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Culture of Mouse Enteric Organoids using Cultrex® Basement Membrane Matrix.

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Introduction

Organoid cultures represent the next generation of tissue culture models. These cultures are extracted directly from living tissues similar to primary cultures; however, they are never subjected to an artificial, tissue culture-treated plastic environment. Instead, 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 mouse gastric, small intestine or colon, derived from normal, healthy mouse intestinal tissues using Cultrex Organoid Qualified Basement Membrane Matrix as a scaffold.

Protocol

This protocol provides the procedure for subculturing normal mouse enteric organoids which was derived from the submerged method as described in Yin, X., et al. (2014, Nature Methods). The "Method" section of this protocol includes a series of tips on how to prepare culturing 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.

1. Materials

Table 1. Ready-to-use materials needed for mouse small intestine organoid culture.

Reagent name	Supplier	Cat No.	Storage
Cultrex® Organoid Harvesting Solution	Trevigen	3700-100-01	4 °C
Cultrex® Organoid Qualified BME*	Trevigen	3533-005-02, 3433-005-R1	-80 °C
Advanced DMEM/F-12 Cell Culture Medium	Thermo Fisher	12634-010	4 °C
GlutaMAX-I	Thermo Fisher	35050-079	4 °C
HEPES, 1M solution	Thermo Fisher	15630-080	4 °C
Penicillin/Streptomycin	Thermo Fisher	15140-122	-20 °C
B27 supplement	Thermo Fisher	17504-044	-20 °C
N2 supplement	Thermo Fisher	17502-048	-20 °C
N-Acetylcysteine	Sigma-Aldrich	A9165	4 °C
Valproic Acid	Sigma-Aldrich	P4543-10G	4 °C
Recombinant Mouse EGF	Thermo Fisher	PMG8041	-80 °C
Recombinant Mouse Noggin	Peprotech	250-38	-80 °C
Chir 99021, (GSK-3 inhibitor)	R&D Systems	4423	-20 °C
Recombinant Human FGF10	Peprotech	100-26	-80 °C

***Trevigen recommends to use Cultrex Organoid Qualified BME Type 2 (3533-005-02) for robust organoid cultures and Cultrex Orgnaoid Qualified BME Type R1 (3433-005-R1) for difficult to grow organoid cultures.**

Table 2. Conditioned media needed for mouse small intestine organoid culture.

Name	Cell line used	Supplier of cell line	Cat No.	Storage of conditioned medium
R-Spondin1 conditioned medium 10X	Cultrex® Rspo1 cells	Trevigen	3710-100-K	-80 °C
Wnt3A conditioned medium 2X	L Wnt3A	ATCC	CRL-2647	4 °C

To produce R-Spondin1 (Rspo1) conditioned medium follow the instructions provided in the Trevigen Technical Tip called "Production of R-Spondin1 conditioned medium using Cultrex® Rspo1 cells." Concentrate the conditioned medium to produce a 10X solution, which should have an activity comparable to a reference solution of R-Spondin1 at a concentration of 10 mg/ml.

Instructions on how to obtain 2X L Wnt3A conditioned medium are available at the ATCC website.

2. Equipment

1. Cell Culture Incubator (37 °C, 5% CO2)
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 Pipets (5 ml)
8. Micropipets and Tips (2-200 µl)
9. Conical tubes, 10 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

3. Method

Note: Use aseptic technique at all times during this protocol. Reagents and cultures should only be opened within the Cell Culture Incubator to prevent contamination.

1. Prepare stock solutions for mouse enteric organoid culture:

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Table 3. Preparation of stock solutions for mouse enteric organoid culture media.

