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SOP-ADSI-1.0	Author: **R. Gstir; P. Filipek; D. Lesiak** Approved: **Lukas Huber**	Issued: **22/03/2022** Revised:

GENERATION AND MAINTENANCE OF INTESTINAL ORGANOIDS (WT/TUMOR ORGANOIDS)

Purpose

The SOP-ADSI-1.0 was issued to describe how to generate organoid cultures from colon cancer and the respective healthy tissue samples.

Scope

SOP-ADSI-1.0 is intended to cover the complete procedure how to generate organoid cultures from colon cancer and the respective healthy tissue samples. In addition, this SOP contains the protocol for cell viability assessment of organoid cultures and procedures for their maintenance.

Introduction

Therapeutic strategies that are based on the individual genetic profile of a patient represent a new frontier of applied cancer research. These strategies are expected to reduce the socio-economic impact of current cancer therapies that are cost-intensive and often ineffective, thus releasing pressure on regional health systems. Especially in cancer research, standard cell culture conditions fail to proper mimic the parental tumor architecture and microenvironment. In this context, tumor-organoids are of special relevance. Tumor-organoids have the special property to mirror the key-features of the original patient's tumor with its microenvironment. Thus, tumor-organoids are an ideal tool to identify patient-specific therapies by performing drug-screenings on primary patient material.

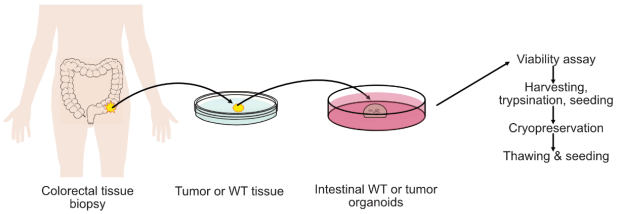

Figure 1. Schematic procedure – generation of WT or tumor organoids and their maintenance.

1. Reagents, solutions, and cell culture media

1.1 Reagents

- Real Time – GloTMMT Cell Viability Assay (#G9711 Promega)
- Matrigel (#356231 Corning)
- 0.25 % Trypsin-EDTA (#T4049-100ML Sigma-Aldrich)
- Liberase DH (#5401089001 Roche)
- Freezing Medium (#12648010 GIBCO- Thermo Fisher)
- DPBS, 1 x no calcium, no magnesium (Gibco#14190-094)
- Pen/Strep (100X) (#15070063 GIBCO)
- Primocin – 500mg (Cat.Code: ant-pm-1 InvivoGen)
- Advanced DMEM/F-12(#12634028 Gibco-Thermo Fisher)
- Glutamax 100 x (#35050038 GIBCO)
- HEPES (1 M) (#15630080 GIBCO)
- Fetal Bovine Serum (#S1810-500 Biowest)
- BSA – Albumin FraktionV (#8076.4 Roth)
- EDTA (#431788-25G Sigma Aldrich)
- WCM – Wnt3a-conditioned media (2016 Broutier)*
- RCM – R-Spondin-conditioned media (2016 Broutier)**
- NCM – Noggin-conditioned media (2016 Broutier)***
- R-Spondin (#120-38 PEPROTECH) optional instead of RCM (100 ng/mL)
- Noggin (#250-38 PEPROTECH) optional instead of NCM (1000 ng/mL)
- Wnt3a – if required 100 ng/mL (PEPROTECH)
- Nicotinamide (#N0636-100G Sigma-Aldrich)
- N-Acetylcyteine (#A9165-25G Sigma-Aldrich)
- B-27 (#17504044 Thermo Fisher)
- m-EGF (#315-09 PEPROTECH)
- SB 202190 (S7067- Sigma-Aldrich 5MG)
- A83-01 (#2939 R&D Systems Europe)
- Gastrin (#3006/1 R&D Systems Europe)
- Prostaglandin E2 (#P0409 Sigma-Aldrich)
- Y-27632 (#120129 Abcam)

*Wnt3a conditioned medium was obtained from L-Wnt3a cells. MTA for the use of producer cell line was obtained from the Hubrecht Institute, Uppsalalaan 8, 3584 CTUtrecht, The Netherlands

**R-spondin1-conditioned medium was produced from 293T-HA-Rspol-Fc producer cell line; MTA for the use of producer cell line was obtained from the Hubrecht Institute, Uppsalalaan 8, 3584 CTUtrecht, The Netherlands

***Noggin-conditioned medium from HEK293-mNoggin-Fc cell line. MTA for the use of producer cell line was obtained from the Hubrecht Institute, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands

1.2 Compositions

- **DPBS + Pen/Strep**

500 mL DPBS (1x)

5 mL P/S 100x

Filter sterilize (0.22 µm)

Store at -20 °C

- **DPBS + Pen/Strep + Primocin**

500 mL DPBS (1x)

