Culture of Mouse Enteric Organoids

Using Cultrex™ UltiMatrix Basement Membrane Extract

Organoid cultures represent the next generation of tissue culture models. These cultures are extracted directly from living tissues. Stem cell populations are maintained using a feeder layer-free extracellular matrix environment under non-differentiating conditions. When subjected to differentiating conditions, these organoids exhibit expression of tissue-specific genes and differentiation of stem cells into tissue-specific architecture and cell types. The protocol provided below is intended to culture organoid progenitor cells from normal and healthy mouse gastric, small intestine, or colon tissues using Cultrex™ UltiMatrix RGF Basement Membrane Extract as a scaffold.

This protocol provides a procedure for subculturing normal mouse enteric organoids, modified from the original submerged method published by Yin, X. *et al.* (2014) Nat. Methods **11**:106. The protocol includes a series of tips on how to prepare culture media for each type of enteric organoid culture (gastric, small intestine, and colon) as well as a general guide to start and passage these organoids.

The majority of reagents used in this protocol were sourced from the Bio-Techne brands of R&D Systems™ and Tocris Bioscience™.

Equipment

1. Cell culture incubator (37 °C, 5% CO_2)
2. Cell culture hood with laminar flow
3. Centrifuge with refrigeration and swinging bucket rotor
4. 37 °C water bath
5. Ice bucket
6. Laboratory refrigerator
7. Pipet aid and serological pipettes (5 mL)
8. Micropipettes and tips (2–200 µL)
9. Conical tubes, 15 mL and 50 mL, sterile
10. 24-well plate, tissue-culture treated, sterile
11. Vacuum pump
12. Medium filtration unit, 0.1 µm, 500 mL, sterile
13. Syringe, 50 mL, sterile
14. Syringe filter, 0.2 µm, sterile
15. Cell culture waste container
16. 20-gauge needle, sterile

Materials

TABLE 1.

Materials Needed for Mouse Enteric Organoid Culture

Product Name	Supplier	Catalog #
Cultrex Organoid Harvesting Solution	R&D Systems	3700-100-01
Cultrex UltiMatrix Reduced Growth Factor Basement Membrane Extract	R&D Systems	BME001-05
Advanced DMEM/F-12 Cell Culture Medium	Thermo Fisher	12634010
GlutaMAX™	Thermo Fisher	35050061
HEPES	Tocris Bioscience	3173
Penicillin/ Streptomycin	Various	Various
N21-MAX Supplement	R&D Systems	AR008
N-2 MAX Supplement	R&D Systems	AR009
N-Acetylcysteine	Tocris Bioscience	7874

Product Name	Supplier	Catalog #
Valproic Acid	Tocris Bioscience	2815
Y-27632 dihydrochloride (Rho kinase inhibitor)	Tocris Bioscience	1254
CHIR 99021 (GSK-3 inhibitor)	Tocris Bioscience	4423
Recombinant Human EGF	R&D Systems	236-EG
Recombinant Human R-Spondin 1	R&D Systems	4645-RS
Recombinant Human Noggin	R&D Systems	6057-NG
Recombinant Human FGF-10	R&D Systems	345-FG
Recombinant Human Wnt-3a	R&D Systems	5036-WN

Other Required Reagents

1. Distilled (DW) or deionized water (DI)
2. Phosphate buffered saline (PBS)
3. 1% Ammonium
4. 1% BSA/PBS
5. DMSO

Reagent Preparation

1. Use aseptic technique at all times during this protocol.
2. Prepare stock solutions for mouse enteric organoid culture, as indicated in TABLE 2.

TABLE 2.

Preparation of Stock Solutions for Enteric Organoid Culture Medium

Reagent Name	Solvent	Stock solution	Preparation	Storage
N-Acetylcysteine	DI water	500 mM = 81.6 mg/mL	200 mg in 2.4 mL	4 °C
GlutaMAX™	PBS	100 mM = 14.6 mg/mL	0.73 g in 50 mL	4 °C
Recombinant Human EGF	1% BSA/PBS	500 µg/mL	200 µg in 400 mL	-80 °C
Recombinant Human R-Spondin 1	1% BSA/PBS	1 mg/mL	1 mg in 1 mL	-80 °C
Recombinant Human Noggin	1% BSA/PBS	100 µg/mL	100 µg in 1 mL	-80 °C
Recombinant Human Wnt-3a	1% BSA/PBS	600 µg/mL	500 µg in 833 µL	-80 °C
Valproic Acid	DI water	200 mM = 33 mg/mL	100 mg in 3 mL	-20 °C
CHIR 99021	DMSO	20 mM = 9.3 mg/mL	10 mg in 1.08 mL	-20 °C
Y-27632 dihydrochloride	PBS	10 mM = 3.2 mg/mL	10 mg in 3.1 mL	4 °C
HEPES	DI water	1 M = 238.3 mg/mL	11.9 g in 50 mL	4 ° C

3. Prepare 10X Solution M1, as indicated in Table 3.

TABLE 3.

