

# GENERATION OF INTESTINAL ORGANOIDS (WT/ TUMOR ORGANOIDS)



## Purpose

The SOP-ADSI-2.0 was issued to describe how to generate organoid cultures from colon cancer and the respective healthy tissue samples.

## Scope

SOP 2.0 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.

## 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 properly 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.

## 1. Procedures 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 PBS + 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 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.

## 2. Establishing an intestinal organoid culture from fresh normal tissue samples

### 2.1

#### Material, reagents and reagent set-up

- Matrigel: thaw aliquot 1 ml matrigel on ice (keep on ice)
- Pre-warm 24 well plates
- Prepare Chelating Solution
  - 10 ml PBS- (without Ca/Mg)
  - + 200  $\mu$ l 0.5 M EDTA
  - keep on ice
- GF- medium
  - Always use a 50 ml aliquot GF-; keep on ice
- Intestinal Seeding Medium
  - This medium is stable for about 1-2 week at 4°C.
  - Pre-warm at 37°C just before use

For reference on media, reagents and instrumentation, see SOP-ADSI-1.

### 2.2

#### Procedure

#### 2.2.1

##### Wash the tissue

- Transfer the tissue into a 50 ml tube with 20 ml cold PBS + Pen/Strep (4°C)
- Shake the tube vigorously to wash the tissue; the supernatant turns red and fat droplets appear
- Pour the tissue along with the supernatant into a sterile 10 cm petri dish

- Use sterile forceps to transfer the tissue into a new 50 ml falcon with 20 ml cold PBS + Pen/Strep (4°C). Repeat washing until the supernatant stays clear.
- After washing transfer the tissue into a fresh 10 cm petri dish with 20 ml PBS + Pen/Strep.
- Carefully dissect the epithelium from the connective tissue and muscle layers.
- 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 PBS + Pen/Strep (4°C) by pipetting.
- Invert the tube several times to remove last bits of blood and fat.

### 2.2.2 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 PBS + 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 biopsies 8 - 10 times (avoid air bubbles).
- Check for crypts in the supernatant under the microscope (4x).
- 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 PBS + Pen/Strep 4°C and repeat crypt-isolation 2-3 times until majority of crypts are released and supernatant stays clear.

### 2.2.3 Wash

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

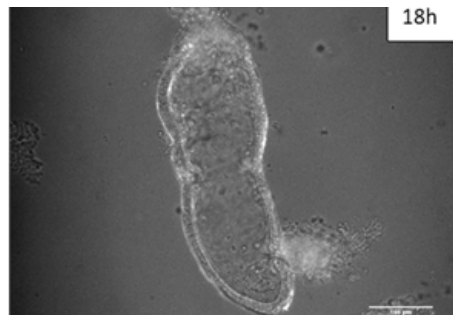
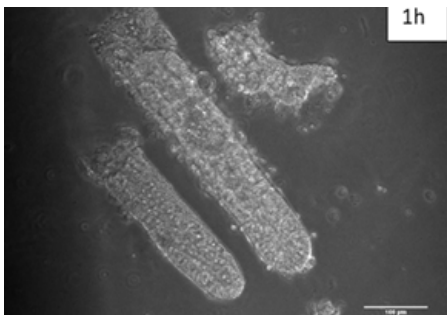
### 2.2.4 Embedding crypts in Matrigel