5 mL P/S 100x

100 µL of Primocin in 50 mL

Store at -4 °C

- **DMEM + Pen/Strep + Primocin**

500 mL DMEM

5 mL P/S (100X)

100 µL of Primocin in 50 mL

Store at 4 °C

- **DMEM + Pen/Strep + FBS**

500 mL DMEM

5 mL P/S (100X)

60 mL FBS

100 µL of Primocin in 50 mL

Store at 4 °C

- **GF-**

500 mL Advanced DMEM/F12

5 mL P/S (100X)

5 mL of HEPES (1M = 100X)

5 mL of Glutamax (100X)

Store at 4 °C

- **Chelation Solution**

10 mL DPBS (1X)

200 µL 0.5 M EDTA

- **RBC Lysis Buffer 1X**

300 µL of RBC Lysis Buffer

2.7 mL of sterile H2O

- **RCM**

Store at -20 °C

Thaw on ice

(4 mL for the generation of 40 mL media)

- **NCM**

Store at -20 °C

Thaw on ice

(2 mL for the generation of 40 mL media)

- **WCM (50%)**

Freshly prepared (every 2 weeks)

Store at 4°C

- **Nicotinamide (1 M = 100X)**

1,464 g

Fill up to 12 mL with 1 x PBS

Dissolve by shaking and leave at RT

Filter sterilize 0,22 µm

Make aliquots 500 µl and store at -20°C

(5000 µl for 500 mL culture medium = 10 mM)

- **N-Acetyl cysteine solution**

3 mL sterile water

245 mg N-Acetyl cysteine

Dissolve by shaking and leave at RT

Filter sterilize 0,22 µm

Make 500 µl aliquots in Eppendorf tubes and store at -20 °C

(1250 µl for 500 mL culture medium)

- **B27 (50X)**

Thaw on ice

Prepare 2 mL aliquots

Store at -20°C

(1000 µl for 500 mL culture medium)

- **m-EGF (500 µg/mL = 10000x)**

Dissolve 500 µg m-EGF in 1000 µl PBS/0.1 % BSA

Make 50 µl aliquots, store at -20 °C

(4 µl in 40 mL culture medium or 50 µl in 500 mL culture medium = 50 ng/mL)

- **PBS/0.1 % BSA**

10 mL PBS + 10 mg BSA

- **Y-27632 (10 mM = 1000x)**

10 mg

Dissolve in 3122 µl sterile water

Make 400 µl aliquots (10 mM)

(40µl in 40 mL culture medium = 10 µM)

Store at -20°C

- **A83-01 (major stock = 50 mM and 1,5 mM = 3000x)**

Stock:

Dissolve 10 mg A83-01 in 474 µl DMSO

Make 18 µl aliquots (50 mM)

Store at -20°C

Pre-dilution stock:

Quickly thaw an 18 µl major stock aliquot

Dilute with 582 µl DMSO

Make 13,3 µl aliquots (1,5 mM = 3000 x)

(13,3 µl for 40mL culture medium)

Store at -20°C

- **SB202190 (30 mM = 3000x)**

Dissolve 5 mg SB202190 in 503 µl DMSO

Make 13,3 µl aliquots

(13,3 µl for 40 mL culture medium)

Store at -20°C

- **Gastrin (100 µM = 10000x)**

Dissolve 1 mg gastrin

in 4,77 mL PBS (without Calcium and Magnesium)

Make 50 µl aliquots or smaller

(4 µl for 40 mL culture medium = 10 nM)

Store at -20°C

- **PGE2 (100 µM = 10000x)**

Dissolve 1 mg PGE2 in 4,77 mL PBS (without Calcium and Magnesium)

Make 50 µl aliquots or less

(4 µl for 40 mL culture medium = 10 nM)

Store at -20°C

- **Primocin (500x)**

(80 µl for 40 mL culture medium = 2x)

Store at -20°C

1.3 Preparation of media for intestinal WT organoids

1.3.1 Preparation of basal medium

Prepare a sterile 500 mL Erlenmeyer flask. Thaw RCM, NCM, B27, organoids Nicotinamide and N-Acetylcysteine on ice. Add the required amounts as indicated in *Table 2*. N-ACETYLCYSTEINE IS VERY ACIDIC – ADD AT THE END! Make 20 mL aliquots in 50 mL tubes. Label properly (Intestinal-SM/EM without growth factors, date, operator) and freeze at -20 °C. Aliquots are stable for up to 12 months at -20 °C.

1.3.2 Preparation of complete ready to use media

Thaw respective basal medium aliquot on ice, add growth factors (thaw at RT) as indicated in *Table 1*. Mix thoroughly and keep at 4 °C. Avoid repeated cycles of warming up. WCM must not be kept longer than 2 weeks at 4 °C.