Preparation of 10X Solution M1

Reagent Name	[STOCK]	[FINAL]	Volume
N21-MAX Supplement	50X	10X	20 mL
GlutaMAX™	100 mM	20 mM	20 mL
HEPES	1 M	100 mM	10 mL
Penicillin/ Streptomycin	100X	10X	10 mL

Dispense 4.5 mL per tube into sterile 15 mL conical tubes, label, and store at ≤–20 °C.

Reagent Name	[STOCK]	[FINAL]	Volume
N-2 MAX Supplement	100X	10X	10 mL
N-Acetylcysteine	500 mM	10 mM	2 mL
Advanced DMEM/F-12 Cell Culture Medium	N/A	N/A	28 mL
		Total	100 mL

4. Prepare 10X Solution M2, as indicated in Table 4.

TABLE 4.

Preparation of 10X Solution M2

Reagent Name	[STOCK]	[FINAL]	Volume
Recombinant Human Noggin	100 µg/mL	1 µg/mL	1 mL
Recombinant Human EGF	500 µg/mL	50 ng/mL	100 µL
Advanced DMEM/F-12 Cell Culture Medium	N/A	N/A	98.9
		Total	100 mL

Dispense 4.5 mL per tube into sterile 15 mL conical tubes, label, and store at <-70 °C.

5. Prepare 1000X Solution M3 by dissolving 500 µg of Wnt-3a in 833 µL of 1% BSA/PBS, and store at ≤–70 °C.

6. Prepare 1000X Solution M4 by dissolving 1 mg of R-Spondin 1 in 1 mL of 1% BSA/PBS, and store at ≤–70 °C.

7. Prepare 10X Solution M5, as indicated in Table 5.

   Dispense 4.5 mL per tube into sterile 15 mL conical tubes, label, and store at ≤–20 °C.

TABLE 5.

Preparation of 10X Solution M5

Reagent Name	[STOCK]	[FINAL]	Volume
Valproic Acid	200 mM	20 mM	10 mL
CHIR 99021	20 mM	25 uM	125 µL
Advanced DMEM/F-12 Cell Culture Medium	N/A	N/A	89.9 mL
		Total	100 mL

8. Prepare 500X Solution M6 by suspending FGF-10 at a concentration of 100 µg/mL in 1% BSA/PBS, and store at ≤–70 °C.

9. Prepare Organoid Culture Medium - To prepare each type of Mouse Enteric Organoid Culture Medium, add the appropriate Stock Solution to a 50 mL conical tube, and complete with Advanced DMEM/F12 medium to a final volume of 45 mL. Filter sterilize and keep medium stored at 4 °C for no longer than 2 weeks, as growth factors and supplements lose activity after prolonged storage. Use Table 6 as a reference.

TABLE 6.

Summary of Stock Solution Volumes Needed to Prepare Mouse Enteric Organoid Culture Medium

Solution	Name	Storage	Concentration	Gastric	Small Intestine	Colon	Aliquot volume
M1	**Supplements**	-20 °C	10X	X	X	X	4.5 mL
M2	**EGF/Noggin**	-80 °C	10X	X	X	X	4.5 mL
M3	**Wnt-3a**	-80 °C	1000X	X		X	45 µL
M4	**R-Spondin 1**	-80 °C	1000X	X	X	X	45 µL
M5	**Valproic Acid**	-80 °C	10X	X	X		4.5 mL
M6	**FGF-10**	-80 °C	500X	X			9 µL

Methods for Culturing Mouse Enteric Organoids

1. **Starting Organoids from a Cryovial**

   a. Thaw Cultrex UltiMatrix RGF BME on ice for four hours or overnight at 2 - 8 °C (on ice in the refrigerator).

   b. Thaw cryovial containing organoids in a 37 °C water bath.

      Note: The contents should thaw in 2–3 minutes; do not allow the cryovial to remain at 37 °C any longer than is necessary.

   c. Transfer the contents of the cryovial to a 15 mL conical tube and add 9 mL of Advanced DMEM/F12 with 10% FBS. Gently pipet up and down three times using a serological pipette to resuspend the organoids.

