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UNIUD-CCO-SOP-1.0

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INTESTINAL ORGANOID GENERATION AND MAINTENANCE

Purpose

The purpose of SOP 1.0 is to describe how to generate and maintain intestinal organoids from human intestinal tissues.

Scope

SOP 1.0 is intended to cover all resources, personnel and equipment in the intestinal organoids laboratory.

Introduction

Colonic tissues were obtained upon surgical resection from the University Hospital Santa Maria della Misericordia of Udine. All patients were diagnosed with colorectal cancer. Normal and cancer intestinal tissue have been passed to UniUD collaborators to provide material for the cultivation of organoids.

Collaborators at UniUD have used normal and tumor material to establish organoid cultivation protocol as following described.

1. Cell culture media, reagents and solutions

- PBS without Ca^{2+}, Mg^{2+} (PBS) (#BE17-512F Lonza)
- Penicillin-Streptomycin (P/S) (#15070063 Life Technologies)
- Primocin (#ant-pm-1 Invivogen)

- Fetal Bovin Serum (FBS) (#S1810-500 BioWest)
- DMEM (#BE12-707F Lonza)
- Advanced DMEM/ F12 (#12634028 Life Technologies)
- GlutaMAX™ Supplement (Glutamax) (#35050038 Life Technologies)
- HEPES (#15630056 Life Technologies)
- Liberase (#05401089001 Roche) disolve powder in sterile H_2O (26 units/ml), avoid freeze&thaw
- RBC Lysis Buffer 10X (# 420301 BioLegend) dilute in sterile H_2O
- Matrigel® (#356231 Corning®)
- B-27® Supplement, serum free (#17504044 Life Technologies)
- N-Acetylcysteine (#A9165-25G Sigma Aldrich disolve powder in sterile H_2O)
- Nicotinamide (#N0636-100G Sigma Aldrich) disolve powder in PBS)
- SB202190 (#S7067-5MG Sigma Aldrich)disolve powder in DMSO)
- m-EGF (#315-09 PEPROTECH) disolve powder in PBS + 0.1% BSA
- m-Noggin (#250-38 PEPROTECH) disolve powder in PBS + 0.1% BSA
- h-R-Spondin-1 (#120-38 PEPROTECH) disolve powder in PBS + 0.1% BSA
- h-Gastrin I (#3006/1 TOCRIS) disolve powder in PBS
- m-Wnt-3a (#1324-WN-002 R&D System) disolve powder in PBS + 0.1% BSA
- A83-01 (#2939/10 R&D System) disolve powder in DMSO
- Prostaglandin E2 (#3632464 Peprotech)
- Y-27632 (#120129 Abcam) disolve powder in sterile H_2O
- Fetal Bovine Serum (#S1810-500 BioWest)
- Freezing medium: Recovery™ Cell Culture Freezing Medium (#12648010 Life Technologies)
- DTT (#10197777001 Sigma Aldrich)
- EDTA (#E5134 Sigma Aldrich)
- DMSO (#154938 Sigma Aldrich)
- Bovine Serum Albumin (BSA) (#05470 Sigma Aldrich)
- Isopropanol (#I9516 Sigma Aldrich)
- sterile H_2O

2. Equipment

- Pipetaid (SN603280198 Euroclone)
- Dissection tweezers (#11150-10, #11254-20, #11274-20 F.S.T.)
- Scissors (#14058-09, #14059-09, #14110-15 F.S.T.)
- Centrifuge (5810R

Eppendorf, Sorvall Legend Micro21R ThermoScientific)

- Brightfield microscope (MC170 HD Leica)
- Rocker in cold room (Multi Bio RS-24 Biosan)
- Shaking incubator (Excella E25 New Brunswick Scientific)
- 37°C, 5% CO_2 cell culture incubator (Safe Grow Pro Euroclone)
- Tissue culture hood (MSC Advance ThermoScientific)
- Mr Frosty (#5100-0001 ThermoFisher)