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

## 2.2.5

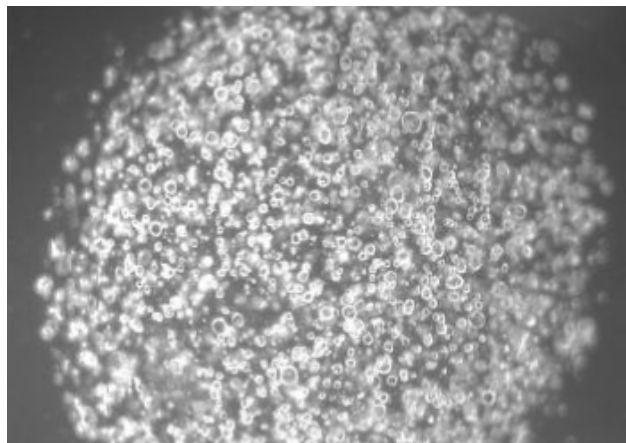
### Seed of crypts into 24 well plate

- Use pre-warmed 24 well plates
- Make 30  $\mu$ l droplets of crypts in matrigel per well
- Incubate 10-15 min at 37°C (5% CO<sub>2</sub>)
- Gently add 0.5 ml pre-warmed Intestinal Seeding Medium per well
- Incubate at 37°C (5% CO<sub>2</sub>)
- 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 behaviour). Usually the crypts are dissociated and passaged for the first day after 5 days.



**Figure 1** Isolated crypts, 1 h and 18 h after seeding in matrigel. Crypts close and round up.

**Figure 2** Intestinal organoids culture consisting of stem cell only.



# 3.

## Isolation of intestinal tumor cells from tumor tissue to generate tumor organoid cultures

### 3.1

#### Material, reagents and reagent set-up

- Pre-warm 24 well plates
- Petri dishes 10 cm
- 100 µm cell strainer
- Sterile tweezers and scissors for dissection
- Pre-cool centrifuge to 4°C
- Pre-warm rocker or roller shaker to 37°C
- Box with crashed ice
- thaw matrigel aliquot on ice
- PBS + Pen/Strep + Primocin
- DMEM + Pen/Strep + Primocin
- DMEM + P/S + FBS
- Matrigel: thaw 1 ml aliquot of matrigel on ice (keep on ice).
- Pre-warm 2 x 24 well plates and a 96 well plate.
- Real Time-Glo™ Viability Assay Kit
- 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.

For reference on media, reagents and instrumentation, see SOP-ADSI-1.

#### • **PBS + Pen/Strep + Primocin**

500 ml DPBS  
5 ml Pen/Strep  
make aliquots (50 ml tube)  
add 100 µl of Primocin in 50ml

#### • **DMEM + Pen/Strep + Primocin**

500 ml DMEM  
5 ml Pen/Strep  
make aliquots (50 ml tube)  
add 100 µl of Primocin in 50 ml

#### • **DMEM + P/S + FBS**

500 ml DMEM  
5 ml Pen/Strep  
60 ml FBS  
make aliquots (50 ml tube)  
add 100 µl of Primocin in 50 ml

#### • **RBC Lysis Buffer 1X**

300 µl of RBC Lysis Buffer  
2.7 ml of sterile H<sub>2</sub>O

- **GF-**

- Advance DMEM/F12 500 ml
- Pen/Strep (100X) 5 ml
- HEPES (1M=100x) 5 ml
- Glutamax (100X) 5 ml

Liberase: use 50 µg/ml (0.26 units/ml; dilute 1:100 from 26 units/ml)

Primocin: dilute 1:500

Y-27632: dilute 1:1000

## 3.2

### Procedure

#### 3.2.1

#### Washing

- Transfer the tumor into a 50 ml tube with 20 ml PBS + Pen/Strep 4°C
- Vigorously shake the tube to remove blood and fat; the supernatant turns red and fat droplets can be distinguished
- Pour the tissue along with the supernatant into a 10 cm petri dish
- Use sterile forceps to transfer the tissue into a new 50 ml falcon with 20 ml cold PBS + Pen/Strep 4°C. Repeat washing until the supernatant stays clear.
- Transfer the tumor into a fresh 10 cm petri dish. Weigh the tumor.

#### 3.2.2

#### Homogenization of the tumor

- Cut the tumor 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 + Liberase + Y-27632 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 PBS + 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 5 minutes 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 PBS + 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.

