiplicity of infection (MOI). Then, PDCOs are disrupted into a single-cell suspension solution by TrypLE, after which they are transduced with TransDux MAX reagent for 72 hr in InstestiCult (Fig. 4A). A notable transduction has been achieved at an MOI of 4000 (Fig. 4B for MOIs of 1000 and 4000; other MOIs are shown in supplementary Figure 1; lower magnification for MOIs 1000 to 6000 is shown in supplementary Figure 2; see Supporting Information). Finally, the single-cell suspension is pelleted and resuspended in Cultrex to enable the seeding of Cultrex-domes containing transduced intestinal crypts. After 8 days, the antibiotic selection starts as shown in Figure 4C. Given the length and complexity of Basic Protocol 4, detailed time considerations are provided directly within the respective protocol sections.

Materials

HEK293T cells (ATCC, cat. no. CRL-3216)
DMEM, low glucose, pyruvate (ThermoFisher Scientific, cat. no. 31885023)
Fetal bovine serum (FBS) (ThermoFisher Scientific, cat. no. 26140079)
Penicillin-streptomycin, Gibco (ThermoFisher Scientific, cat. no. 15140122)
H_2O, milliQ

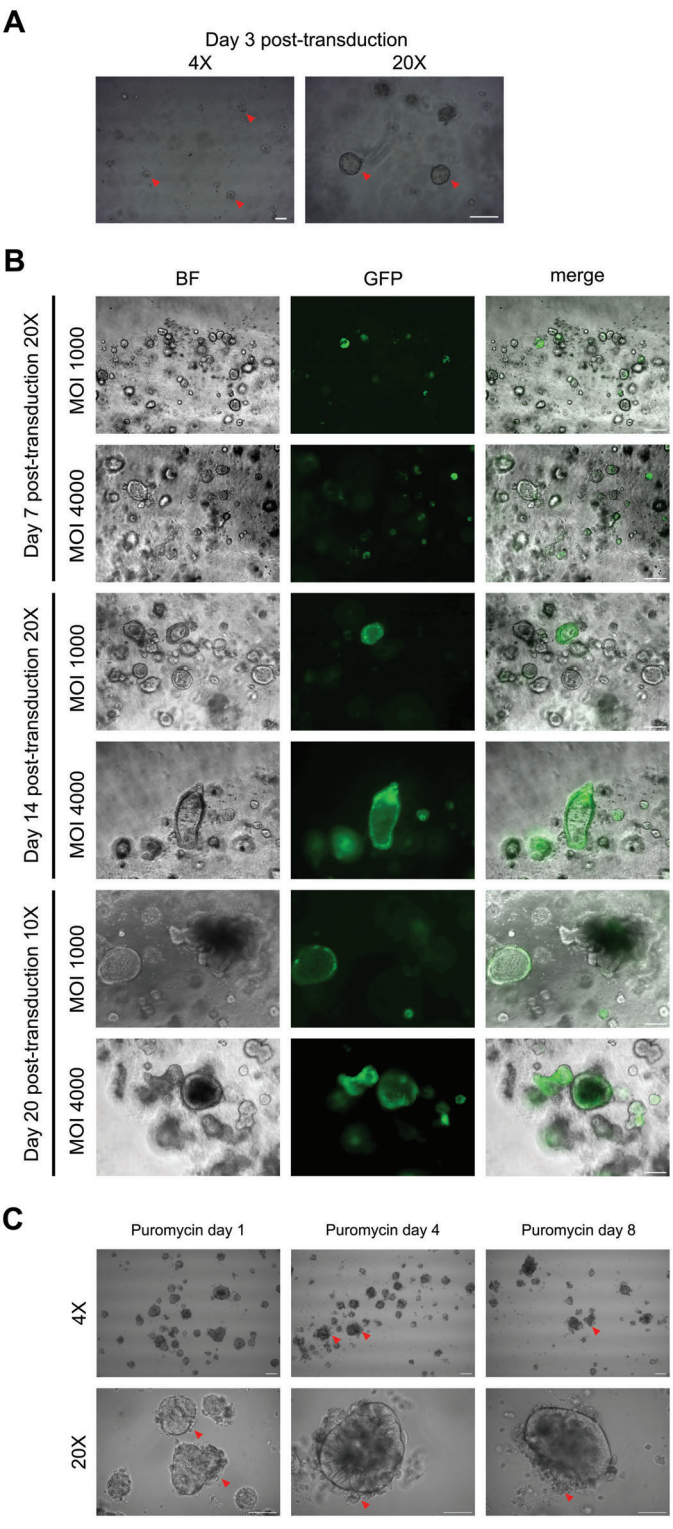

Figure 4 Lentiviral transduction of PDCOs. (**A**) PDCOs 3 days post-transduction, observed with 4× and 20× objectives. Red arrows indicate the first signs of a lumen development. (**B**) Representative images of PDCO growth and expression of GFP from the lentiviral vector after 7-, 14-, and 20-days post-transduction with MOIs of 1000 and 4000. (**C**) Brightfield images over time of PDCO cultures after the addition of 5 μg/ml puromycin. Of note, cell debris seem to accumulate over time after the addition of puromycin, indicating that selection is efficient (shown by red arrows). Scale bars, 100 μm for 4× and 20×, 200 μm for 10×.

pLV-eGFP (AddGene, cat. no. 36083)
pCMV-DR8.91 (Delta 8.9) (AddGene, cat. no. 8455)
VSV-G plasmid (AddGene, cat. no. 8454)
$CaCl_2$, 2 M solution (see recipe)
HEPES-buffered saline (HeBS), 2× (see recipe)
NucleoSpin RNA virus, mini kit for viral RNA from cell-free fluids (Macherey-Nagel, cat. no. 740956.50)
Lenti-X RT-qPCR titration kit (Takara Bio, cat. no. 631235)
DPBS (see recipe)
TrypLE Express enzyme, 1×, no phenol red (ThermoFisher Scientific, cat. no. 12604039)
DMEM/F-12 (ThermoFisher Scientific, cat. no. 11320033)
HEPES, 1 M (ThermoFisher Scientific, cat. no. 15630056)
Bovine serum albumin (BSA) fraction, protease-free, TSE-/BSE-free (Carl Roth, cat. no. T844.3)
TransDux MAX lentivirus transduction enhancer (SanBio, cat. no. LV860A-1)
Y-27632, ROCK inhibitor (see recipe)
IntestiCult organoid growth medium (human) (StemCell Technologies, cat. no. 06010)
Antibiotic-antimycotic (100×) (ThermoFisher Scientific, cat. no. 15240062)
Cultrex UltiMatrix reduced growth factor basement membrane extract (RGF BME) (Bio-techne R&D Systems, cat. no. BME001-05)
Geltrex LDEV-free reduced growth factor basement membrane matrix (ThermoFisher Scientific, cat. no. A1413201)
Accutase (StemCell Technologies, cat. no. 07920)
Recombinant human Wnt-3a protein (Bio-techne R&D Systems, cat. no. 5036-WN)
Wnt-3a, murine (see recipe)
Puromycin, sterile, ≥98%, HPLC, 10 mg/ml, liquid (InvivoGen, cat. no. ant-pr-1)

T150 flask (Corning, cat. no. 734-1719)
Assorted pipettor and tips
15-ml centrifuge tube (Corning, cat. no. 430791)
Vortex
Incubator for cell culture, CellXpert C170 (Eppendorf, cat. no. 6734000011)
0.2-µm filter (Sarstedt AG&Co, cat. no. 83.1826.001)
Cryogenic vials (VWR, cat. no. 479-1220)
Tube revolver rotator (ThermoFisher Scientific, cat. no. 88881001)
Centrifuges, 5702 and 5425 (Eppendorf, cat. nos. 5702000010 and 5405000310)
24-well clear multiple well plates, flat bottom with lid, sterile, Corning CellBIND (Corning, cat. no. 3337)
1.5-ml Eppendorf microtubes (Merck, cat. no. Z606340)

Homemade production of lentiviral particles

Day 1: Seed HEK293T cells (∼20 min)

1. Seed one T150 flask of HEK293T per condition. The next day, cells must reach 50% of confluency. The T150 is filled up with 30 ml of DMEM containing 10% of FBS and penicillin-streptomycin (pen-strep, 100 U/ml).

   Seed HEK293T at a passage between P3 and P8 to guarantee the production of a large number of lentiviral particles. Do not seed too many cells to avoid over-confluency and the impairment of lentiviral particle production at day 4 and day 5.

   Budget-friendly note: In-house production of lentiviruses, whenever allowed by adequate infrastructures, is a significant budget-saving approach. Besides, the use of a bigger material format (i.e., T150 flasks) can maximize the amount of obtained particles thereby minimizing batch variability.

Day 2 (end of the day): Calcium phosphate transfection of HEK293T cells with plasmids allowing the production of lentiviral particles (∼1 hr)

2. Prepare the transfection mix in a 15-ml conical tube per condition (i.e., per T150 flask):

   1100 µl milliQ H_2O
   8 µg of plasmid of interest (shRNA or lentiviral GFP as a control)
   6 µg of pCMV-dR8.91(Delta 8.9) packaging expression vector for lentivirus production encoding the *gag*, *pol*, and *rev* genes
   4 µg of VSV-G plasmid expressing the viral envelope
   170 µl of 2 M $CaCl_2$

3. Add dropwise 1200 µl of 2× HEPES-buffered saline (HeBS) to the mix; vortex immediately.

   Vortexing immediately is important; therefore, process samples sequentially one at a time.

4. Incubate for 20 min at room temperature.

5. Add the mix dropwise to the T150 flask. Gently shake the flask to homogenize the solution.

6. Incubate overnight in the incubator at 37°C under 5% CO_2.

   Budget-friendly note: The authors' choice to use the "traditional" transfection technique with calcium phosphate relies on 2 strong assets. (1) It is the cheapest approach to generate lentiviral particles. (2) It works very efficiently to transfect HEK293T cells, leading to an extremely cost-effective step.

Day 3 (morning): Medium refresh (∼10 min)

Manipulation of lentivirus requires to perform experiments below a dedicated safety cabinet (BSL2+) and to be properly equipped and protected while handling lentivirus (days 3, 4 and 5).