3. Plastics

- 24-multiwell TC-Plate (#GR662160 Greiner Bio-One)
- Pipettes (#EPS05N, #EPS10N, #EPS25N Euroclone)
- 10 µl, 200 µl and 1000 µl pipette tips (#ECTD50010RN, #ECTD50200RN, #ECTD51000RN Euroclone)
- 1.5 ml microcentrifuge tubes (#ET3415 Euroclone)
- 2 ml cryogenic vials (#ECC3212RB Euroclone)
- 100 mm petri dish (#ET2100 Euroclone)
- 50 ml and 15 ml conical tubes (#ET5050B, #ET5015B Euroclone)
- 100 µm cell strainer (#ET6100 Euroclone)

4. Solutions

- PBS + P/S:
  500 ml PBS
  5 ml of P/S 100X
- PBS + P/S + DTT:
  500 ml PBS
  5 ml of P/S 100X
  25µl of 1M DTT (final concentration 0.5 mM)
- GF-:
  500 ml Advance DMEM/F12
  5 m of P/S 100X
  5 ml of HEPES 1M
  5 ml of Glutamax 100X
- GF- + FBS:
  50 ml GF-
  5 ml of FBS 100%

5. Media composition

Legend:

RCM R-Spondin1 Conditioned Medium*

NCM Noggin Conditioned Medium**

WCM Wnt3a Conditioned Medium***

PGE2 Prostaglandin E2

* R-Spondin1-conditioned medium was produced from HEK293T-HA-Rspo1-Fc cells; MTA for the use of producer cell line was obtained from Stanford University (Prof. Calvin Kuo).

** Noggin-conditioned medium was produced from HEK293-mNoggin-Fc cells. MTA for the use of producer cell line was obtained from the Hubrecht Institute (Prof. Jeroen den Hertog).

*** Wnt3a conditioned medium was produced from L-Wnt3a cells. MTA for the use of producer cell line was obtained from the Hubrecht Institute (Prof. Jeroen den Hertog).

Stock	Reagent	Medium +Wnt3a	Medium -Wnt3a
	GF-	1X	1X
50X	B27	1X	1X
500mM	N-acetylcysteine	1.25mM	1.25mM
1M	Nicotinamide	10mM	10mM
10uM	Gastrinl	10nM	10nM
10uM	PGE2	10nM	10nM
10mM	Y-27632	10uM	10uM
50mg/ml	Primocin	100ug/ml	100ug/ml
1.5mM	A83-01	500nM	500nM
100%	NCM	10%	10%
30mM	SB202190	3uM	3uM
50ug/ml	EGF	50ng/ml	50ng/ml
100%	RCM	20%	20%
100%	WCM	50%	

6. Procedure

6.1 Generation of normal intestinal organoid

6.1.1 Pre-warm 24-well at 5% CO_2, 37°C for 24 hours before the beginning of the procedure.

Perform all procedures on ice unless indicated otherwise.

6.1.2 Place the normal tissue in a 50 ml conical tube (use sterile tweezer) with 10 ml ice-cold PBS + P/S and shake 10 times. Allow tissue to settle down and remove supernatant Repeat this process.

6.1.3 Normal tissue is then collected in a petri dish. Using scissors, dissect epithelial layer from muscle and fat layers and cut into around 2 mm pieces. Place tissue fragments in in a 15 ml conical tube.

6.1.4 Suspend the tissue fragments with 5ml of PBS + P/S + DTT using a 5-ml pipette and pipet up and down (8-10 times). Allow biopsies to settle down and remove supernatant. Add 5 ml of PBS + P/S + DTT and repeat this process 2-3 times until the supernatant no longer contains any visible debris.

6.1.5 Incubate tissue fragments with 10ml of PBS + P/S + DTT + 200 µl 0.5 M EDTA for 1 hours at 4˚C (cold room shaking slowly). Check if crypts are visible under microscope.

6.1.6 Prepare a new 15ml canonical tube with 2ml of FBS. Allow tissue fragments to settle down and remove supernatant. Add 2 ml of of PBS + P/S + DTT and pipette and pipet up and down with P1000 after cutting off tip. Crypts become loose in supernatant. Transfer supernatant into a 15ml tube with 2ml of FBS. Add 2 ml of of PBS + P/S + DTT and repeat this process until you have a total of 10ml.