      Note: Organoids may be counted at this time if needed to determine seeding volumes.

   d. Centrifuge the vial at 500 × g for 3 minutes to pellet the organoids, and aspirate the medium.

   e. Resuspend the organoids in Cultrex UltiMatrix RGF Basement Membrane Extract at 10,000 organoids per mL (500 organoids per 50 µL). Pipet up and down three times using a serological pipette to disperse the organoids in the Cultrex UltiMatrix RGF Basement Membrane Extract, and dispense 50 µL of the Cultrex UltiMatrix RGF Basement Membrane Extract/organoid mixture in the center of each well of a 24-well plate or arrange domes placing 6 to 8 domes in a well of a 6-well plate (FIGURE 1).

      Note: The Cultrex UltiMatrix RGF Basement Membrane Extract-contained organoids should not touch the sides of the well.

   f. Incubate the plate in the cell culture incubator for 25 minutes to polymerize the Cultrex UltiMatrix RGF Basement Membrane Extract.

   g. Calculate the volume of Organoid Starting/Passaging Medium needed. Each well of a 24-well plate requires 500 uL of Organoid Starting/Passaging Medium, while 3 mL per well is required for a 6-well plate.

   h. Prepare Organoid Starting/Passaging Medium by adding Y-27632 dihydrochloride to the Organoid Culture Medium at a final concentration of 10 µM.

   i. Add 500 µL of Organoid Starting/Passaging Medium per well of a 24-well plate or 3 mL per well of a 6-well plate.

Figure 1. (A) Placement of Cultrex UltiMatrix RGF BME/organoid mixture in the center of the well of a 24-well plate. (B) Placement of multiple domes within a well of a 6-well plate.

Note: Medium should be gently pipetted into the corner of the well away from the Cultrex UltiMatrix RGF Basement Membrane Extract domes to prevent their disruption.

j. Return the plate containing the organoid cultures to the cell culture incubator to promote organoid growth.

2. Enteric Organoid Culture Maintenance

a. The culture medium should be aspirated from each well and replaced with fresh Organoid Culture Medium every other day. Mouse enteric organoids can be cultured for up to two weeks before passaging, depending on the cell seeding density (see the protocol below).

Note: Medium should be gently aspirated from and pipetted into the corner of the well away from the Cultrex UltiMatrix RGF BME/ organoids to prevent their disruption.

3. Passaging Organoids

a. View organoids under the microscope. Each well of a 24-well plate should contain approximately 100 to 500 organoids for optimal growth. Organoid cultures exhibiting rapid growth may be split 1:4 during passaging, while slow growing cultures may benefit from a 1:1 split. Make this determination prior to harvesting to estimate reagent needs before starting.

Note: Organoid density is important for optimal growth; too many organoids will strain culture resources, while too few organoids lack paracrine signaling necessary to sustain growth.

b. Aspirate the medium without disturbing the organoids at the bottom of the wells.

c. Wash each well with 10 volumes of cold (4 °C) PBS, and aspirate without disturbing the Cultrex UltiMatrix RGF Basement Membrane Extract dome.

d. Add 10 volumes of cold (4 °C) Cultrex Organoid Harvesting Solution to each well to depolymerize the Cultrex UltiMatrix RGF Basement Membrane Extract. If each well contains 50 uL of Cultrex UltiMatrix RGF Basement Membrane Extract, 500 uL of Organoid Harvesting Solution will be needed per well in the plate.

e. Place the plate(s) in a 4 °C cooler with moderate shaking for one hour to depolymerize the Cultrex UltiMatrix RGF Basement Membrane Extract.

Note: Most of the Cultrex UltiMatrix RGF Basement Membrane Extract should be visibly depolymerized during this incubation; however, some small amount may remain.

f. Pipet up and down three times with a serological pipette across the well to solubilize any remaining gel.

g. Pass the organoid solution through a 20-gauge needle into a conical tube to fragment the organoids.

h. Centrifuge the tube at 500 × g at 4 °C for 5 minutes.

i. Aspirate the supernatant but be careful not to disturb the organoid pellet.

j. Resuspend the pellet in 10 volumes of cold (4 °C) PBS.

k. Centrifuge the tube at 500 × g at 4 °C for 5 minutes.

l. Aspirate the supernatant but be careful not to disturb the organoid pellet.

m. Repeat the centrifugation and aspiration to remove all of the liquid to prevent dilution of the Cultrex UltiMatrix RGF Basement Membrane Extract.

n. Resuspend the segmented organoids in Cultrex UltiMatrix RGF Basement Membrane Extract and dispense 50 µL of the Cultrex UltiMatrix RGF Basement Membrane Extract/ organoid mixture into the center of each well of a 24-well plate to form a dome.

