Tissue collection and storage

Pig intestinal organoids were generated from intestinal tissue of 5 month-old Large White pigs collected at slaughter. Two-cm sections were dissected from the different portions: duodenum was collected at 20 cm from the pilori, ileum at 20 cm from caecum, jejunum at the middle, and colon (ascendant) at 20 cm from caecum.

Colon sections were washed extensively under tap water to remove faeces. All sections were opened longitudinally and washed at least twice in ice-cold PBS and incubated on ice for 30 min in decontamination medium: PBS containing 5% of Vétédine solution (Med Vet), 0.2% fungizone (500 pg/mL amphotericin B final concentration), 50 U/mL penicillin and 50 µg/mL streptomycin and 50 µg/mL gentamycin (all from Gibco). Intestinal tissue sections were then transferred to PBS and kept on ice for up to 6 h.

Samples were moved to the cell culture laboratory where, under sterile condition and working on ice, they were incubated for 30 min in decontamination medium and then washed twice in ice-cold PBS. Villi were removed by gentle scrapping with a scalpel. Small sections of around 1 cm^2 were divided into small cubes of around 1mm^3 and transferred into cryovials containing 1 mL of fetal bovine serum (FBS, Gibco) with 10% DMSO (Sigma Aldrich). Cryovials were frozen slowly (in a freezing container with isopropanol placed at -80 °C for 24-48 h) and then transferred to liquid nitrogen for long-term storage.

Generation of organoids from frozen tissues and cryopreservation of organoids

For the isolation of intestinal crypts, frozen biopsies were thawed, put on ice and washed twice with ice-cold PBS containing 5% of FBS. Biopsies were then incubated for 30 min on ice with decontamination medium and then incubated for 10 min at room temperature on a rocking platform (about 40 rpm) in 10 mL PBS – 30 mM EDTA. Supernatant was removed and 10 mL PBS – 30 mM EDTA were added for a 10 min-incubation at 37 °C. Supernatant was discarded and biopsies were washed twice with ice-cold DMEM with antibiotics (50 U/mL penicillin and 50 µg/mL streptomycin, Gibco). Tissue was then disrupted by vigorous vortexing and pipetting up and down, placed on a 100 µm cell strainer filter and crushed using a syringe piston. After washing with DMEM (Gibco) with antibiotics, isolated cells were centrifuged (5 min, 4 °C, 300 *g*) and finally suspended in 100-200 µL of medium. Cell suspension was diluted in Matrigel™ (Corning® Matrigel® Growth Factor Reduced Basement Membrane Matrix, Phenol Red-free, REF 356231) and 50 µL domes were plated on a 24-well culture plate with 600 µL per wells of organoid culture medium A: human IntestiCult™ (Organoid Growth Medium, STEMCELL Technologies, Vancouver, Canada) with antibiotics (50 U/mL penicillin, 50 µg/mL streptomycin and 50 µg/mL gentamycin) and 10 µM RHO/ROCK pathway inhibitor Y-27632 (STEMCELL, Technologies, Vancouver, Canada). Medium was replaced by medium without Y-27632 every 2-3 days and organoids were cultured for 7-10 days until reaching 100-200 µm, as observed under an inverted IX83 microscope.

Passage for organoids amplification and storage

Medium was discarded and organoids were collected in 1.5mL Eppendorf tubes after incubation with ice-cold PBS for 5 min and pipetting up and down to dissolve domes of Matrigel. After centrifugation (5 min, 4°C, 300 *g*), supernatant was carefully discarded and the pellet resuspended with 650µL of Tryple express was incubated 10 min at 37°C.

Mechanical dissociation was performed by pipetting up and down at the beginning and at the end of this incubation. 750µL of DMEM /F12 (Gibco) containing 5% FBS were added and after centrifugation (5 min, 4°C, 300 *g*), supernatant was carefully discarded. Cells were washed with 1.5mL of DMEM /F12, 5% FBS and counted. Cell suspension was adjusted to a final concentration of 10,000 cells / 20 µL and further mixed with Matrigel on ice (6-8 mg/mL final concentration) and 50 µL domes were plated on a 24-well culture plate with 600 µL per well of organoid culture medium A (human IntestiCult™ Organoid Growth Medium, STEMCELL Technologies, Vancouver, Canada) with antibiotics (50 U/mL penicillin and 50 µg/mL streptomycin) and 10 µM RHO/ROCK pathway inhibitor Y-27632 (STEMCELL, Technologies, Vancouver, Canada). Medium was replaced by medium without Y-27632 every 2-3 days. Organoids were passaged again after 7 days of culture (37°C, 5% CO_2) following the same protocol. After those 2 passages, organoids were collected as for a passage, counted, dispatched in cryovials containing 100,000 cells in 200µL of Cryostor CS10 (STEMCELL), frozen slowly (in a freezing container with isopropanol placed at -80 °C for 24-48 h) and then transferred to liquid nitrogen for long-term storage.

Culture of organoids

After rapid thawing, cells were washed in 1.2mL of PBS containing 5% of FBS and transferred into a 1.5mL Eppendorf tube. After centrifugation (5 min, 4°C, 300 *g*), supernatant was carefully discarded and cells were mixed with Matrigel on ice (6-8 mg/mL final concentration). 3 domes of 50 µL were plated on a 24-well culture plate with 600 µL per well of basal culture medium (BCM) (van der Hee *et al.*, 2018) containing 10 µM RHO/ROCK pathway inhibitor Y-27632 (STEMCELL, Technologies, Vancouver, Canada) and incubated at 37°C, 5% CO_2. Medium was replaced by BCM without Y-27632 every 2-3 days. Organoids were passaged after 7days of culture, cell number was adjusted at each passage (10,000 cells seeded in 50µL droplets with 6-8 mg/mL of Matrigel). Conditioned media used in BCM were from the same batches and their optimal concentrations were experimentally tested. Organoids were used for experiments only after at least one passage after thawing and maximum four.