7. Change the medium of HEK293T cells, with 30 ml fresh complete DMEM medium complemented with 10% FBS and pen-strep, to prevent toxicity due to the transfection reagent.

Day 4 (morning): Collection of lentiviral particles at ∼40 hr post-transfection (∼2 hr)

8. Collect the supernatant of HEK293T containing the lentiviral particles and filter it using a 0.2-µm membrane.

9. Divide the lentivirus-containing medium into 500 µl aliquots labeled beforehand and kept on ice.

10. Freeze the cryovials at −80°C.

    This will constitute your stock of lentiviral particles collected at ∼40 hr post-transfection.

11. Add 30 ml of fresh medium to HEK293T cells (DMEM with 10% FBS and pen-strep)

Day 5 (morning): Collection of lentiviral particles at 72 hr post-transfection (∼2 hr)

Lentiviral particles harvested at the different times post-transfection originate from the same HEK293T flask; only the medium is changed at step 11.

12. Repeat the same steps on day 4 above (steps 8 to 10).

    This will constitute your stock of lentiviral particles collected at 72 hr post-transfection.

Budget-friendly note: (1) Collecting both at ∼40 hr- and 72 hr-post transfection, instead to only once, allows to increase at least by 2-fold the number of lentiviral particles that will be produced within a single experiment. It is a gain of time and money, since between the 2 collection days, the only requirement is to add the low-cost DMEM complete medium. (2) Because of their sensitivity to freeze-thaw cycles, the lentiviral particles must be frozen and can be thawed only once to be able to perform all the experiments required, on the short-term and on the long-term, under the same conditions, meaning with the same potency of infection. That is the reason why lentiviral particles are frozen before the titration.

Titration of lentiviral particles with the Lenti-X RT-qPCR titration kit (0.5 day)

The titration of lentiviral particles is a prerequisite to the calculation of the MOI value, which is the number of particles that can potentially infect a single cell, essential to the transduction process. First, RNA will be extracted and purified from the lentiviral particles. Then, the subsequent lentiviral genomic RNA will be amplified and quantified by qRT-PCR.

Extraction and purification of lentiviral genomic RNA

13. Follow the guidelines of NucleoSpin RNA virus kit.

    This kit is an additional purchase, it is not included in the Lenti-X RT-qPCR titration kit.

    Pay attention to treat your RNA samples with DNaseI to remove residual plasmid DNA coming from the transient transfection of HEK293T cells. This step will dilute your RNA sample at 1:2 (important to keep in mind for the final calculation of the titration).

Titration of lentiviral genomic RNA by RT-qPCR

14. Follow the guidelines of Lenti-X RT-qPCR Titration kit.

Lentiviral transduction and culture of colon organoids

Day 1: Generation of a single cell suspension of colon organoids (∼1.5 hr)

This step aims to gently break the maturing colon organoids (7 to 14 days old) into a single cell suspension in order to monitor with accuracy the number of cells/ml, leading to the establishment of an accurate MOI.

15. Depending on your experimental design (i.e., the number of conditions), select the appropriate number of domes to be passaged.

    For example, in our hands, we have dilution ratios ranging from 1:2 and 1:3 depending on the density of the organoids inside each dome, meaning that we need 3 or 2 domes, respectively, if we want to seed 6 domes with transduced cells.

    Do not forget to consider the killing control condition in the experimental design, in which cells will not be transduced. This is a crucial control for the antibiotic-based selection step (at day 12).

16. Wash the selected domes twice with DPBS.
17. Add 500 µl of TrypLE on top of the exposed dome in each well. Pipet the TrypLE in the well up and down 3 to 4 times to break up the dome and the organoids. Using the same pipette tip, transfer the organoid mixture to a 15-ml conical tube.
18. Repeat the previous step with 150 µl of TrypLE to ensure all pieces of BME have been recovered from the plate.
19. Incubate for 20 min at 37°C on an orbital tube rotator (∼400 rpm).
20. Centrifuge the cells 4 min at 500 × *g*, room temperature. Gently pour off and discard the supernatant.

Table 2 Details of the Transduction Mix Using TransDux MAX Technology in a 24-Well Plate Format (Basic Protocol 4)

Reagents	1 well (ml)	6 well (ml)
TransDux MAX	2.5	15
MAX enhancer	100	600
Y-27632 (10 mM)	0.25	1.5
IntestiCult	200	1200
Volume of the mix/well	~300 ml	
Total volume/well	~500 ml	

21. Add 1 ml/dome passed of DMEM/F-12 + 15 mM HEPES + 1% BSA to each 15-ml conical tube and count the cells.

    On average, we reach a suspension of 1.5 × 10^6 cells/ml.

22. In a 24-well plate, seed in IntestiCult, 80,000 cells/well for a total volume of 200 µl in each condition (i.e., for each batch of lentiviral particles and/or MOI).

23. Prepare the mix of transduction in an Eppendorf tube, or a 15-ml conical tube (depending on the final volume), as shown in Table 2.

    Always prepare a larger volume of the mix preparation (e.g., corresponding to the desired number of wells + 1 well).

    It is crucial to add the Y-27623 to the mix to increase the survival of the freshly seeded cells. We observed important cell death when it was not included.

    Budget-friendly note: Polybrene, which is a cheap transduction reagent, was first used to achieve the transduction of the colon single-cell suspension solution. However, each attempt failed. That is the reason why we have switched to the use of TransDux MAX lentivirus transduction reagent, which is more expensive, but it is worth the price (as designed here, with 1 kit you can perform 100 transductions).

24. Add the proper number of lentiviral particles.

    Using lentiviral particles (bearing a plasmid for GFP expression) ranging from a MOI of 100 to 6000, we have identified MOI 4000 as a relevant dose to achieve lentiviral transduction of the colon single cell suspension (Fig. 4B for MOIs of 1000 and 4000; other MOIs are shown in supplementary Figure 1; lower magnification for MOIs 1000 to 6000 are shown in supplementary Figure 2).

25. Incubate the single-cell suspension with the lentiviral particles for 72 hr at 37°C (*Fig. 4A*). Gently tap with your finger on the 24-well plate to homogenize the solution.

Day 4: Back to 3D-dome culture (~1 hr)

At this step, the single-cell suspension is very heterogeneous with some of the transduced cells in suspension and others adherent. Most of them present clusters of cells with the formation of a lumen (Fig. 4A).

a. Preparation

26. Warm at 37°C, at least 2 hr prior the beginning of the experiment, a tissue-culture treated 24-well plate.

27. Prepare complete IntestiCult organoid growth medium (human) following the supplier guidelines, and warm it up to room temperature (15° to 25°C).

28. Prepare DMEM/F-12 with 15 mM HEPES and 1% BSA and keep it on ice.

    BSA is crucial to prevent the biopsy pieces to stick on the plastics and consequently lose material.

29. Thaw on ice Cultrex, for each well of a 24-well plate.

Budget-friendly note: Alternatively, Cultrex can be replaced by Geltrex LDEV-free reduced growth factor basement membrane matrix.

b. Dome seeding

30. Transfer the supernatant of each well in a sterile Eppendorf tube, to collect the cells in suspension.

31. Rinse each well with 200 µl of DPBS at room temperature and transfer the supernatant to the corresponding Eppendorf tube.

32. Add 150 µl of Accutase in each well to detach gently the adherent cells. Incubate 5 min at 37°C. Under the microscope, double-check that all cells are detached and transfer the supernatant to the corresponding Eppendorf tubes.

At this step, Accutase is preferred over TrypLE, because Accutase has a milder action. It preserves cell clusters that appear 72 hr after transduction, which allows faster growth and regeneration of organoids. TrypLE has a stronger digestive activity, which would destroy cell clusters.

33. Centrifuge the cells 4 min at 500 × g, room temperature. Gently pour off and discard the supernatant.

34. Add 60 µl of Cultrex per condition. Gently homogenize the cell pellet by doing up and down with the pipette.

35. Seed each dome in a well of 24-well plate.

36. Incubate the plate for 30 min at 37°C under 5% CO_2 to allow dome solidification.

37. Once dome solidification is achieved, remove the 24-well plate from the incubator and add 500 µl of IntestiCult medium supplemented with 10 µM of the ROCK inhibitor Y-27632 to each single well, as well as 50 ng/ml of Wnt3a for healthy tissue exclusively.

38. Refresh the medium as described previously in Basic Protocol 2:

    a. 750 µl of IntestiCult from Monday to Friday.
    b. 500 µl of IntestiCult from Friday to Monday.

Of note, the fresh medium is IntestiCult alone for tumor organoids and IntestiCult supplemented with Wtn3a for healthy organoids.

Day 12: Selection (∼15 min)

The selection process was performed at day 8 post-seeding, to allow organoid recovery before starting antibiotic treatment.

39. Start the selection by adding puromycin at 5 µg/ml in IntestiCult.

The selection process can last from 3 to 7 days, depending on the outcome of the killing control (organoids without plasmid). When all the cells in the killing control condition have died, the selection is considered effective.

Here, puromycin was used because the plasmid we used displayed a resistance marker to this antibiotic. While using other antibiotics, their concentration will need to be optimized.

40. Refresh the medium as described above with puromycin at 5 µg/ml:

    a. 750 µl of IntestiCult + puromycin from Monday to Friday.
    b. 500 µl of IntestiCult + puromycin from Friday to Monday.