### 3.2.3 Cell counting

- Re-suspend the pellet with 3 ml of GF-.
- Count the cells.
- Centrifuge at 300 g for 5 minutes. Discard supernatant. Re-suspend the cells in GF- at the concentration required to generate organoids (100000 cells / 10 µl).
- Approximately 2,5 million cells are required to generate a stable and comprehensive tumor organoid culture.

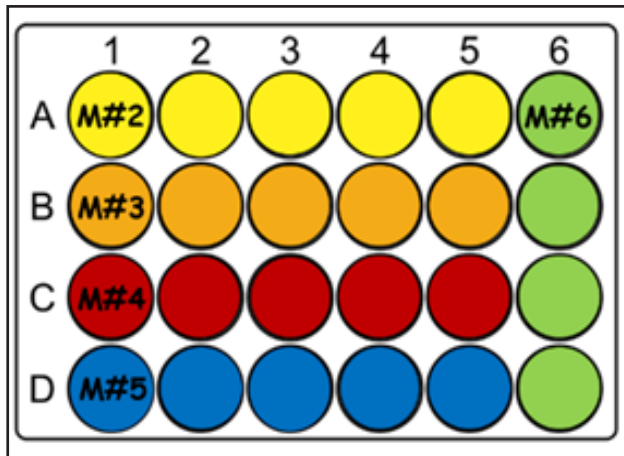
### 3.2.4 Seeding of cells: tumor organoid generation

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  $2,5 \times 10^6$  cells in a 15 ml tube (organoids need to be seeded on an entire 24 well plate (layout see image below) to generate a comprehensive tumor organoid culture)
- Divide the amount of cells as required for tumoroid seeding ( $2,5 \times 10^6$  cells for tumoroid culture and  $5 \times 10^5$  for Cell Viability Assay) using 15 ml tubes
- Add GF- medium up to 10-12 ml. Centrifuge the single cells at 1500 rpm for 5 minutes at 4°C
- Remove the supernatant – keep the cells on ice
- Resuspend 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
- Use pre-warmed 24 well plates
- Make 30 µl droplets / well
- Incubate 10-15 min at 37°C (5% CO<sub>2</sub>) in the cell incubator
- Carefully add 500 µl pre-warmed medium (Medium #2, #3, #4, #5, #6) per well

- Check pipetting scheme for the medium selection in the image below
- Incubate at 37°C (5% CO<sub>2</sub>) in the cell incubator
- Refresh medium every 2-3 days
- Carefully add 500 µl pre-warmed medium (Medium #2, #3, #4, #5, #6) per well.
- Check pipetting scheme for the medium selection in the *Figure* below

**Figure 3** Pipetting scheme for the four media strategy M#2, M#3, M#4, M#5, M#6 in the 24 well plate. Seed 30µl droplets with 1x10<sup>6</sup> cell per well.



### 3.2.5 Real Time-Glo™ – Viability Assay

To determine which media conditions to use to propagate tumor organoid growth a viability assay is performed.

#### 3.2.5.1 Material, reagents and reagent set-up

Put the Real Time Glo (RTG) Kit at RT

Prepare 600µl 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 600µl of each medium (#2-#6). Put back in the water bath until use.