RNA extraction from tissues

Tissues stored in FBS (Gibco) with 10% DMSO were thawed and washed 3 times in ice-cold PBS. RNA was extracted using Power Bead, QIAGEN (REF 13 113-50) and NucleoSpin RNA Kit Macherey Nagel (REF 740955.250).

- Add in centrifuge tube (Power bead) 400 µL Buffer RA1 and 3.5 µL ß-mercaptoethanol.
- Disrupt up to 40 mg of tissue with TissueLyser II and Power Bead
- Perform two cycle of TissueLyser (4°C), 2 min at 25 Hz.
- Centrifuge for 1min full speed and check if the tissues are homogenized.
- Reduce viscosity and clear the lysate by filtration through NucleoSpin® Filter (violet ring): Place NucleoSpin®Filter in a Collection Tube (2 mL), transfer 350 µl of mixture, and centrifuge for 1 min at 11,000 x g.
- Transfer flowthrough into a new 1.5 mL microcentrifuge tube (not provided), add 350 µL ethanol (70 %), and mix by vortexing (2 x 5 s).
- For each preparation take one NucleoSpin® RNA Column (light blue ring) placed in a Collection Tube. Pipette lysate up and down 2–3 times and load the lysate to the column. Centrifuge for 30 s at 11,000 x g. Place the column in a new Collection Tube (2 mL).

- Add 350 µL MDB (Membrane Desalting Buffer) and centrifuge at 11,000 x g for 1 min to dry the membrane.
- Prepare DNase reaction mixture in a sterile 1.5 mL microcentrifuge tube (not provided): For each isolation, add 10 µL reconstituted rDNase (also see section 3) to 90 µL Reaction Buffer for rDNase. Mix by flicking the tube. Apply 95 µL DNase reaction mixture directly onto the center of the silica membrane of the column. Incubate at room temperature for 15 min.
- Add 200 µL Buffer RAW2 to the NucleoSpin® RNA Column. Centrifuge for 30 s at 11,000 xg. Place the column into a new Collection Tube (2 mL).
- Add 600 µL Buffer RA3 to the NucleoSpin® RNA Column. Centrifuge for 30 s at 11,000 xg. Discard flowthrough and place the column back into the Collection Tube.
- Add 250 µL Buffer RA3 to the NucleoSpin® RNA Column. Centrifuge for 2 min at 11,000 xg to dry the membrane completely. Place the column into a nuclease-free Collection Tube.
- Elute the RNA in 40 µL RNase-free H2O, and centrifuge at 11,000 xg for 1 min. Repeat this step with elution to obtain better concentration.
- The entire sample may be used immediately or stored at –80°C until use.

RNA extraction from organoids

Medium was discarded and organoids from one 50µL dome were collected in 1.5mL Eppendorf tubes after incubation with ice-cold PBS for 5 min and pipetting up and down to dissolve Matrigel. After centrifugation (5 min, 4°C, 300 *g*), supernatant was carefully discarded and the pellet was washed twice in ice-cold PBS, centrifugation (5 min, 4°C, 300 *g*), and removal of supernatant. Pellet was finally resuspended in 1,5 mL of TRIzol Reagent (Invitrogen, REF 15596026), split into 2 tubes, and frozen at -80°C before further RNA extraction.

RNA extraction was performed using Arcturus PicoPure RNA isolation kit (ThermoFisher, REF 12204-01) after a first step with TRIzol/chloroform:

- Thaw samples stored in TRIzol at -80°C.
- Incubate 5 min at room temperature.
- Add 150 µL of chloroform to the 750 µL of sample in Trizol.
- Shake vigorously by hand for 15 s.
- Incubate 2 to 3 min at room temperature.
- Centrifuge 15 min at 4°C, 12000 *g*
- Recover the upper aqueous phase (approximately 300 µL).
- Add 70% ethanol (V/V, about 300 µL).
- Shake vigorously by hand and incubate 5 min at room temperature.
- Pre-condition the RNA Purification Column: pipette 250 µL Conditioning Buffer (CB) onto the purification column filter membrane ; incubate the RNA Purification Column with CB for 5 minutes at room temperature ; centrifuge the purification column in the provided collection tube at 16,000 g for one minute.
- Pipette 300µL of sample into the pre-conditioned purification column.
- To bind RNA, centrifuge for 2 minutes at 1000 g.
- Pipette the remaining 300µL of sample into the pre-conditioned purification column.
- To bind RNA, centrifuge for 2 minutes at 1000 g.
- Centrifuge for 30 s, 16,000 g to remove ethanol.

- Pipette 100 µL Wash Buffer 1 (W1) into the purification column and centrifuge for one minute at 8,000 g.
- Pipette 100 µL Wash Buffer 2 (W2) into the purification column and centrifuge for one minute at 8,000 g.
- Pipette another 100 µL Wash Buffer 2 (W2) into the purification column and centrifuge for two minutes at 16,000 g. Check the purification column for any residual wash buffer. If wash buffer remains re-centrifuge at 16,000 g for one minute.
- Transfer the purification column to a new 0.5 mL microcentrifuge tube provided in the kit.
- Pipette 15µL of Elution Buffer (EB) directly onto the membrane of the purification column (Gently touch the tip of the pipette to the surface of the membrane while dispensing the elution buffer to ensure maximum absorption of EB into the membrane).
- Incubate the column for one minute at room temperature.
m. Centrifuge the column for one minute at 1,000 g to distribute EB in the column, and then spin for one minute at 16,000 g to elute RNA.
- The entire sample may be used immediately or stored at –80°C until use.