IMMUNOBLOT AND EVALUATION OF VARIABILITY BETWEEN DONORS

Originally, this immunoblotting protocol was developed to be able to extract and detect proteins from a single Cultrex-organoid dome as shown in Figure 5A. However, our first experiments revealed that the abundance of clathrin (CHC) varies from one dome to another. Since clathrin is a ubiquitously expressed protein involved in a canonic endocytic mechanism which ensures housekeeping cellular functions, its protein abundance generally correlates with the amount of cellular material present in a sample. In theory,

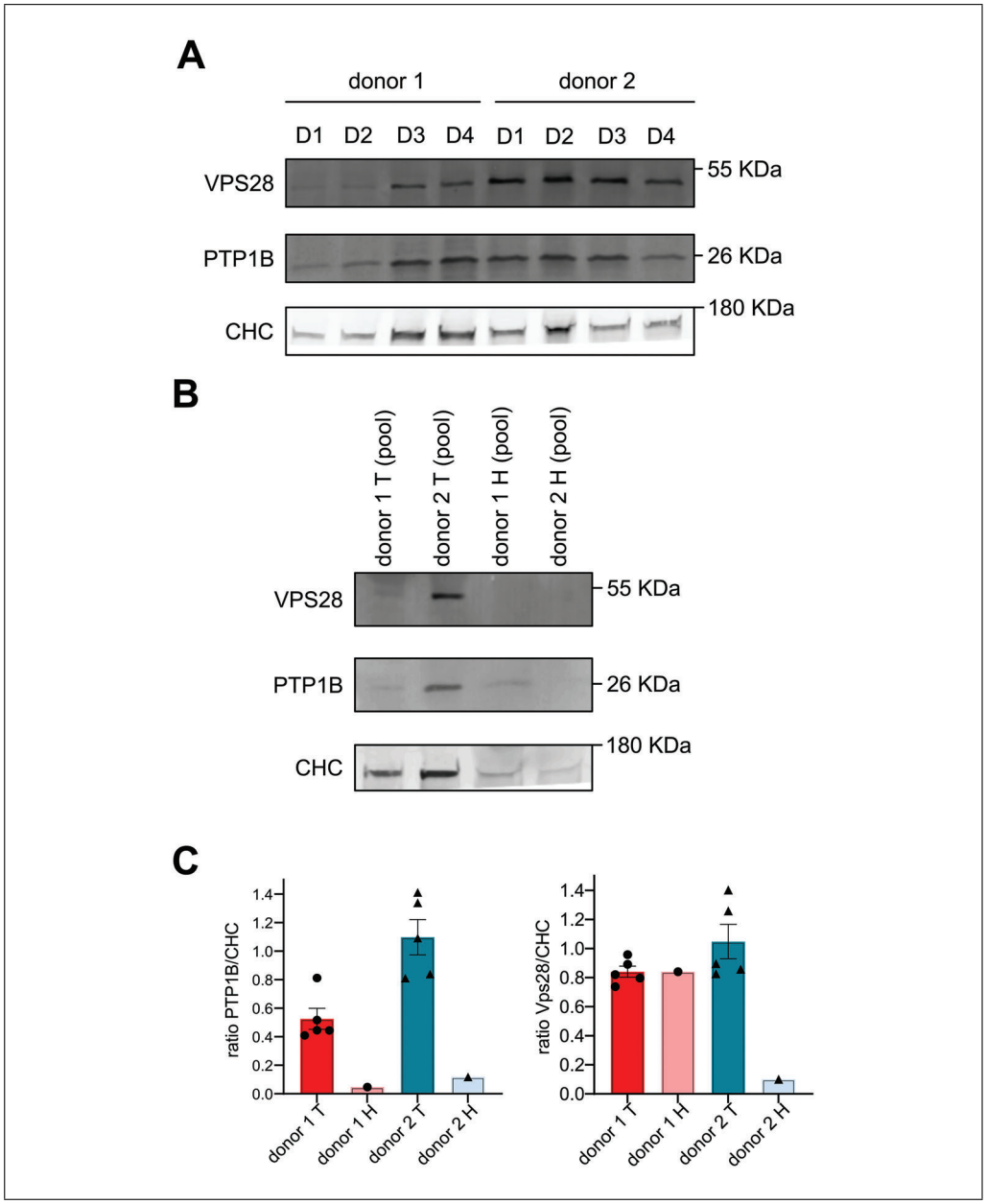

Figure 5 Evaluation of protein expression between domes and donors by immunoblotting. Expression levels of VPS28 and PTP1B proteins were assessed with specific antibodies. Clathrin heavy chain (CHC) was used as a loading control. (**A**) Immunoblots on four domes (D1 to D4) originate from the tumor tissue of two different donors (1 and 2). Of note, one can observe variations in protein abundance from one dome to another originating from the same patient. (**B**) Immunoblots after pooling of 3 domes for each donor (1 and 2) and healthy (H) or tumor (T) tissue. (**C**) Quantifications of western blot signals for PTP1B and VPS28 in tumor and healthy PDCOs originating from the two donors (1 and 2). PTP1B and VPS28 signals were normalized with CHC band intensities (ratios PTP1B/CHC and VPS28/CHC). Number of samples: n = 5 for tumor PDCOs, n = 1 for healthy PDCOs. Data are mean ± SEM.

CHC abundance should thus remain constant from dome to dome, given that the same volume of crypt solution has been seeded in each dome. Thus, the observed variations in the CHC abundance between domes likely reflect some variability in the number of organoids which effectively implanted in each dome and in their growth speed. Therefore, we eventually propose an alternative protocol in which 3 domes are pooled together to compensate this inter-dome variability and reach more homogeneous protein concentrations as shown in Figure 5B. This protocol can be applied to cancer biomarker discovery studies. Briefly, PDCO-domes are disrupted using TrypLE, washed and then incubated with a lysis buffer in reducing conditions to enable protein extraction. Then, proteins from the lysates are separated by SDS-PAGE and transferred onto a membrane using a Trans-Blot Turbo transfer system. Finally, the proteins of interest are detected by western blot and revealed by chemiluminescence.

Materials

DPBS (see recipe)
TrypLE Express enzyme, 1×, no phenol red (ThermoFisher Scientific, cat. no. 12604039)
RIPA lysis buffer, 10× (Merck, cat. no. 20-188)
Loading buffer 5× (see recipe)
Migration buffer (see recipe)
4% to 15% Mini-PROTEAN TGX precast protein gels, 10-well, 50 µl (Bio-Rad, cat. no. 4561084)
Color pre-stained protein standard, broad range, 10 to 250 kDa (New Englands BioLabs Inc, cat. no. P7719S)
Trans-Blot Turbo RTA Mini LF PVDF transfer kit, 40 blots (Bio-Rad, cat. no. 1704272)
Wet-transfer buffer (see recipe)
Bovine serum albumin (BSA) fraction, protease-free, TSE-/BSE-free (Carl Roth, cat. no. T844.3)
Tween 20 (Carl Roth, cat. no. 9127.1)
Milk powder (Régilait 0.8 MG)
Pierce ECL western blotting substrate (ThermoFisher Scientific, cat. no. 32109)
SuperSignal West Femto maximum sensitivity substrate (ThermoFisher Scientific, cat. no. 34095)
Purified mouse anti-clathrin heavy chain clone 23 (BD Transduction Laboratories, cat. no. 610500)
Anti-VPS28 rabbit polyclonal antibody (Proteintech, cat. no. 15478-1-AP)
Anti-PTP1B antibody [EP1841Y] (Abcam, cat. no. ab75856)
Goat anti-mouse immunoglobulins/HRP (Agilent Technologies, cat. no. P044701-2)
Goat anti-rabbit immunoglobulins/HRP (Agilent Technologies, cat. no. P044801-2)

1.5-ml Eppendorf microtubes (Merck, cat. no. Z606340)
15-ml centrifuge tube (Corning, cat. no. 430791)
Tube revolver rotator (ThermoFisher Scientific, cat. no. 88881001)
Centrifuges, 5702 and 5425 (Eppendorf, cat. nos. 5702000010 and 5405000310)
Criterion vertical electrophoresis cell (Bio-Rad, cat. no. 1656001)
SureLock tandem midi blot module (Thermo Scientific, cat. no. S2001)
Western blotting filter paper, 0.83-mm thick, 8-cm × 13.5-cm (Thermo Scientific, cat. no. 84784)
Immobilon-FL PVDF, 0.45 µm, 8.5-cm × 10-m roll (Merck, cat. no. IPFL85R)
Trans-Blot Turbo transfer system (Bio-Rad, cat. no. 1704150)
Chemiluminescence imaging system

Protein extraction

1. Wash the domes of interest once with cold DPBS.
2. Add 500 µl of TrypLE on top of the dome in each well. Pipet the TrypLE in the well up and down 3 to 4 times to break up the dome and generate a single-cell suspension. Using the same pipette tip, transfer the organoid mixture to a 1.5-ml Eppendorf tube.

   Alternatively, pool the single cell suspension coming from 3 domes together in a 15-ml centrifuge tube.
3. Repeat the previous step with 150 µl of TrypLE to rinse it and to ensure all pieces of organoids embedded in the dome of Cultrex have been recovered from the well.

   Alternatively, pool together the washing solution of 3 domes (same as step 2).
4. Incubate for 20 min at 37°C on a tube rotator at ∼400 rpm.
5. Centrifuge the cells 4 min at 500 × *g*, 4°C. Carefully discard the supernatant.
6. Resuspend the cell pellet in 1 ml of ice-cold DPBS within the same Eppendorf tube.

   Alternatively, when dealing with a pool of 3 domes, resuspend the pellet in 1 ml of ice-cold DPBS and transfer the solution into a 1.5-ml Eppendorf tube.
7. Repeat step 5.
8. Resuspend the cell pellet in 20 µl of lysis buffer.

   Alternatively, resuspend the pellet resulting in the pooling of 3 domes into 60 µl of lysis buffer.
9. Incubate for 15 to 30 min on a shaker at 4°C.
10. Spin 10 min at 14,000 × *g*, 4°C.
11. Transfer the supernatant to a new Eppendorf tube.

    At this step, you can stop and store the sample at −20°C for up to a year.

Electrophoresis separation and semi-dry transfer

12. Add the loading buffer (1× final) to each sample to denature the proteins in reductive conditions and heat at 95°C for 10 min.
13. Fill up the electrophoresis tank with migration buffer (1×) containing SDS to allow proteins to migrate according to their molecular weight.
14. Load the samples on 4% to 15% Mini-PROTEAN TGX precast protein gels using color pre-stained protein standard ladder as a molecular weight marker.

    Budget-friendly note: Alternatively, homemade Tris·HCl 8% or 10% gels can be used.
15. Allow the migration at 160 to 180 V for 35 to 50 min depending on the size of the proteins of interest.
16. Perform semi-dry transfer using the Trans-Blot Turbo transfer system and following Bio-Rad guidelines (3 to 10 min).