### 3.2.5.2

#### Procedures

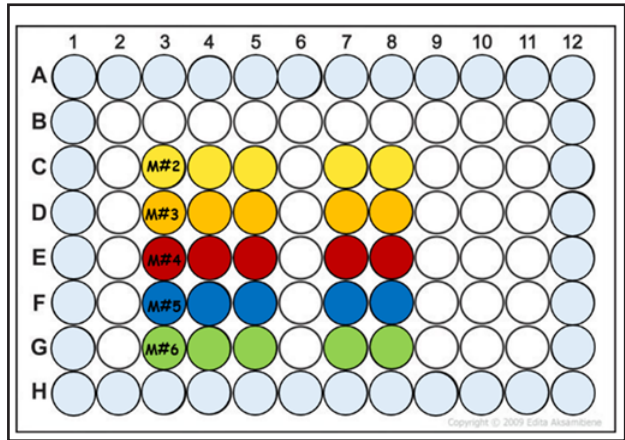
- Prepare  $5 \times 10^5$  cells in a 15 ml tube
- Centrifuge the single cells at 1500 rpm for 5 minutes at 4°C
- Resuspended cells in 50  $\mu$ l ice cold GF- and 100  $\mu$ l ice cold Matrigel (try to avoid generating air bubbles)
- Make 10  $\mu$ l droplets per 96 well – see pipetting scheme below (use a pre-warmed 96 well plate (Corning #3603))
- For the media control, add 40 $\mu$ l GF- in a precooled 1.5 ml tube and mix with 80  $\mu$ l of matrigel
- Seed 10  $\mu$ l droplets per well as suggested in the pipetting scheme below
- Incubate the plate for 15 min at 37°C (5% CO<sub>2</sub>)
- Gently add 100  $\mu$ l pre-warmed Medium (Medium #2, #3, #4, #5, #6) supplemented with RTG enzyme and substrate according the pipetting scheme
- Incubate at 37°C (5% CO<sub>2</sub>)
- Take pictures of every well with organoids at time points: 1h, 24h, 48, 72h, 96h, 120h, 144h;
- Perform luminescence measurement at time points: 1h, 4h, 24h, 48h, 72h;
- Refresh medium after time point 72 hours to medium without enzyme and substrate

### 3.2.5.3

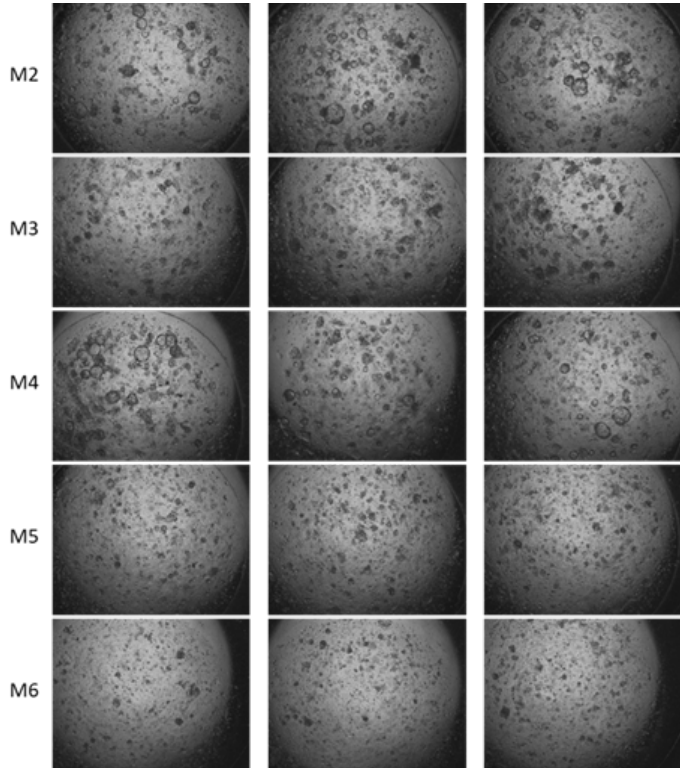
#### Luminescence measurement

- 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 s and 0.5 s), Mithras will heat up to 37°C (takes approximately 1 hour)
- 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 to be 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 of image table below, *Figure 4*)

**Figure 4** Plate layout for the assessment of medium strategy for tumor organoid cultivation. 10  $\mu$ l droplets of matrigel mixed with GF- and  $\sim 33.3 \times 10^3$  seeded accordingly. The different media conditions are applied as indicated by the color code per well. Outer wells (indicated with light blue wells) are filled up with 150  $\mu$ l PBS to prevent evaporation.



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



# 4.

## Applicable references



ADSI –CCO-SOP-10