Table 1. Media composition for intestinal WT organoids (Colon, Rectum)

Compound	Seeding Medium (SM)	Expansion Medium (EM)	Basal medium	Complete ready to use medium
GF- medium	217 mL	217 mL	X	
RCM	100 mL	100 mL	X	
NCM	50 mL	50 mL	X	
Nicotinamide	10 mL	10 mL	X	
N-acetyl	2.5 mL	2.5 mL	X	
B27	20 mL	20 mL	X	
WCM	20 mL	20 mL		X
m-EGF	4 µL	4 µL		X
Y-27632	40 µL			X
A83-01	13.3 µL	13.3 µL		X
SB202190	13 µL	13 µL		X
Gastrin	4 µL	4 µL		X
PGE2	4 µL	4 µL		X
Primocin	80 µL	80 µL		X

1.4 Preparation of media for intestinal tumor organoids

1.4.1 Preparation of basal medium

Preparation of basal medium Prepare a sterile 500mL Erlenmeyer flask. Thaw RCM, NCM, B27, Nicotinamide and N-Acetylcysteine on ice. Add the required amounts as indicated in the table. N-ACETYLCYSTEINE IS VERY ACIDIC – ADD AT THE END! Make 20 mL aliquots in 50 mL tubes. Label properly (BM Basal Medium without growth factors, date, operator) and freeze at -20°C. Aliquots are stable for up to 12 months at -20°C.

1.4.2 Preparation of complete ready to use media

Thaw respective basal medium aliquot on ice, add growth factors (thaw at RT) as indicated in *Table 2*. Mix thoroughly and keep at 4° C. Avoid repeated cycles of warming up. WCM must not be kept longer than 2 weeks at 4° C.

Compound	M#2	M#3	M#4	M#5	M#6	Basal medium	Complete ready to use medium
GF- medium	217 mL	217 mL	217 mL	217 mL	727 mL	X	
RCM	100 mL	100 mL	100 mL	100 mL	100 mL	X	
NCM	50 mL	50 mL	50 mL	50 mL	50 mL	X	
Nicotinamide	10 mL	10 mL	10 mL	10 mL	10 mL	X	
N-acetyl	2.5 mL	2.5 mL	2.5 mL	2.5 mL	2.5 mL	X	
B27	20 mL	20 mL	20 mL	20 mL	20 mL	X	
WCM	-	-	-	-	20 mL	X	
m-EGF		4 µL	-	4 µL	4 µL		X
Y-27632	40 µL	40 µL	40 µL	40 µL	40 µL		X
A83-01	-	-	13.3 µL	13.3 µL	13.3 µL		X
SB202190		13 µL		13 µL	13 µL		X
Gastrin	4 µL	4 µL	4 µL	4 µL	4 µL		X
PGE2					4 µL		X
Primocin	80 µL	80 µL	80 µL	80 µL	80 µL		X
GF-medium	20 mL	20 mL	20 mL	20 mL			X

Table 2. Media composition for intestinal tumor organoids (Colon, Rectum)

2. Equipment

- Incubator Binder APT. lineTM (150CE2)
- Laminar Air-Flow Labculture Plus ESCO, Class II BSC
- Micro Plate Reader (Mithras LB940)
- Centrifuge (4°C) Hettich Zentrifugen ROTIXA A50 RS
- Brightfield microscope Motic AE31 and camera ProgRes CF cool JENOPTIKA
- Table centrifuge VWR Mini Star
- Analog Vortex Mixer (VWR)
- Waterbath
- Mr. Frosty freezing-container (ThermoFisher Scientific #5100-0001)
- Polystyrene box
- Box with crashed ice
- Pipette Aid
- Set of pipettes 10 µL, 200 µL, 1000 µL and pipette tips
- Liquid nitrogen
- Dissection tweezers (#11150-10, #11254-20, #11274-20 Fine Science Tools)
- Scissors (#14058-09, #14059-09, #14110-15 Fine Science Tools)
- Sterile scalpel + blades
- Roller shaker

3. Plastics

- 24-well-plates (Greiner bio-one #662160)
- 96-well-plates (Corning #3603)
- 100 cm petri dish #83.3902 SARSTEDT))
- 50 mL conical tubes (#ET5050B Euroclone)
- 15 mL conical tubes (#ET5015B Euroclone)
- Cryovials (#E.3110-6122 STAR LAB)
- pluriStrainer 100µm (#43-50100-03 pluriSelect)
- pluri-Connector Ring (#41-50000-03 pluriSelect)
- Sterile syringes, 10 mL (#4606108V Braun)
- Sterile 0,5 mL, 1,5 mL, 2mL and 5 mL tubes
- Pipettes 10 mL, 25 mL, 50 mL
- Bottle Top Filter (#83.1823.101 SARSTEDT)

4. Procedure for the collection of normal and tumor tissue

Collect the dissected healthy tissue and tumor tissue directly from the operation theatre and put it on ice! The tumor is immediately transferred to the pathology. Contact the pathologist to do the routine inspection of the tissue. Collect tissue from the tumor and healthy intestinal tissue independently in DPBS + Pens/Strep on ice (take care to prevent cross contamination of the tumor and the healthy tissue). Transfer tissue and patient agreement as fast as possible to the lab (not longer than 1-2 hours). The isolation of the CAFs as well as tumor cells and intestinal crypts should be performed in parallel by independent laboratory staff to minimize the time of ischemia. Perform all procedures on ice unless indicated otherwise.