    Budget-friendly notes: (1) Be aware that the transfer stacks and the transfer buffer sold in the kit are both reusable under the following conditions. The transfer buffer must be filtered to remove the remaining pieces of gel, and the transfer stacks must be extensively but cautiously washed under tap water and then rinsed once with deionized water. Following these recommendations, the transfer stacks can be used a dozen of times if their handling has been gentle. (2) As an alternative to the purchase of the kit and the Bio-Rad transfer buffer, a homemade transfer buffer can be prepared (see Reagents and Solutions). The homemade transfer buffer proposed is adapted to wet transfer systems. Wet transfer is much slower (2 hr) than Turbo transfer, but it can often give better results for proteins that are difficult to transfer (e.g., high molecular weight). In addition, instead of using the

transfer stacks found in the kit, SureLock tandem midi sponge kit (reusable) or western blotting filter paper can be used for similar high efficiency of transfer on both PVDF and nitrocellulose membranes.

17. Incubate the membranes in a blocking buffer containing 5% BSA with 0.05% of Tween 20 in DPBS (referred to DPBST 5% BSA), for at least 45 min at room temperature, on an orbital shaker.

    Alternatively, an overnight incubation at 4°C can be performed on a rocker.

18. Dilute the primary antibody (anti-clathrin heavy chain, anti-VPS28 and anti-PTP1B) in DPBST 5% BSA at the concentration recommended by the company.

    A dilution of 1:1000 was used for each of the three primary antibodies that we used (CHC, VPS28, PTP1B). The dilution must be optimized for each new antibody.

19. Incubate the primary antibody on the membrane overnight at 4°C.

    Alternatively, and for non-phosphorylated proteins only, DPBST with 5% milk can be used as a stronger blocking buffer to decrease non-specific antibody binding. But be aware that milk powder can sometimes go sour on your membrane, especially during summertime.

20. Wash the membrane 3 times for 5 min, at room temperature in DPBST 5% BSA.

21. Repeat steps 17 to 19 with a secondary antibody (goat anti-mouse or anti-rabbit) coupled to HRP and incubate the membrane for 30 min to 45 min at room temperature.

    A dilution of 1:5000 was used for HRP-coupled secondary antibodies that we used.

Chemiluminescence reaction and revelation

22. Use Pierce ECL western blotting substrate for revelation in chemiluminescence, if your proteins are well expressed and you expect a strong signal, such as for clathrin or tubulin. Alternatively, use SuperSignal West Femto substrate for revelation if your proteins have a weaker expression or if you expect a weak signal. Follow the supplier guidelines.

23. Monitor chemiluminescence signals with any western blot imager commercially available.

IMMUNOFLUORESCENCE LABELING AND HIGH-RESOLUTION MICROSCOPY OF PDCOS

BASIC PROTOCOL 6

This protocol aims to easily achieve high resolution confocal microscopy on PDCOs in order, for instance, to assess their maturation status or determine the localization of specific markers at the subcellular level (Fig. 6A-B). An important step in this protocol is to use the adapted devices developed for microscopy. Consequently, after passage, Cultrex-organoid dome cultures are seeded in Lab-Tek chambers (Fig. 6C), which have a glass bottom adapted to imaging by confocal and high-resolution microscopy devices displaying an inverted configuration. Once PDCOs grown in IntestiCult in the Lab-Tek containers have reached maturation, they are fixed with 4% PFA at 4°C, residues of PFA are quenched with NH_4Cl, and cells are permeabilized with 0.5% Triton X-100. Then, primary and secondary antibodies are incubated with the Cultrex-domes to label the PDCOs. After extensive washing steps, PDCOs are ready for imaging.

Materials

Cultrex UltiMatrix reduced growth factor basement membrane extract (RGF BME) (Bio-techne R&D Systems, cat. no. BME001-05)

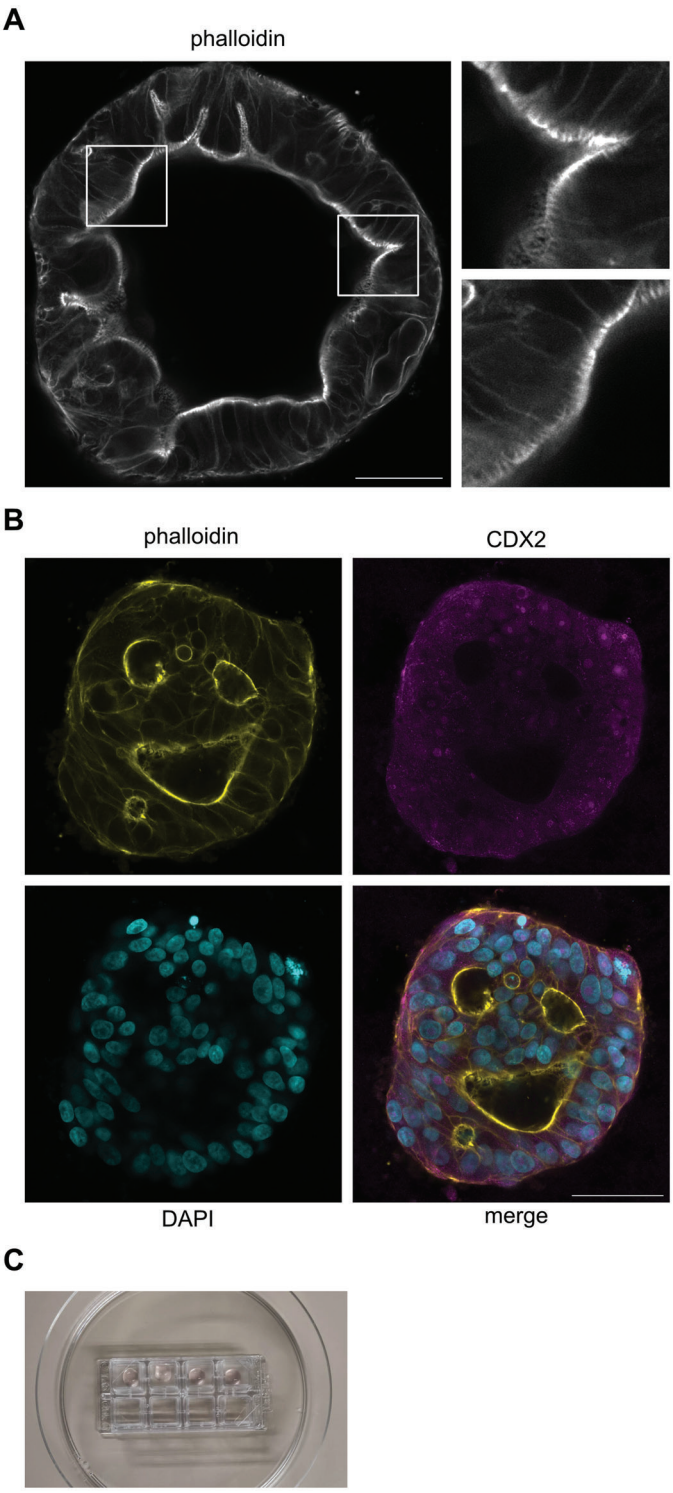

Figure 6 PDCOs imaged by high resolution confocal microscopy after immunofluorescence labeling. (**A**) Representative confocal microscopy image of a tumor PDCO, labeled with phalloidin to reveal its actin cytoskeleton and the subsequent microvilli brush borders of enterocytes. (**B**) Immunofluorescence labeling of the actin cytoskeleton (by phalloidin, in yellow), CDX2 (intestinal progenitor marker, in magenta), and DAPI (nuclei, in cyan) on a tumor PDCO. Scale bars, 50 μm. **(C)** Example of Cultrex-domes seeded in a LabTek chambered slide designed for imaging by inverted confocal microscopy.

IntestiCult organoid growth medium (human) (StemCell Technologies, cat. no. 06010) with antibiotic-antimycotic (1×) (ThermoFisher Scientific, cat. no. 15240062)
DPBS, no calcium, no magnesium (ThermoFisher Scientific, cat. no. 14190144)
4% PFA (see recipe)
NH_4Cl (ammonium chloride) (Merck, cat. no. A9434)
Triton X-100 (Merck, cat. no. 9036-19-5)
Blocking buffer (see recipe)
Bovine serum albumin (BSA) fraction, protease-free, TSE-/BSE-free (Carl Roth, cat. no. T844.3)
Fetal bovine serum (FBS) (ThermoFisher Scientific, cat. no. 26140079)
Invitrogen Alexa Fluor 633 phalloidin (ThermoFisher Scientific, cat. no. A22284)
Anti-CDX2 mouse monoclonal antibody, clone 5A4E6 (Proteintech, cat. no. 60243-1-IG)
Anti-MUC2 rabbit polyclonal antibody (Proteintech, cat. no. 27675-1-AP)
Anti-Sox9 mouse monoclonal antibody (Proteintech, cat. no. 67439-1-IG)
Anti-CGA rabbit polyclonal antibody (Proteintech, cat. no. 10529-1-AP)
Anti-ZO-1 rabbit monoclonal antibody, D6L1E (Bioke, cat. no. 13663)
Anti-EPCAM rabbit polyclonal antibody (Proteintech, cat. no. 21050-1-AP)
DAPI (Merck, cat. no. D9542)
Appropriate secondary antibodies conjugated to Alexa Fluor 488/568 dyes (Invitrogen)

Nunc Lab-Tek chambered coverglass, #1.5 borosilicate glass (ThermoFisher Scientific, cat. no. 155411)
Zeiss LSM900 confocal microscope equipped with Airyscan 2 (or equivalent technology)
Cell culture dish, polystyrene, 100/20 mm (Greiner Bio-one, cat. no. 664160)
Corning Pasteur pipettes, non-sterile (Merck, cat. no. CLS7095B5X)
Plan-Apochromat 20×/NA 0.8 objective (Zeiss, cat. no. 440640-9903-000)

PDCO seeding in Lab-Tek chambered slides

1. Passage PDCOs following Basic Protocol 2 and seed 30 µl of the Cultrex-organoid suspension per well of a Lab-Tek chambered coverglass, #1.5 borosilicate glass.

   Nunc Lab-Tek chambered coverglass, #1.5 borosilicate glass are specially developed for immunofluorescence labeling assays and can be optimally used with confocal and high-resolution microscopes, such as the Zeiss LSM900 Airyscan 2 high-resolution confocal microscope (or equivalent).