Note: The Cultrex UltiMatrix RGF Basement Membrane Extract-contained organoids should not touch the sides of the well.

o. Incubate the plate in the cell culture incubator for 25 minutes to polymerize the Cultrex UltiMatrix RGF Basement Membrane Extract.

p. Add 500 µL of Organoid Starting/Passaging Medium per well of a 24-well plate, or 3 mL per well of a 6-well plate.

Note: Medium should be gently pipetted into the corner of the well away from the Cultrex UltiMatrix RGF BME to prevent its disruption.

q. Return the plate containing organoid cultures to the cell culture incubator to promote organoid growth.

4. Cryobanking Organoids

a. View organoids under the microscope. Each well of a 24-well plate should contain approximately 100–500 organoids.

b. Aspirate the medium without disturbing the organoids at the bottom of the wells.

c. Wash each well with 10 volumes of cold (4 °C) PBS, and aspirate without disturbing the Cultrex UltiMatrix RGF Basement Membrane Extract dome.

d. Add 10–20 volumes of cold (4 °C) Organoid Harvesting Solution to each well to depolymerize the Cultrex UltiMatrix RGF Basement Membrane Extract.

e. Place the plate(s) in a 4 °C cooler with moderate shaking for one hour to depolymerize the Cultrex UltiMatrix RGF Basement Membrane Extract.

Note: Most of the Cultrex UltiMatrix RGF Basement Membrane Extract should be visibly depolymerized during this incubation; however, some small amount may remain.

f. Pipet up and down three times with a serological pipette across the well to solubilize any remaining gel.

g. Pass the organoid solution through a 20-gauge needle into a conical tube to fragment the organoids.

h. Centrifuge the tube at 500 × g at 4 °C for 5 minutes.

i. Aspirate the supernatant but be careful not to disturb the organoid pellet.

j. Resuspend the pellet in 10 volumes of cold (4 °C) PBS.

k. Centrifuge the tube at 500 × g at 4 °C for 5 minutes.

l. Aspirate the supernatant but be careful not to disturb the organoid pellet.

m. Repeat the centrifugation and aspiration to remove all the liquid to prevent dilution of the cryopreservation medium.

n. Resuspend the segmented organoids in 90% FBS, 10% DMSO, and 10 µM Y-27632, and dispense 500 µL of the organoid mixture into each labeled cryovial.

o. Place the cryovials in a freezing container, and store at –80 °C for 24 hours.

p. Transfer the cryovials to a liquid nitrogen tank for long term storage.

Data Examples

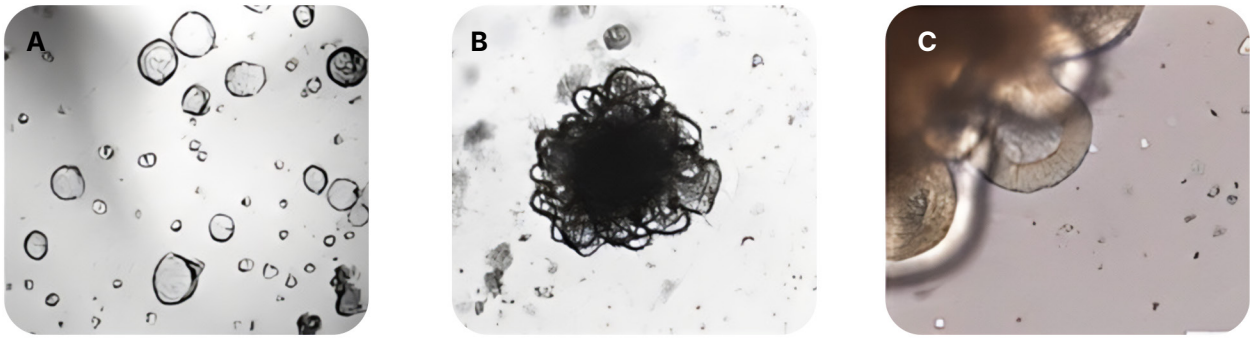

Figure 2. Mouse Small Intestine Organoids. Representative brightfield images of undifferentiated (A) and differentiated (B, C) mouse small intestine organoids that were cultured using Cultrex UltiMatrix RGF Basement Membrane Extract (R&D Systems, Catalog # BME001-05) and the other reagents listed in this protocol.

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