6.1.7 Centrifuge at 800 rpm for 5 min at 4˚C. Discard supernatant.

6.1.8 Resuspend the pellet with 10 ml of GF-. Count crypts.

6.1.9 Centrifuge at 800 g for 5 minutes at 4˚C. Discard supernatant.

6.1.10 Resuspend the pellet with GF- at the concentration needed. Usually mix 500 crypts in 10µl with 20 µl of Matrigel and plate 3 x 10 µl drops in 24-well.

After polymerization of Matrigel (10-15 min at 37˚C), add 500 ml of Medium +Wnt3a medium. In 1-well add Medium -Wnt3a medium as your negative control.

6.1.11 Refresh medium every 2-3 days.

6.1.12 Passage organoids every 1-2 weeks (1:2).

(See ***Figure 1***)

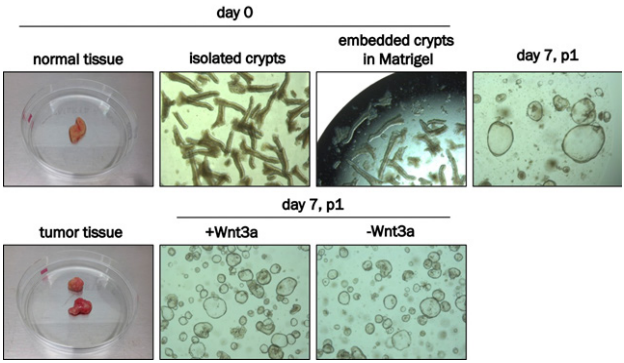

Figure 1 Generation of normal and tumor human colon organoids. Representative images of normal and tumor colon organoids. In order from left to right normal tissue, isolated crypts, crypts embedded into Matrigel and normal organoids cultured in Medium +Wnt3a on day 7 (p1=passage 1). In order from left to right tumor tissue, tumor organoids cultivated in Medium +Wnt3a and Medium -Wnt3a media on day 7 (p1=passage 1). Magnification 5X.

6.2 Generation of tumor intestinal organoids

6.2.1 Pre-warm 24-well at 5% CO_2 37°C 24 hours before the beginning of the procedure. Perform all procedures on ice unless indicated otherwise.

6.2.2 Place the piece of tumor in a 50 ml conical tube (use sterile tweezer) with 10 ml ice-cold PBS + P/S. Shake 10–15 times, remove the PBS+ P/S and replace with 10 ml of ice-cold PBS + P/S. Repeat this process 2 more times until the supernatant no longer contains any visible debris.

6.2.3 Tumor is then collected in a petri dish and weight.

6.2.4 Homogenise tumor tissue with a pair of scissors and place in a 50 ml conical tube with DMEM + P/S + Primocin 100ug/ml + Liberase 50 ug/ml (0.26 units/ml) + Y-27632 10uM for 1 hour at 37 °C at 250 rpm (1 g of tissue in 10 ml). Shake vigorously every 15 min

6.2.5 Pass this fraction through a 100 µm cell strainer to remove debris. Add 2.5-5 ml of PBS + P/S to wash the cell strainer. Collect the filtered solution in a 50 ml conical tube.

6.2.6 Centrifuge single cells at 1200 rpm for 5 minutes at 4°C. Discard supernatant.

6.2.7 Resuspend the pellet with 3 ml of RBC Lysis Buffer 1X (diluite in sterile H_2O). Incubate for 10 min at room temperature in the dark. Neutralize lysis adding 10 ml of GF- with FBS.

6.2.8 Centrifuge at 1200 rpm for 5 minutes at 4°C. Discard supernatant.

6.2.9 Resuspend the pellet with 5 ml of GF- and pass this fraction through a 100 µm cell strainer to remove debris (optional). Collect the filtered solution in a 50 ml tube.

6.2.10 Centrifuge at 1200 rpm for 5 minutes at 4°C. Discard supernatant.

6.2.11 Resuspend the pellet with 1 ml of GF- and count cells.

6.2.12 Centrifuge at 1200 rpm for 5 minutes. Discard supernatant.

6.2.13 Resuspend the pellet with GF- at the concertation needed. Usually mix 200 000 cells in 10µl with 20 µl of Matrigel and plate 30 µl drop in 24-well.