   Since the lid of the Lab-Tek is very loose and subsequently, not very handy, put the Lab-Tek in a 10-cm dish to avoid any storage issue in the incubator, such as an accidental opening. In addition, it will protect the Lab-Tek from scratches on the glass bottom, which is crucial for microscopy.

2. Maintain the Cultrex-organoid domes from 2 to 4 weeks until you reach PDCO maturation, that can be assessed by monitoring organoid morphology (see Fig. 6A-B). Refresh the medium every other day with 250 µl of complete IntestiCult.

   It was noticed that Cultrex domes are less adherent on glass than on plastic. Consequently, carefully remove the medium to be discarded, using a Pasteur pipette with an elongated glass tip to ensure less disturbance of the dome. Moreover, perform smooth movements to avoid flushing the dome.

PDCO fixation

3. On the day of the experiment, carefully wash the dome cultures once with DPBS at room temperature.

4. Carefully discard DPBS using a Pasteur pipette as mentioned above.
5. Fix with 4% PFA 30 min at 4°C.

   Do not incubate with PFA longer than 30 min to avoid potential destruction of the Culrex-domes.

6. Quench PFA remnants with a 15 min incubation in 50 mM NH_4Cl solution at room temperature.

   At this step, you can keep the plate at 4°C for up to 3 weeks on a flat and still shelf. Every other day, double-check on the volume present in each well. In case of evaporation, refill the wells with the same NH_4Cl solution.

   To keep on long-term storage and avoid potential contamination, it is also possible to add sodium azide.

PDCO permeabilization and blocking

7. Then, permeabilize the Cultrex-organoid domes with a solution of DPBS containing 0.5% Triton X-100 for 1 hr at room temperature.
8. Carefully discard the Triton X-100 solution.
9. Incubate the Cultrex-organoid domes with blocking buffer for 1.5 hr at room temperature. The blocking buffer is composed of 10% FBS, 1% BSA, and 0.1% Triton X-100 in DPBS.

   Alternatively, if more convenient, the blocking step can be performed at 4°C for 4 hr.

PDCO fluorescence labeling

10. Carefully discard the blocking buffer solution without disturbing the domes.
11. Incubate primary antibodies diluted in the blocking buffer on top of the Cultrex-organoid domes for 16 hr at 4°C on a flat and still shelf.

    In Figure 6A, phalloidin was used at 1:500 to visualize microvilli brush borders of enterocytes. In addition, in Figure 6B, an anti-CDX2 antibody was used at 1:200 to reveal intestinal progenitor cells. Of note, you are free to use any other classical markers at this stage, such as MUC2 (goblet cell marker), Sox9 (crypt marker), CGA (enteroendocrine cell marker), ZO-1 (tight-junction marker), or EPCAM (basolateral epithelium marker).

12. Carefully discard the primary antibody solution without disturbing the domes. Gently wash once with 350 µl per well of blocking buffer.

    This step does not require extensive washing considering how fragile is the adherence of the domes on the Lab-Tek glass bottom.

13. Incubate Cultrex-organoid domes with DAPI and the secondary antibodies diluted in the blocking buffer for 2 hr at room temperature on a flat and still shelf, protected from light.

    In Figure 6B, DAPI was used at a final concentration of 0.5 µg/ml to visualize the nucleus and secondary antibodies Alexa fluor were used at 1:1000.

    DAPI staining is included in all PDCO immunofluorescence labeling assays to visualize the nucleus of all the cells present in the PDCO. It is also used to set up the focus of the confocal microscope.

14. Carefully discard the secondary antibody solution without disturbing the domes. Carefully, wash three times with 350 µl per well of blocking buffer.

    This step requires extensive, but cautious washes to remove the free antibodies.

    At this step, Cultrex-organoid domes can be stored in DPBS at 4°C, on the flat and still shelf up to 2 weeks, protected from light.

PDCOs high-resolution imaging with Zeiss LSM900 Airyscan 2

15. Perform the focus settings with DAPI staining through the objectives.

Since PDCOs are embedded in a dome of Cultrex, long working-distance objectives are required. In our case, we imaged PDCOs with a dry plan apochromatic 20× objective with a numerical aperture of 0.8. Airyscan 2 module allows to reach lateral resolution beyond the diffraction limit, up to 120 nm. For the best resolution images, higher magnification objectives (e.g., 63×) can be used. Of note, alternative confocal microscopy devices can also be used.

TRANSCRIPTOMIC ANALYSES OF PDCOS BY RT-QPCR

BASIC PROTOCOL 7

This protocol enables the extraction of mRNA from organoid-domes and the subsequent analysis of target gene expression through RT-qPCR. It allows for the comparison of gene expression under various conditions, such as tumor organoids vs healthy organoids after a specific treatment (Figure 7). In summary, PDCO-domes are disrupted using TrypLE, washed, and then incubated with a lysis buffer to facilitate mRNA extraction. Starting from mRNA, cDNA is generated using reverse transcriptase and specific primers. The cDNA is then analyzed using qPCR. For this type of analysis, it is recommended to pool at least three domes together and perform the extraction on the pooled sample. This approach ensures there is sufficient material and reduces variability between domes derived from the same donor, as previously mentioned in Basic Protocol 5.

Materials

10% SDS solution, ultrapure (VWR, cat. no. J77504)
DPBS (see recipe)
TrypLE Express enzyme, 1×, no phenol red (ThermoFisher Scientific, cat. no. 12604039)
ReliaPrep RNA tissue miniprep system (Promega, cat. no. Z6111)
GoScript reverse transcriptase mix + random primer (Promega, cat. no. A2801)
GoScript reverse transcriptase mix + oligo(dT) (Promega, cat. no. A2791)

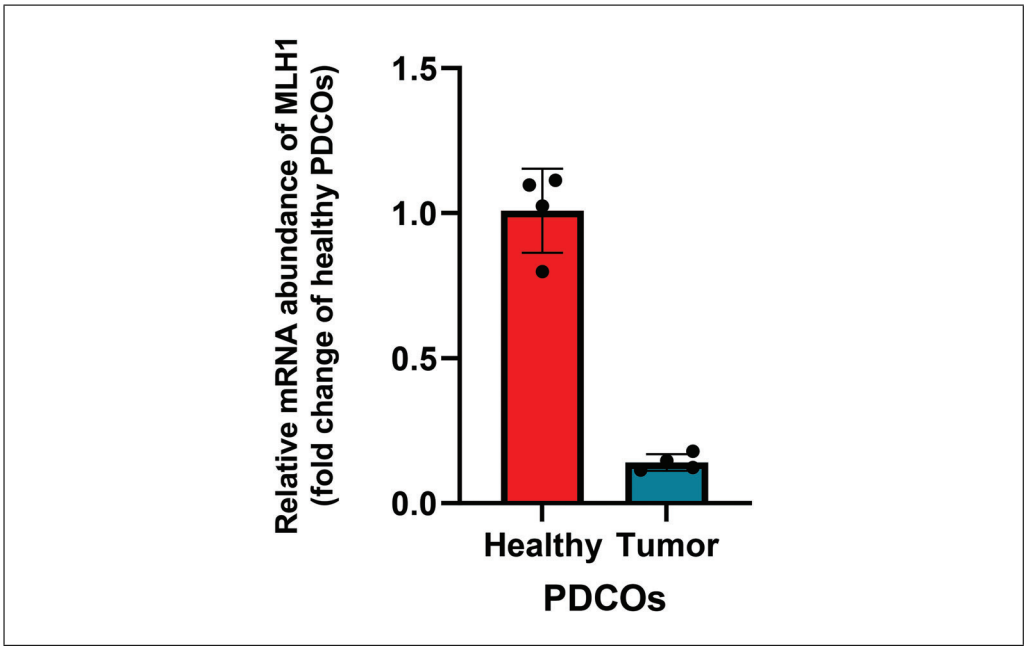

Figure 7 Evaluation of mRNA expression in tumor and healthy PDCOs by RT-qPCR. Relative mRNA levels of MLH1 gene in tumor and healthy PDCOs quantified using RT-qPCR and normalized to 23 kD. Results are expressed in fold change of healthy PDCOs. Single experiment, four technical replicates. Data are mean with SD. Of note, MLH1 is a DNA damage repair protein whose expression is often lower in colon cancer due to mutations.

GoTaq qPCR and RT-qPCR systems (Promega, cat. no. A6001)
H_2O, milliQ

Assorted pipettors and RNase-free filter tips
15-ml centrifuge tube (Corning, cat. no. 430791)
Tube revolver rotator (ThermoFisher Scientific, cat. no. 88881001)
Centrifuges, 5702 and 5425 (Eppendorf, cat. nos. 5702000010 and 5405000310)
1.5-ml Eppendorf microtubes (Merck, cat. no. Z606340)
NanoPhotometer N50 (Westburg Life sciences, cat. no. IM N50)
8-tube strips and caps, PCR, polypropylene, 0.2-ml (Greiner Bio-One, cat. no. 60827)
Savant SpeedVac DNA 130 integrated vacuum concentrator system (ThermoFisher Scientific, cat. no. DNA130-230)
MyBlock HL Mini dry bath with heated lid (Merck, cat. no. Z742495)
SimpliAmp thermal cycler (ThermoFisher Scientific, cat. no. A24811)
Vortex ZX3 (VWR, cat. no. 444-2000)
Applied Biosystems MicroAmp Fast optical 96-well reaction plate, 0.1-ml (Fisher Scientific, cat. no. 43-469-07)
MicroAmp optical adhesive film (Fisher Scientific, cat. no. 43-119-71)
ViiA 7 real-time PCR system with 96-well block (ThermoFisher Scientific, cat. no. 4453534)

NOTE: For this protocol, it is important to work in an RNase-free environment. To achieve this, clean the bench and all materials with a 1% SDS solution diluted in deionized water. Use appropriate gloves and RNase-free filter tips.

mRNA extraction

1. Wash the domes of interest once with cold DPBS.
2. Add 500 µl of TrypLE on top of the dome in each well. Pipette up and down with the TrypLE in the well 3 to 4 times to break up the dome and generate a single-cell suspension. Using the same pipette tip, transfer the organoid mixture of each dome in a common 15-ml centrifuge tube.
3. Repeat the previous step with 150 µl of TrypLE to rinse the well and to ensure all pieces of organoids embedded in the dome of Cultrex have been recovered from the well. Add the washing solution to the 15-ml centrifuge tube.
4. Incubate for 20 min at 37°C on a tube rotator at ∼400 rpm.
5. Centrifuge the cells 4 min at 500 × *g*, 4°C. Carefully discard the supernatant.
6. Resuspend the cell pellet in 1 ml of ice-cold DPBS and transfer it into a 1.5-ml Eppendorf tube.
7. Repeat step 5 to have a dry pellet at the end.
8. Follow the guidelines of ReliaPrep RNA tissue miniprep system.