5. Isolation of intestinal cells from healthy tissue to generate wildtype organoid cultures

5.1 Preparation of material and reagents

- Matrigel: thaw 1 mL aliquot on ice (keep on ice until usage)
- Pre-warm 24 well plates in incubator at 37 °C
- Prepare Chelating Solution
- Keep GF- (50 mL aliquots) on ice
- Pre-warm intestinal seeding medium at 37 °C in waterbath before use
- Sterile tweezers and scissors for dissection
- 10 cm petri dishes
- 100 µm cell strainer
- Pre-cool centrifuge to 4 °C
- Pre-warm rocker or roller shaker to 37 °C
- Box with crashed ice
- DPBS + Pen/Strep

5.2 Washing of intestinal patient tissue

- Transfer tumor tissue with sterile forceps in a sterile 50 mL conical tube with 10 mL ice-cold DPBS + Pen/Strep (4°C). Shake 10-15 times, remove the DPBS + Pen/Strep and replace with 10 mL ice-cold DPBS + Pen/Strep. Repeat this process 2 more times until the supernatant no longer contains any visible debris. The supernatant turns red and fat droplets appear.

- Pour the tissue along with the supernatant into a sterile 10 cm dish. Carefully dissect the epithelium from the connective tissue and muscle layers with sterile scissors and forceps. Cut the epithelial layer into small pieces with 5 mm Ø.
- Cut a 1000 µl (blue) pipette tip in order to transfer the tissue pieces into a 15 mL tube with fresh DPBS + Pen/Strep (4°C) by pipetting.
- Invert the tube several times to remove last bits of blood and fat.

5.3 Isolation of crypts

- Wait 1 minute until tissue pieces settled down.
- Remove the supernatant and add 10 mL chelation solution. Put the tube on a roller shaker for 45 to 60 minutes at 4°C.
- Put tube upright and wait 1 minute until tissue pieces settled down.
- Remove the supernatant and add 3 mL DPBS + Pen/Strep.
- Use a cut blue 1000 µl pipette tip to mechanically set free the crypts from the tissue pieces by pipetting up and down of biopsies 8 - 10 times (avoid air bubbles). Check for crypts in the supernatant under the microscope (4x magnification).
- Allow biopsies to settle down and collect the supernatant containing the crypts into a fresh 15 mL tube and keep on ice. Add another 3 mL DPBS + Pen/Strep 4°C and repeat crypt-isolation 2-3 times until majority of crypts are released and supernatant stays clear.

5.4 Washing of crypts

- Keep the supernatant containing the crypts on ice.
- Centrifuge 5 min at 800 rpm and 4°C. Remove supernatant as much as possible.

5.5 Embedding of crypts in Matrigel

- Resuspend the crypts in 240 µl ice-cold GF-.
- Add 480µl of Matrigel, mix carefully by pipette up and down (avoid air bubbles) and keep the mix on ice.

5.6 Seeding of crypts into a 24-well-plate

- Take the pre-warmed 24-well-plate from incubator and apply 30 µL droplets of Matrigel-crypt-mix per well. Incubate 24-well-plate with Matrigel-crypt-mix for 10-15 min at 37 °C, 5 % CO2 and 95 % r.H.
- Gently add 0.5 mL pre-warmed Intestinal Seeding Medium per well and incubate at 37 °C, 5 % CO2 and 95 % r.H.

Crypts will start to round up and close within the first 3 hours. Replace medium 2-3 times per week. Passage the crypts after 5-7 days (depending on their grow behavior). Usually, the crypts are dissociated and passaged for the first day after 5 days.

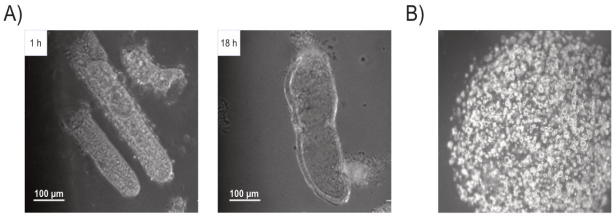

Figure 2. A) Isolated crypts (1 h and 18 h after seeding in Matrigel) and B) intestinal organoid culture consisting of stem cells embedded in Matrigel.