6.2.14 After polymerization of Matrigel (10-15 min at 37°C), add 500 µl of Medium +Wnt3a and Medium -Wnt3a.

6.2.15 Refresh medium every 2-3 days.

6.2.16 Passage organoids every 1-2 weeks (1:4). (See ***Figure 1***).

6.3 Passage intestinal organoids (mechanical methods)

Passage organoids 1:2 or 1:4 every 1-2 weeks. Referesh medium every 2-3 days.

6.3.1 Pre-warm 24-well at 5% CO_2 37°C 24 hours before the beginning of the procedure. Thaw Matrigel on ice 1 hour before use.

Perform all procedures on ice unless indicated otherwise

6.3.2 Remove medium. Add 1 ml of cold GF- and make Matrigel loose with p1000 tip. Pipet up and down. Recover in a 15 ml canonical tube. Repeat once. Usually put together 8 wells.

6.3.3 Centrifuge at 800 rpm for 3 min at 4°C. Discard supernatant.

6.3.4 Attach a p200 tip on the p1000 tip. Add 1 ml GF- to the pellet. Pipet 20 times gently up and down to disrupt organoids.

6.3.5 Add GF- until 5 ml.

6.3.6 Centrifuge at 800 rpm for 3 min at 4°C. Discard supernatant.

6.3.7 Resuspend the pellet with GF- at the concentration needed. Mix organoids in 10µl with 20 µl of Matrigel and plate 3 x 10 µl drops (normal organoids) or 30 µl drops (tumor organoids) in 24-multiwell plate.

6.3.8 After polymerization of Matrigel (10-15 min at 37°C), add 500 µl of culture medium. (See ***Figure 2***).

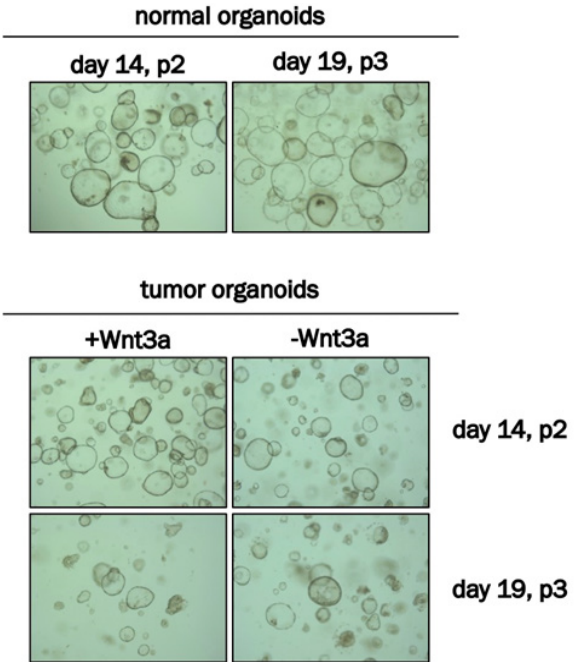

Figure 2 Maintenance of normal and tumor intestinal organoids - Passage intestinal organoids (mechanical methods). Representative brightfield microscope images of healthy and tumor organoids on day 14 (passage 2, p2) and day 19 (passage 3, p3) from the generation. Normal organoids are cultured in Medium +Wnt3a medium. Tumor organoids are cultured in Medium +Wnt3a and Medium -Wnt3a. Magnification 5X.

6.4 Passage intestinal organoids (enzymatic methods)

Passage organoids 1:2 or 1:4 every 1-2 weeks. Change medium every 2-3 days.

6.4.1 Pre-warm 24-well at 5% CO_2 37°C 24 hours before the beginning of the procedure. Thaw Matrigel on ice 1 hour before use.

Perform all procedures on ice unless indicated otherwise.

6.4.2 Remove medium. Add 1 ml of cold GF- and make Matrigel loose with p1000 tip. Pipet up and down. Recover in a 15 ml canonical tube. Repeat once. Usually put together 8 wells.