   Budget-friendly note: Extraction of mRNA is also possible using the classic phenol/chloroform method, but the yield is poorer.

9. After RNA extraction, keep the samples on ice.
10. Measure the mRNA concentration using a nanophotometer.

    mRNA can be stored long-term at −80°C.

Reverse transcription

In this part of the protocol, it is crucial to work on ice, as mRNA is less stable compared to cDNA.

Table 3 Details of the Reverse Transcription Mix (Basic Protocol 7)

Reagents	1 sample (µl)	n sample + 1
H_2O, RNase free	2	2 × (n + 1)
Buffer random primers	2	2 × (n + 1)
Buffer oligo dT	2	2 × (n + 1)
Enzyme mix	2	2 × (n + 1)
Volume of the mix/microtube	8 µl	8 × (n + 1) µl

11. Dilute mRNA in 0.2-ml microtubes to reach 2 µg per condition in a maximum volume of 12 µl.

    It is possible to reduce the mRNA amount to 1 µg per condition, with a maximum volume of 12 µl. If the mRNA is too dilute, it can be concentrated using a vacuum concentrator system.

12. Heat the microtubes containing mRNA in a dry bath for 5 min at 70°C.

13. Prepare the Reverse Transcription Mix in an Eppendorf tube on ice, as shown in Table 3. Gentle vortex the mix and add 8 µl of the mix in each 0.2 ml microtube on ice.

    Always prepare a larger volume of the mix preparation (e.g., corresponding to the desired number of microtubes + 1 microtube).

    Please note that the reverse transcription mix is a mix of random primer and oligo(dT) that, in our hands, was more efficient.

14. Spin the microtubes briefly at maximum speed, room temperature, and start the reverse transcription reaction in a controlled-temperature block or in a programmed thermal cycler.

    The temperature and time of each cycle is provided in the protocol of GoScript reverse transcriptase kit.

15. After reverse transcription, spin the 0.2-ml microtubes briefly at maximum speed, room temperature. cDNA can be stored at 4°C for direct analyses or at −20°C for long-term storage.

    For long-term storage, transfer the products to 1.5-ml Eppendorf tubes.

Real-time PCR

For the real-time PCR, it is important to not forget a housekeeping gene (e.g., GAPDH) and a blank condition corresponding to PCR mix + H_2O (without cDNA). Moreover, each sample needs to be done in triplicate. For the RT-PCR, we designed and validated our own primers, however validated commercial primers can be used.

16. Dilute cDNA 100-fold with miliQ H_2O and dilute the qPCR primers at a concentration of 3 µM.

    If the mRNA amount used at the step 11 was 1 µg per condition, the recommended dilution is 50-fold.

17. Prepare the PCR mix in an Eppendorf tube, as shown in Table 4. Vortex the PCR Mix.

    At this step, it is important to thaw the GoTaq qPCR master mix at room temperature and vortex 3 to 5 s before using it.

    Do not forget to measure all your conditions in triplicate.

Table 4 Details of the PCR Mix (Basic Protocol 7)

Reagents	For each gene (µl)	n sample + 1
H_2O, milliQ	2	2 × (n + 1)
Forward primer 3 µM	2	2 × (n + 1)
Reverse primer 3 µM	2	2 × (n + 1)
GoTaq qPCR master mix (2×)	10	10 × (n + 1)
Volume of the mix/gene	16 µl	16 × (n + 1) µl

18. In a 96-well plate designed for real-time PCR, transfer 16 µl of PCR mix in each well (depending on the number of genes and conditions to test). Add 4 µl of cDNA diluted 100-fold in the corresponding wells, or 4 µl of milliQ H_2O for the blank condition.
19. Seal the 96-well plate with an adhesive film and centrifuge the plate 1 min at 250 × *g*, room temperature.
20. Run the real-time PCR using the ViiA 7 real-time PCR system with standard cycling conditions.

REAGENTS AND SOLUTIONS

Blocking buffer

5 ml FBS
0.5 g BSA
50 µl Triton X-100
45.950 ml of DPBS (see recipe)
Store up to 1 month at 4°C

$CaCl_2$, 2 M

Add 14.7 g of $CaCl_2 \cdot 2H_2O$ in 50 ml of ultrapure H_2O. Make fifty 1 ml aliquots into Eppendorf tubes and store indefinitely at −20°C. $CaCl_2$ is in a liquid form at this temperature.

DPBS, 1×

9 g NaCl
20 ml 0.5 M phosphate buffer, pH 7.4
Bring up to 1 L with deionized H_2O
Store indefinitely at 4°C

HEPES-buffered saline (HeBS), 2×

8 g NaCl (274 mM)
0.37 g KCl (10 mM)
0.1065 g $Na_2HPO_4 \cdot 2H_2O$ (1.5 mM)
1 g dextrose (12 mM)
5 g HEPES (50 mM)
450 ml deionized H_2O
Adjust to pH 7 with NaOH
Bring up to 500 ml with deionized H_2O
Store up to several years at 4°C

Loading buffer, 5×

10 ml SDS 20% (6%)
5 ml β-mercaptoethanol

10 ml 85% glycerol (24.3%)
17.5 mg bromophenol blue
35 ml H_2O
Store indefinitely at −20°C

Migration buffer, 10×

For 1 L:
30.03 g Tris (250 mM)
143 g glycine (1.9 M)
100 ml 10% SDS
Bring up to 750 ml with H_2O
Adjust pH to between 8 and 9 with NaOH or HCl
Bring up to 1 L with H_2O
Store up to 6 months at 4°C

PFA, 4%

Break the 10 ml ampule of 16% formaldehyde solution, methanol-free (ThermoFisher Scientific, cat. no. 28908) and transfer the solution to a 50-ml centrifuge tube. Add 30 ml DPBS (see recipe). Due to PFA instability, aliquot PFA in 10-ml centrifuge tubes and store up to 6 months at −20°C. Alternatively store up to 3 weeks at 4°C. Avoid freeze/thaw cycles.

RIPA buffer

50 mM Tris·HCl
50 mM NaCl
0.25% sodium deoxycholate
1% Triton X-100
1 mM EDTA
0.1% SDS
Dilute in deionized H_2O
Adjust to pH 7.4 using NaOH or HCl
Store up to 1 year at 4°C

Wet-transfer buffer, 10×

For 1 L:
30.03 g Tris (250 mM)
143 g glycine (1.92 M)
Bring up to 750 ml with deionized H_2O
Adjust pH to 8.4 with NaOH or HCl
Bring up to 1 L with deionized H_2O
Store up to 6 months at 4°C

Wnt3a, murine

Prepare a solution of DPBS (see recipe) with 0.2% BSA and filter it using a 0.2-µm filter. Reconstitute recombinant murine Wnt-3a (PeproTech, cat. no. 315-20) stock solution at 10 µg/ml by adding 1 ml of sterile DPBS containg 0.2% BSA in the vial containing 10 µg of Wnt3a. Use a working concentration of 50 ng/ml (dilution 1:200). Once reconstituted, make 50 µl aliquots and store up to 1 year at −80°C.

Y-27632, ROCK inhibitor

The Y-27632 (dihydrochloride) (StemCell Technologies, cat. no. 72304) stock solution is 20 mM and used at 10 µM. Add 0.5 µl to prepare 1 ml of IntestiCult supplemented in Y-27632. Once reconstituted store up to 1 year at −20°C protected from light. Make small aliquots of 50 µl to avoid thaw/freeze cycles.

COMMENTARY

Background Information

For decades, much of the knowledge we have accumulated in human biology has been from 2D culture and animal models. However, these models suffer from limitations: 2D-culture models, while being technically convenient, remain a simplistic view of cells growing independently to physiological parameters, which are crucial for integrating all the complexity of the human body. This level of complexity is inherent to animal models, being subsequently the clinical model of predilection. However, it is facing recurrent ethical issues and constant questioning about whether its discoveries are 100% transferrable and consequently relevant to human biology, based on the failure of a significant number of clinical trials (Harrison, 2016; Seruga et al., 2015). In 2009, Sato and colleagues revolutionized the disease model field with their hallmark study revealing how to grow a "mini-gut" from stem cells (Sato et al., 2009). Two years later, they published another breakthrough work reporting the generation of 3D organoids from patient biopsies, paving the way for personalized medicine therapy (Sato et al., 2011). These PDCOs are grown from colon-restricted adult stem cells resulting from healthy or diseased tissue resections. They are able to self-organize in vitro, self-renew, and proliferate. In addition, they display the features of the tumor or the healthy surrounding tissue, such as the complex cell hierarchy and spatial distribution highlighting their ability to recapitulate at best the phenotypic and molecular heterogeneity found in the colon. As such, they represent a growing interest in pre-clinical trial phases and personalized therapies (Chew et al., 2021; Gonzalez-Exposito et al., 2019; Pamarthy & Sabaawy, 2021). However, their generation, maintenance and study in the laboratory are confronted with budget contstraints and reproducibility issues. Moreover, the number of protocols for some techniques to study these PDCOs (including immunoblot, high-resolution confocal microscopy, and lentiviral transduction) is limited.

Critical Parameters

Access to a fresh source of biopsies near the lab

Considering that PDCOs must be generated from fresh biopsies, the most critical parameter that should be considered while starting any patient-derived organoid project, is the access to a hospital linked to a biobank located within a close perimeter from the laboratory. For instance, the authors were 30 min away from the biobank providing the biopsies.

Primary tissues used in this study were obtained from the Biobank of the CHU UCL Namur (coordinated by Dr. Fabienne George). The Biobank organized and supervised the controlled transit of the samples from the hands of the surgeons directly to the hands of the researchers under strict standard operating procedures.