6. Isolation of intestinal tumor cells from tumor tissue to generate tumor organoid cultures

6.1 Preparation of material and reagents

- Box with crashed ice
- Matrigel: thaw 1 mL aliquot on ice (keep on ice until usage)
- DPBS + Pen/Strep + Primocin
- DMEM + Pen/Strep + Primocin
- DMEM + Pen/Strep + FBS
- Pre-warm 2 x 24 well plates and a 96 well plate
- Pre-cool centrifuge to 4°C
- Pre-warm rocker or roller shaker to 37°C
- Prepare Tumor culture Medium-Expansion medium (Rectum/Colon) (Medium #2, #3, #4, #5, #6). These medias are stored at 4°C and are stable for about 1-2 week.
- 10 cm petri dishes

- 100 µm cell strainer
- Sterile tweezers and scissors for dissection
- Liberase: dilute 1:100
- Primocin: dilute 1:500
- Y-27632: dilute 1:1000

6.2 Washing of intestinal patient tissue

- Transfer tumor tissue with sterile forceps in a sterile 50 mL conical tube with 10 mL ice-cold DPBS + Pen/Strep (4°C). Shake 10-15 times, remove the PBS + Pen/Strep and replace with 10 mL ice-cold DPBS + Pen/Strep. Repeat this process 2 more times until the supernatant no longer contains any visible debris.
- Collect tumor tissue then in a 10 cm petri dish. Dissect tumor tissue with sterile scissor into approximately 2-5 mm cubes. A portion of fragments is used for isolation of CAFs. The remaining ones are used for generation of cancer associated fibroblasts (SOP-ADSI-2.0).

6.3 Homogenization of the tumor

- Cut the tumor fragment into small tissue pieces (as small as possible) by using a pair of small scissors and forceps.
- Transfer the tissue pieces into a 50 mL tube with DMEM +Pen/Strep + Primocin 100 µg/mL+ Liberase 50 µg/mL (0.26 units/mL) + Y-27632 10 µM and shake the tissue for 1 hour at 37 °C with 250 rpm.
- Pass the fraction through a 100 µm cell strainer to remove bigger debris. Finally apply 5 mL DPBS + Pen/Strep to wash off all cells from the cell strainer. Collect all flow through with the single cells in a 50 mL tube.
- Centrifuge the 50 mL tube with the single cells at 300 g for 5minutes at 4°C. Discard the supernatant.
- Resuspend the pellet in 3 mL RBC Lysis Buffer. Incubate for 10 min at RT in the dark! Neutralize the lysis buffer by adding 10 mL of DMEM + P/S + Primocin with FBS.
- Centrifuge at 300 g for 5 minutes at 4°C. Discard supernatant.
- Resuspend the pellet in 5 mL DPBS + Pen/Strep + Primocin

and pass this fraction through a 100 µm cell strainer to remove remaining debris (optional). Collect the filtered solution in a 50 mL tube.

- Centrifuge at 300 g for 5 minutes at 4°C. Discard supernatant.

6.4 Cell counting

- Re-suspend the pellet with 3 mL of GF- and count cells.
- Centrifuge at 300 g for 5 minutes. Discard supernatant. Re-suspend the cells in GF- at the concentration required to generate organoids (100 000 cells / 10 µl).
- Approximately 2.5 million cells are required to generate a stable and comprehensive tumor organoid culture.

6.5 Seeding of cells: tumor organoid generation

Seed about 100 000 cells per well (24 well plate). This high seeding density of the cells promotes tumor organoid formation and growth. Populate at least one entire 24 well plate to have enough wells to establish a comprehensive tumor organoid culture.

- Prepare 2,5 x 10^6 cells in a 15 mL tube (organoids need to be seeded on an entire 24 well plate (layout see *Figure 3*) to generate a comprehensive tumor organoid culture)
- Divide the number of cells as required for tumoroid seeding (2,5 x 106 cells for tumoroid culture and 5 x 105 for Cell Viability Assay) using 15 mL tubes
- Add GF- medium up to 10-12 mL. Centrifuge the single cells at1500 rpm for 5 minutes at 4°C
- Remove the supernatant – keep the cells on ice, and re-suspend the cell pellet in 240 µl ice-cold GF- and 480 µl ice-cold Matrigel (10 µL GF- and 20 µL Matrigel per 24-well-plate) (try to avoid generating air bubbles).
- Take pre-warmed 24-well-plate from incubator and apply 30 µL droplets of Matrigel-cell-mix per well. Incubate 24-well-plate with Matrigel-cell-mix for 10-15 min at 37 °C, 5 % CO2 and 95 % r.H.
- Carefully add 500 µL pre-warmed medium (Medium #2, #3, #4, #5, #6) per well (layout see *Figure 3*).

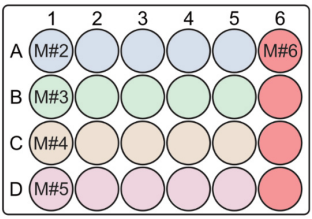

Figure 3. Pipetting scheme for the four media strategy M#2, M#3, M#4, M#5, M#6 in 24-well-plate format.