6.4.3 Centrifuge at 800 rpm for 3 min at 4°C. Discard supernatant.

6.4.4 Add 500 µl of Trypsin 0.25% and incubate 5 min at 37°C.

6.4.5 Attach a p200 tip on the p1000 tip. Pipet 20 times gently up and down to disrupt organoids.

6.4.6 Add 2.5 ml GF- + 10% FBS. Centrifuge 1200 rpm for 5 min at 4°C. Discard supernatant.

6.4.7 Resuspend the pellet with GF- at the concentration needed. Mix organoids in 10µl with 20 µl of Matrigel and plate 3 x 10 µl drops (normal organoids) or 30 µl drops (tumor organoids) in 24-multiwell plate.

6.4.8 After polymerization of Matrigel (10-15 min at 37°C), add 500 µl of culture medium. (See ***Figure 3***).

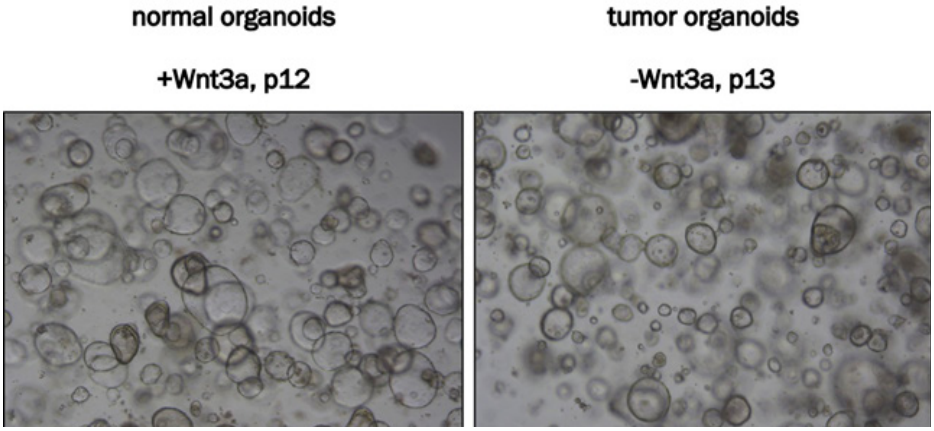

Figure 3. Long-term culture of normal and tumor intestinal organoids - Passage intestinal organoids (mechanical methods). Representative brightfield images of healthy and tumor organoids after 4 months from the generation. Normal organoids cultured in Medium +Wnt3a medium, passage 12 (p12). Tumor organoids cultured in Medium -Wnt3a medium, passage 13 (p13). Magnification 5X.

6.5 Freeze organoids

Freeze 1 or more well of a 24-multiwell plate in 1 cryovial.

Reagent:

Freezing medium: Recovery™ Cell Culture Freezing Medium #12648010 Life Technologies (or GF- + 10% DMSO)

6.5.1 Prepare freezing medium

6.5.2 Remove medium. Add 1 ml of freezing medium and make Matrigel loose with p1000 tip. Pipet 10 times up and down. Recover in a cryovial.

6.5.3 Place as fast as possible in Mr Frosty and then place it at -80°C o/n.

6.5.4 Store cryovials in liquid nitrogen.

6.6 Thaw organoids

Thaw 1 cryovial in 1 well of 24-multiwell plate (or in more wells when indicated).

6.6.1 Pre-warm 24-well at 5% CO_2 37°C 24 hours before the beginning of the procedure. Thaw Matrigel on ice 1 hour before.

Perform all procedures on ice unless indicated otherwise.

6.6.2 Thaw cryovials on waterbath. Move the content of the cryovials in a 15 ml conical tube. Add 1 ml of cold GF-.

6.6.3 Centrifuge at 800 rpm for 3 min at 4°C. Discard supernatant.

6.6.4 Resuspend the pellet with GF-. Mix organoids in 10µl with 20 µl of Matrigel and plate 3 x 10 µl drops (normal organoids) or 30 µl drops (tumor organoids) in 24-multiwell plate.

6.6.5 After polymerization of Matrigel (10-15 min at 37°C), add 500 ml of culture medium.

7. Applicable references to Uni Udine SOPs

UniUD – CCO - SOP – 2.0, UniUD – CCO - SOP – 3.0

8. References

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