BME selection

Basic Protocol 1 and 2 describe the establishment, maintenance, and expansion of PDCOs embedded in a dome of BME. The choice of BME is crucial to maintain a constant growth of the organoids. In addition, it is crucial to use BME showing consistency among batches and resistance over days in culture on different plastic- and glassware. That is the reason why Cultrex UltiMatrix RGF BME, and alternatively Geltrex LDEV-free reduced growth factor basement membrane matrix, have been chosen.

PDCO culture work with InstestiCult and cryopreservation with CryoStor CS10

In literature, several homemade conditioned media are described for the culture of patient-derived organoids. However, given the number of reagents composing these media and the associated risk accountable to variations between batches, in addition to the complexity to make them, the authors decided to choose InstestiCult. This medium is commercially available and ensures reproducibility. Moreover, InstestiCult has been developed by the Dutch biotech company HUB Organoid Technology to set up optimal culture conditions for efficient PDCO growth. It is crucial to use this culture medium for experimental reproducibility and to secure an efficient PDCO culture.

Another crucial medium is the one used for cryopreservation, as all patient-derived organoids are intended for biobanking. CryoStor CS10 offers the advantage of excellent recovery post-thaw, a crucial factor for organoid regeneration and growth.

Accurate titration of lentivirus and efficient transduction

The crucial step of PDCO lentiviral transduction is to define the MOI resulting in the most efficient transduction of PDCOs. Lentiviral particle titration must be extremely

accurate, which can be achieved with Lenti-X RT-qPCR titration kit from Takara, as described in Basic Protocol 4. The operator must be extremely cautious to work under RNAse-free conditions. Moreover, it is vital to perform the titration on an aliquoted sample that is thawed to remain in the same condition (1 freeze/thaw cycle) as the lentiviral particles that will be used to transduce PDCOs.

Antibody specificity

Before their use, it is crucial to validate the specificity of each antibody. An siRNA approach targeting the protein of interest can be adopted, and the specificity of the antibody monitored by immunoblot or immunofluorescence. The antibodies mentioned in Basic Protocols 5 and 6 have been validated.

In addition to antibody specificity, it is essential to assess whether the antibody of interest can be used for different assays, such as immunofluorescence labeling or immunoblot. It is not guaranteed that an antibody working for immunofluorescence labeling will work also for immunoblot, and vice versa.

Adapted plastic- and glassware to each single downstream experiment

Before seeding Cultrex-domes containing organoid fragments, it is crucial to take time to consider whether the plastic- or glassware the users want to employ is adapted for the downstream analysis. For instance, these authors have reported in Basic Protocol 6 that utilizing Lab-Tek #1.5 borosilicate glass is optimal for confocal microscopy with LSM900 Airyscan 2.

Troubleshooting

Each step of the protocols has been troubleshooted to address the most frequently encountered issues during the process. Detailed troubleshooting information can be found in Tables 5 to 10. These tables primarily address common problems related to organoid-dome handling. For certain protocols, particularly Basic Protocol 7 that involves the use of commercial kits, it is advisable to consult the troubleshooting tables provided with those kits.

Understanding Results

This article described a step-by-step workflow to generate PDCOs from biopsies as presented in Figure 1. This results in the establishment of PDCO cultures that undergo morphological and size changes over the course of weeks, which is inherent in their growth as shown in Figure 2A. Indeed, after 2 to 3 days, small doughnut-like organoids appear in the dome and grow to become larger spheres/ovals containing a lumen after 1 to 2 weeks. Then, some buds become visible and the PDCOs start their complex folding to reach their mature morphology ~4 to 6 weeks. Figure 2B displays the different PDCOs coming from tumor or healthy tissues, generated by the authors following Basic Protocols 1 and 2. It is important to highlight that depending on the donor and the nature of the tissue used to establish the organoid culture, the growth rate will vary in speed. Therefore, the proposed timeline offers ranges and not defined values (Fig. 2A).

Basic Protocol 4 aims to tackle the technical challenge of establishing silenced PDCO cultures. As reported in Figure 3, PDCOs are first dissociated to generate a single cell culture suspension solution. The latter is transduced with lentiviral particles containing the shRNA of interest, here a GFP lentiviral vector to visualize the efficiency of the proposed strategy. After 72 hr (Fig. 4A), cells are seeded back in domes and their growth and GFP expression are monitored over 21 days using inverted epifluorescence microscope (Fig. 4B). Green fluorescence can be noticed already 7 days post-transduction for an MOI of 1000, 4000 and 6000. The reader may think that these MOI are very high, but it must be considered that all the particles will not necessarily infect the cells due to the complex medium and the interference it can induce. Remarkably, it was reported that after 21 days in culture, the transduced PDCOs with the 3 different MOI display mature morphology. An MOI of 4000 reveals a PDCO population expressing medium levels of GFP compared to an MOI of 1000 (low) and an MOI of 6000 (high). These data led the authors to conclude that an MOI of 4000 seems to be a relevant MOI to trigger efficient silencing in PDCOs. Of note, it is important to adjust this MOI to the different lentiviral vectors used, investigating different titrations, and following the same example as the ones tested in this article. Finally, the antibiotic selection was addressed and showed notable death of the PDCOs with 5 μg/ml of puromycin (Fig. 4C).

In the literature, the study of potential therapeutic target genes often begins with classical biochemistry investigations. This is exactly what the authors encourage to do in PDCOs with the description of Basic Protocols 5 to 7. First, the immunoblot approach was under the spotlight revealing a variation in protein abundance between domes of the same donor (Fig. 5A). This can be explained by the

Table 5 Troubleshooting Guide for Culture of PDCOs (Basic Protocols 1 and 2)

Problem	Possible cause	Solution
Biopsy cannot be digested into small pieces	The texture of the biopsy is extremely crunchy due to the tumor	Mince the biopsy to the smallest pieces that can be generated and continue the protocol (it will be less efficient)
	Biopsy has been cut into too big chunks	Mince the biopsy into the smallest pieces that can be generated
Healthy tissue organoid culture domes are not growing	Wnt3a forgotten	Add Wnt3a at 50 ng/ml
Fragments extracted from the biopsy are not growing into organoids	Absence of intestinal crypts due to an over-digestion of the tissue or, on the contrary, not enough digestion	Adapt accordingly to the time of digestion
Domes detach from the culture support	Presence of numerous bubbles	Leave a slim layer of medium above the pellet and resuspend it in this liquid phase before adding the Cultrex, mix intestinal crypts gently with Cultrex to avoid bubbles
	Too harsh flushing	Be gentle while refreshing the medium; do not use vacuum aspiration, but only pipet with a Pasteur pipette or with a tip
Absence of organoid fragments in domes freshly seeded after passaging	Loss of material during centrifugation steps	Increase the time of centrifugation steps to get an optimal pellet
		Do not disturb the pellet and do not discard the whole supernatant; always leave a slim layer of liquid above the pellet
Generation of several organoid fragments after passaging but no growth induction	Organoid culture was not in an exponential phase	Carefully select the domes to be passaged; most organoids must display a doughnut-like shape
	Too high concentration of intestinal crypts	Increase the dilution of intestinal crypts
Organoids are growing extremely slowly after thawing	Organoids were not in an exponential phase when seeded	Passage organoid cultures before cryopreservation; do not wait >1 week between passage and cryopreservation
	Not enough intestinal crypts	Cryopreserved organoids should come from doughnut-like organoid cultures and not from too young or too mature cultures, which will result in the absence of Lgr5+ stem cells allowing the regeneration of organoids
Organoids do not recover after passaging	ROCK inhibitor forgotten	Add Y-27632 at 10 mM in the culture medium after dome seeding

various number of organoids inside each dome. That is the reason why it was suggested to pool together 3 domes to tackle this variability and generate representative and reproducible data. In addition, the results highlighted an expected heterogeneity of protein levels between different donors, and between the different types of tissues (tumor versus healthy) (Fig. 5B). The variability between donors is the perfect illustration of what happens in clinical trials. Although it will involve increasing the number of donors to achieve statistically significant data, it strongly supports the fact that PDCOs is a relevant pre-clinical model. Then, the differentially expressed protein levels observed between healthy PDCOs vs tumor PDCOs is an important argument to use this protocol to identify novel colorectal cancer biomarkers. For instance, the immunoblot presented in the article may suggest that PTP1B could be considered as such, although it would require further investigations to be confirmed. Another way to analyze the differences between

Table 6 Troubleshooting Guide for Cryopreservation of PDCOs (Basic Protocol 3)

Problem	Possible cause	Solution
Cryopreserved organoids do not recover after thawing	Toxicity due to too slow freezing and/or thawing steps	Label cryovials in advance and put them on ice
		After 1 min in the water bath, add pre-warmed medium on top of the half-frozen cryovial supernatant and pipet it up and down to induce faster thawing
	ROCK inhibitor forgotten	Add Y-27632 at 10 mM in the culture medium after dome seeding
No cells after thawing	Loss of material during centrifugation steps	Increase the time of centrifugation steps to get an optimal pellet
		Do not disturb the pellet; do not discard the whole supernatant; always leave a slim layer of liquid above the pellet
Organoids are growing extremely slowly after thawing	Organoids were not in exponential phase when seeded	Passage organoid cultures maximum 1 week before cryopreservation
	Not enough intestinal crypts	Cryopreserved organoids should come from doughnut-like organoids cultures and not from too young or too mature cultures, which will result in the absence of Lgr5+ stem cells allowing the regeneration of organoids

Table 7 Troubleshooting Guide for Lentiviral Transduction of PDCOs (Basic Protocol 4)

Problem	Possible cause	Solution
Poor production of lentiviral particles	Too old HEK293T cells	Do not use HEK293T at a passage higher than 15
	Failed transfection of HEK293T with $CaCl_2$	Double-check to add all the needed lentiviral plasmids in the mix
		Add HeBS 2× quickly to the mix, process one sample at a time
Poor recovery of lentiviral particles after thawing	Failed cryopreservation	Cryopreservation must be performed with CryoStor CS10 quickly on ice, cells must be kept in a freezing container at −80°C and be transferred the next day in liquid nitrogen
Not enough cells seeded after the single cell suspension solution	Loss of cells	Be cautious during the recovery of solutions (PBS wash, Accutase steps) and optimize the centrifugation steps
	Cells remained adherent	Increase the incubation time with Accutase at 37°C, assess under a bright-field microscope the number of cells detached before transferring the cell solution and discard the plate
Poor transduction	Transduction reagent	Use TransDux MAX
	Single-cell incubation time too short	It is extremely important to let the transduction mix incubate on the single cell suspension of organoids for 72 hr; 24 and 48 hr have been assessed and failed
	Non-optimal MOI	Depending on the plasmid used, adapt the MOI
Organoids dying after transduction	Apoptosis due to stress	Do not forget to add Rock inhibitor Y-27632 at 10 μM