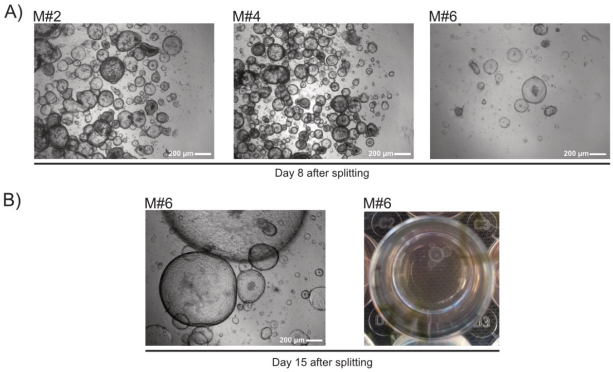

Figure 4. Growth of tumor organoids in different media. A) Tumor organoids 8 days after splitting in M#2, M#4 and M#6 media. B) Tumor organoids with M#6 medium 15 days after splitting. Left image represents visibility of organoids without microscope in a 24-well-plate

7. Viability assay of organoids

The viability assay is applied to determine most efficient media condition required to promote tumor organoid growth.

7.1 Preparation of material and reagents

- Put the Real Time Glo (RTG) Kit at RT
- Prepare 600 µl of media #2, #3, #4, #5, #6 in a 1.5 mL tube
- Shortly before adding the media (#2-#6) to the cells, pre-warm the RTG components (enzyme + substrate) in a water bath at 37 °C. Vortex and centrifuge. Add 0.6 µl of each component to the 600 µl of each medium (#2-#6). Put back in the water bath until use.

7.2 Procedure for Kit

- Prepare 5 x 105 cells in a 15 mL tube and centrifuge single cells at 1500 rpm for 5 minutes at 4 °C.
- Re-suspend cells in 50 µl ice-cold GF- and 100 µl ice-cold Matrigel (try to avoid generating air bubbles).
- Apply 10 µl droplets per 96 well – (layout see figure 7.2) (use a pre-warmed 96-well-plate (Corning #3603)).
- For the media control, add 40µl GF- in a precooled 1,5 mL tube and mix with 80 µl of Matrigel.
- Seed 10 µl droplets per well as suggested in the pipetting scheme below.
- Incubate the plate for 15 min at 37°C (5% CO2).
- Gently add 100 µl pre-warmed Medium (Medium #2, #3, #4,#5, #6) supplemented with RTG enzyme and substrate according to the pipetting scheme (figure 7.2) and incubate at 37 °C, 5 % CO2 and 95 % r.H.
- Take pictures of every well with organoids at time points: 1 h,24 h, 48 h, 72 h, 96 h, 120 h, 144 h.
- Perform luminescence measurement at time points: 1 h, 4 h,24 h, 48 h, 72 h.
- Refresh medium after time point 72 hours to medium without enzyme and substrate.

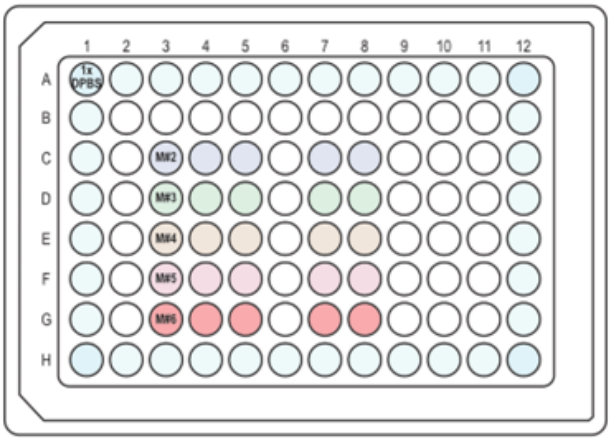

Figure 5. 96-well-plate layout for assessment of media efficacy to promote tumor organoid growth. The different media conditions (M#2, M#3, M#4, M#5, M#6) are applied as indicated by colours per well. Outer wells are filled up with 150 µL of DPBS 1X to prevent evaporation.

7.3 Luminescence measurement

- Switch on luminometer (Mithras) 1 hour before measurement. Turn on the PC and open the MikroWin 2000 program. Open Lumi_1s05s.par (to measure the luminescence for 1 sand 0.5 s), Mitras will heat up to 37°C (takes approximately 1hour). Excitation Filter Slide: A-FLUORESCENCE or B-ABSORBANCE.

  The read out is extremely sensitive to temperature changes.

  To gain most accurate results transfer the 96-well-plate in a polystyrene box from the cell incubator to the Mithras (try tobe as fast as possible to prevent the plate from cooling down).

- Export the data in EXCEL file for further calculations.
- Photo documentation of all wells with organoids is done immediately after the measurement of luminescence (see example figure 7.3).

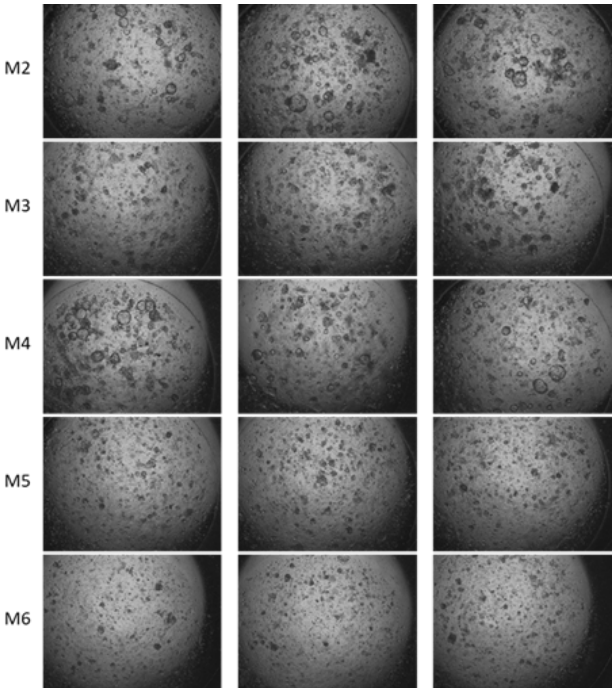

Figure 6. Organoid cultures 5 days after seeding in different media conditions M#2 to M#6. The favored growth conditions can be detected by phase contrast microscopy. This example shows good tumor growth in medium M#2 and M#4.

8. Splitting of WT and tumor organoids for cultivation

8.1 Preparation of material and reagents

- Box with crashed ice
- Thaw required amount of Matrigel on ice, always keep on ice
- 0.25% Trypsin / EDTA, 4°C
- Put GF- on ice
- Pre-warmed 24-well-plates at 37 °C in incubator
- Prepare required culture media
- 15 mL tube
- Pipette tips and pipettes (10µl, 200µl, 1000µl)
- Pipette aid and pipettes (10 mL)
- Pre-cool the centrifuge to 4°C

8.2 Harvesting of organoids

- Remove medium from wells (check if the Matrigel is stable otherwise you might loose the cultures!).
- Add 500 µl ice-cold GF- to every well and detach Matrigel drop with 1000 µl tip by scratching.
- Transfer the detached organoids with GF- to a 15 mL tube (kept on ice).
- Wash wells with 500 µL cold GF- to collect remaining cells and transfer them into the 15 mL tube (kept on ice).
- Add GF- up to 10-14 mL and centrifuge for 3 min, 800 rpm at 4 °C.
- Remove supernatant (Matrigel pellet with the organoids is visible by eye).
- If the Matrigel pellet is very big, remove the supernatant and resuspend the pellet with 3 mL ice-cold GF-. Pipette up and down. Add 7mL of ice-cold GF- and centrifuge again for 3 minutes at 1200 rpm at 4°C.

8.3 Trypsinization of organoids

- Add 200 to 500 µL 0.25% Trypsin-EDTA (depending on size of the pellet) to the 15 mL tube with the cell pellet.
- Pipette up and down and incubate 7 min at 37 °C in water bath.

- Pipette up and down with a 100 µl pipette tip (if cells will not get loose either extend the incubation time or attach a 200 µl pipette tip to the 1000 µl pipette tip)
- Check repeatedly under the microscope (in the tube). Repeat incubation cycles until the majority of cells are single cells.
- If single-cells still do not appear after 3 times of checking under the microscope, add additional 200 µl 0.25% Trypsin-EDTA and extend incubation cycles at 37°C.

8.4 Seeding of organoids

- When single cell suspension is obtained, add 10 mL GF-.
- Centrifuge for 5 min, 1500 rpm at 4°C.
- Remove supernatant and re-suspend in 200 µL of GF-.
- Count cells in duplicates and calculate number of required cells.
- Transfer the required amount to a 15 mL tube and add 10 mL GF-.
- Centrifuge for 3 min, 1500 rpm at 4 °C.
- Remove the supernatant and resuspend in the required amount GF- (example: 10 µl GF-, 20 µl Matrigel per well in 24well plate) and add Matrigel according to the dilution.
- Seed 30 µl droplets per well. Pipette fast to avoid cooling of the pre-warmed plate.
- Incubate for 10-15 min at 37 °C, 5 % CO2 and 95 % r.H.
- Carefully add 500 µl pre-warmed medium per well. Incubate cells at 37 °C, 5 % CO2 and 95 % r.H.
- Replace medium every 2 to 3 days.

9. Cryopreservation of WT and tumor organoids

Organoids as well as tumor organoids can be stored at -80°C (temporary – days/ a few weeks) and in liquid nitrogen (long term). The best way to freeze them is in the Recovery™ Cell Culture Freezing Medium. It is a ready-to-use complete cryopreservation medium for mammalian cell cultures. In order to achieve the ideal cooling rate of -1°C/ min use a freezing container (e.g. Mr. Frosty). Freeze 6 wells of a 24 well plate in 500 µl freezing medium. Perform all procedures on ice unless otherwise indicated.