Table 8 Troubleshooting Guide for Immunoblotting on PDCOs (Basic Protocol 5)

Problem	Possible cause	Solution
Poor extraction: no protein detected after lysate titration	Lysis failed	Increase the incubation time with the lysis buffer (be careful to work at 4°C)
	Low number of organoids per dome	Pool several domes belonging to the same condition altogether
Signal variability in the loading control	Absence of protein titration	Titrate protein levels with a range of BSA
	Selection of a wrong protein as loading control	Use ubiquitous proteins, e.g., cytoskeleton proteins, which exhibit constancy over time and treatments
No migration/transfer	Buffer problem	Double check on the buffer composition, and pay attention to work with the 1×
Weak transfer with Turbo-Transblot	Technical issue with the device	Clean the electrodes of Turbo-Transblot with a solution of acetic acid 20% (in H_2O) and wash several times with water before use
		Be sure that the magnets of Turbo-Transblot are still in place at the back of the cassette
	Transfer time not adequate	Choose the optimal program depending on the size of the proteins of interest: low <25 kDa, mixed for proteins between 25 and 150 kDa, high >150 kDa
Non-specific signal	Problem with blocking buffer/antibody	To be more stringent, use PBST with 5% milk to block instead of PBST with 5% BSA
		Test the antibody specificity using a lysate from cells in which the protein of interest has been depleted by RNA interference (siRNA, shRNA, etc.)

Table 9 Troubleshooting Guide for Immunofluorescence Labeling of PDCOs (Basic Protocol 6)

Problem	Possible cause	Solution
Destruction of Cultrex-domes and loss of the organoids	4% PFA fixation step	Decrease the time of incubation with 4% PFA from 30 min to 20 min at 4°C; if the problem persists, work with 2% PFA at room temperature for 10 min
Detachment and aspiration of Cultrex-domes	Abrupt movement leading to harsh flushing of the dome	Be extremely cautious during washing steps; if using the transfer pipette induces too harsh flows, work with P200 tips or a Pasteur pipette
Autofluorescence	PFA is not pure enough	Use PFA adapted to microscopy
High noise compared to the signal	Non-optimal final washing step	Increase the incubation time of the washing steps
		Add one more washing step
Faint signal	Non-optimal antibody concentration/problem with permeabilization	Increase antibody concentration and/or the incubation time with the permeabilization buffer
No signal under the confocal microscope	Magnification/focus plan problems	Due to the thickness of the domes, objectives with high magnification (≥40×) or small working distance are not usable; prefer the use of lower magnification objectives (e.g., dry objectives ≤20×) or objectives with larger working distance to reveal signal; focus is done using DAPI signal

Table 10 Troubleshooting Guide for RT-qPCR on PDCOs (Basic Protocol 7)

Problem	Possible cause	Solution
Poor extraction of mRNA, concentration of mRNA is low	Lysis failed	Add the right volume of lysis buffer (recommended in Promega kit)
	Elution problem	If the first elution gave a too low concentration of mRNA, a second elution is possible (but mRNA will be more diluted); it is possible to concentrate the mRNA using a vacuum concentrator system
	Low number of organoids per dome	Pool several domes belonging to the same condition altogether
Problem with 260/280 and 260/230 ratios	Poor sample wash	PBS wash after incubation with TrypLE is crucial; take care to eliminate any trace of TrypLE

tumor and healthy tissues is to look at the transcriptomic data. Thanks to RT-qPCR, we can analyze the expression of target-genes depending on several conditions. To conclude, the other biochemical assay investigated is the immunofluorescence labeling monitored by inverted high resolution confocal microscopy. In this article, immunofluorescence labeling has been used to assess PDCO maturation status, but this assay is not restricted to this aspect. Indeed, it could be used to monitor the spatial distribution of different molecular players including the potential therapeutic targets. Confocal imaging microscopy reveals microvilli brush borders of enterocyte cells as well as intestinal progenitor marker (CDX2), showing the cellular complexity of the PDCOs that have been generated (Fig. 6A-B).

Time Considerations

In addition to the timeline described in each basic protocol listed hereabove, the reader must also consider one's level of technical expertise. Indeed, this point is extremely relevant for Basic Protocol 1, dealing with the establishment of PDCOs from biopsies, since someone with expertise in cellular biology techniques will be more efficient due to some automatism acquired with experience than someone starting from scratch. Besides this crucial aspect, the particularity of this protocol is to work with fresh biopsies, meaning that the operator must arrange for the transport of the biopsies and take into consideration this extra time that will define the starting point of Basic Protocol 1. Consequently, it is necessary to work with a biobank located at close range from the laboratory. Once the 2 biopsies (tumor tissue and healthy tissue) arrive under the BSC, the whole steps of Basic Protocol 1 can be completed between 3.5 hr and 5 hr depending on the operator's level of expertise. Then, the timeline of the maintenance of PDCO cultures, as referred in Basic Protocol 2, is detailed in Figure 2A, with the obtention of mature PDCOs after 4 to 6 weeks, which is dependent on each patient and piece of biopsy received. Of note, healthy tissue with the addition of Wnt3a tends to grow a bit faster than tumor PDCOs generated from the same patient.

Each PDCO passage should take <1 hr, with an average of 8 domes to passage. Of course, this time consideration can vary depending on the number of domes to be passaged and then to be seeded.

Considering that when reaching Basic Protocol 3 to perform cryopreservation and/or thawing of PDCOs, the operator would have acquired expertise in PDCO culture through Basic Protocol 1 thus the cryopreservation step should take between 1 hr to 1.5 hr, depending on the number of domes and consequently of samples that will be cryopreserved. The thawing of 4 to 6 cryopreserved PDCO vials should take between 45 min to 1 hr.

Due to the length and complexity of Basic Protocol 4, detailed time considerations are directly given in the different protocol sections. However, for an overview, the generation of lentiviral particles leading to the freezing can be performed in 5 days (usually from Monday to Friday). Then, allow half a day for lentiviral titration, which is usually in the morning prior to performing PDCO transduction in the afternoon. The generation of single cell suspension from dome-organoid culture and transduction take 1.5 hr. Finally, allow between 45 min and 1 hr to generate dome-organoid culture starting from transduced cells.

For Basic Protocol 5, depending on the number of samples, allow between 1 hr and 1.5 hr to perform the protein extraction steps. Then, consider 1.5 hr for both sample

preparation and SDS-PAGE (add 1 hr to this timeline if casting homemade gels). The transfer step will last between 5 and 10 min, plus 10 min of preparation. Finally allow 45 min for the blocking step, overnight incubation for primary antibodies, 1 hr for secondary antibodies (including washes), and an average of 15 to 30 min for the acquisition of the chemiluminescence data (can vary depending on the specificity of the antibody and the level of expression of the protein of interest).

For Basic Protocol 6, the seeding of PDCOs on Lab-Tek takes the same time as the standard passage described in Basic Protocol 2 (<1 hr). Allow one afternoon to fix, permeabilize, and block overnight the PDCOs, allowing 1 hr for the fixation and the permeabilization, respectively. Then allow the next morning to perform the rest of the experiment. Finally, no time consideration can be provided regarding the imaging acquisition step since it depends on the number and the quality of the samples, on the microscope used, as well as on the resolution and the outcomes sought. Finally, for Basic Protocol 7, the extraction of mRNA and the reverse transcription can be done in a half-day and real-time PCR take ∼3 hr (depending on the number of conditions).

A summary of time considerations for each basic protocol is presented in Supplementary Figure 3 (see Supporting Information).

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Author Contributions

Emilie Rigaux: Data curation; formal analysis; investigation; validation; visualization; writing original draft; writing review and editing. **Jia-Wei Chen:** Data curation; methodology; validation; visualization; writing review and editing. **Fabienne George:** Resources; writing review and editing. **Julien Lemaire:** Resources. **Claude Bertrand:** Resources. **Laurence Faugeras:** Resources. **Antoine Fattaccioli:** Investigation. **Quentin Gilliaux:** Resources. **Lionel D'Hondt:** Resources; supervision. **Carine Michiels:** Funding acquisition; supervision; writing review and editing. **Henri-François Renard:** Conceptualization; funding acquisition; project administration; supervision; validation; visualization; writing review and editing. **Natacha Zanin:** Conceptualization; data curation; formal analysis; funding acquisition; investigation; methodology; project administration; supervision; validation; visualization; writing original draft; writing review and editing.

Conflict of Interest

The authors declare no competing interests.

Data Availability Statement

The authors declare that the main data are available within the manuscript. Extra data are available from the corresponding authors on reasonable request.

Supporting Information

cpz1943-sup-0001-FigureS1.pdf

Supplementary Figure 1: Lentiviral transduction of PDCOs. Representative images of PDCO growth and expression of GFP from the lentiviral vector in additional conditions, complementary to Figure 4B. (A) After 7 days post-transduction, MOIs of 100 and 500 (20× objective). Of note, the efficiency of transduction appears lower at these MOIs. (B) After 7-, 14- and 20-days post-transduction, MOI of 6000. Scale bars, 100 μm for 20×, 200 μm for 10×.

cpz1943-sup-0002-FigureS2.pdf

Supplementary Figure 2: Overview of lentiviral transduction of PDCOs observed at lower magnification (4× objective). Complementary to Figure 4B. Representative images of PDCO growth and expression of GFP from the lentiviral vector after 14- and 20-days post-transduction with MOIs of 1000, 4000 and 6000. Of note, the lower magnification allows us to observe a homogeneous expression of GFP at MOIs of 4000 and 6000. Scale bar, 500 μm.

cpz1943-sup-0003-FigureS3.pdf

Supplementary Figure 3: Summary of time considerations for each Basic Protocol.

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