9.1 Preparation of material and reagents

- Box with crashed ice
- Label cryotubes (passage number, date, organoid ID (e.g.TPIO-19), amount of wells frozen, medium, operator)
- Pre-cool cryotubes on ice
- Put Recovery™ Cell Culture Freezing Medium of GIBCO on ice
- Mr. Frosty freezing container should be filled with isopropanol according to the manufacturer's protocol and be kept at RT

9.2 Harvesting of organoids

- Remove medium from wells (check if the Matrigel is stable not to lose the cultures).
- Add 500 µl ice-cold GF- to every well and detach Matrigel drop with 1000 µl tip.
- Transfer detached organoids to 15 mL tube kept on ice.
- Wash wells with 500 µL ice-cold GF- to collect remaining cells into the 15 mL tube.
- Add ice-cold GF- to 15 mL tube up to 10-14 mL.
- Centrifuge 15 mL tube for 3 min at 800 rpm at 4 °C.
- Remove supernatant (Matrigel pellet with the organoids is visible by eye.
- If the Matrigel pellet is very big, remove the supernatant and re-suspend the pellet with 3 mL ice-cold GF-. Pipette up and down. Add 7 mL of ice-cold GF- and centrifuge again for 3 min, 200 rpm at 4°C

9.3 Trypsinization of organoids

- Add 200 to 500 µL 0.25% Trypsin-EDTA (depending on size of the pellet) and pipette up and down.
- Incubate 7 min at 37°C (water bath).
- Pipette up and down with a 100 µL pipette tip (if cells will not get loose either extend the incubation time or attach a 200 µl pipette tip to the 1000 µl pipette tip). Check repeatedly under the microscope (in the tube). Repeat incubation cycles until majority of cells are single cells. If single-cells still do not appear after 3 times of checking under the microscope, add additional 200 µl 0.25 % Trypsin-EDTA and extend incubation cycles at 37 °C

9.4 Freezing of organoids

- Remove supernatant as far as possible and re-suspend pellet carefully in Recovery™ Cell Culture Freezing Medium.
- Transfer 500 µL per vial using a 1000 µL pipette tip (work fast as possible).
- Transfer the cryovials to the Mr. Frosty and store Mr. Frosty at -80 °C for at least 2 days.
- Transfer vials to liquid nitrogen (-196°C) for long term storage. At -80 °C organoids can be stored for up to 4 weeks. Samples should be transferred from -80 °C to liquid nitrogen at least after 2-4 days.

10. Thawing and seeding cryopreserved WT and tumor organoids

Start organoid culture from frozen vials at ratio 1:1 or 1:2 from the number of wells that were frozen. Omit adding apoptosis inhibitor for at least 4 days after thawing organoids. Perform all procedures on ice unless otherwise indicated.

10.1 Preparation of material and reagents

- Thaw required amount of Matrigel on ice, always keep on ice
- Pre-warm 24-well-plates plates in incubator at 37 °C
- Always use GF- aliquots, keep on ice
- Pre-warm water bath to 37 °C
- Put 15 mL tubes on ice
- Pipette tips and Pipettes (10 µL, 200 µL, 1000 µL)
- Pipette aid and pipettes (10 mL)
- Prepare culture medium. Add Y-27632 (concentration according the protocol). Pre-warm at 37 °C just before use

10.2 Thawing of cells

- Pick up cryovial from liquid nitrogen (check Biobank – sign vial out from the database). Keep vial in dry ice or liquid nitrogen until shortly before thawing.
- Thaw at 37 °C in water bath until only a small block of ice is left (Keep lid above water surface to avoid hazard emanating from remaining liquid nitrogen between lid and vial).
- Add 10 mL ice-cold GF- to a 15 mL tube.

- Pipette cell suspension carefully from cryovial into prepared 15 mL tube with 10 mL GF-.
- Rinse the cryovial with 500 µl GF- to collect all cells and add them to the 15 mL tube.
- Centrifuge for 5 min, 1500 rpm at 4 °C.
- Remove supernatant.

10.3 Seeding of cells

- Resuspend the pellet in GF- (10 µl per well) and Matrigel (20µl per well).
- Seed 30 µL droplets of Matrigel with cells per well into the pre-warmed 24-well-plate.
- Incubate 15 min at 37 °C, 5 % CO2 and 95 % r.H.
- Carefully add 500 µl prewarmed desired culture medium (M#2, M#3, M#4, M#5, M#6) with 10 µM Y-27632 per well.
- Incubate at 37 °C, 5 % CO2 and 95 % r.H.
- Record passage number: p (from vial) + 1
- Refresh medium after 2-4 days with culture medium.

11. References

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