Reagent name	Solvent	Stock solution	Preparation	Storage
N-Acetylcysteine	DI water	500 mM = 81.6 mg/ml	816 mg in 10 ml	4 °C
Recombinant Mouse EGF	1% BSA/PBS	500 µg/ml	100 µg in 200 µl	-80 °C
Recombinant Mouse Noggin	1% BSA/PBS	100 µg/ml	100 µg in 1 ml	-80 °C
Valproic Acid	DI water	200 mM = 33 mg/ml	166 mg in 5 ml	-20 °C
Chir 99021 (GSK-3 inhibitor)	DMSO	20 mM = 9.3 mg/ml	10 mg in 1.08 ml	-20 °C
Y-27632 dihydrochloride (Rho Kinase Inhibitor)	PBS	10 mM = 3.2 mg/ml	1 mg in 313 µl	4 °C

a. Prepare 10X Solution M1 as indicated in Table 4:

Table 4. Preparation of 10X Solution M1.

Reagent	[Stock]	[Final]	Volume
B27 supplement	50X	10X	20 ml
GlutaMAX-I	200 mM	20 mM	10 ml
HEPES	1 M	100 mM	10 ml
Penicillin/Streptomycin	100X	10X	10 ml
N2 supplement	100X	10X	10 ml
N-Acetylcysteine	500 mM	10 mM	2 ml
Advanced DMEM/F-12 Cell Culture Medium	NA	NA	38 ml
			100 ml

Dispense 4.5 ml per tube into sterile 15 ml conical tubes, label, and store at -20 °C.

b. Prepare 10X Solution M2 as indicated in Table 5:

Table 5. Preparation of 10X Solution M2.

Reagent	[Stock]	[Final]	Volume
Recombinant Mouse Noggin	100 µg/ml	1 µg/ml	1 ml
Recombinant Mouse EGF	500 µg/ml	500 ng/ml	100 µl
Advanced DMEM/F-12 Cell Culture Medium	NA	NA	98.9 ml
			100 ml

Dispense 4.5 ml per tube into sterile 15 ml conical tubes, label, and store at -80 °C.

c. The 2X Solution M3 is 2X L Wnt-3a conditioned medium (L Wnt3A CM).

d. Prepare 10X Solution M4 as indicated in Table 6:

Table 6. Preparation of 10X Solution M4.

Reagent	[Stock]	[Final]	Volume
Valproic Acid	200 mM	20 mM	10 ml
Chir 99021 (GSK-3 inhibitor)	20 mM	25 µM	125 µl
Advanced DMEM/F-12 Cell Culture Medium	NA	NA	89.9 ml
			100 ml

Dispense 4.5 ml per tube into sterile 15 ml conical tubes, label, and store at -20 °C.

e. The 10X M5 solution is 10X R-Spondin1 conditioned medium.

f. The 500X M6 solution is FGF10 at a concentration of 100 µg/ml in 1% BSA/PBS

g. Summary of stock solutions for mouse enteric organoid culture:

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	L Wnt3A CM	4 °C	2X	X		X	N/A
M4	VC	-20 °C	10X	X	X		4.5 ml
M5	Rspo1	-80 °C	10X	X	X	X	4.5 ml
M6	FGF10	-80 °C	500X	X			9 µl

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.

2. Starting Organoids from a Cryovial

a. Thaw Cultrex Organoid Qualified BME (Cultrex BME from now on) on ice for four hours or overnight 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 10% FBS. Gently pipet up and down three times using a serological pipet to resuspend organoids. **Note: Organoids may be counted at this time if needed to determine seeding volumes.**
d. Centrifuge the vial at 500 x g for 3 minutes to pellet organoids, and aspirate medium.
e. Resuspend organoids in Cultrex BME at 10,000 organoids per ml (500 organoids per 50 µl). Pipet up and down three times using a serological pipet to disperse organoids in Cultrex BME, and dispense 50 µl of the Cultrex BME /organoid mixture in the center of each well of a 24 well plate (Image 1) or arrange domes placing 6 to 8 domes in a well of a 6-well plate (Image 2). **Note: The hydrogel containing organoids should not touch the sides of the well.**

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Placement of Cultrex Organoid Qualified BME /organoid mixture in tissue culture plates.

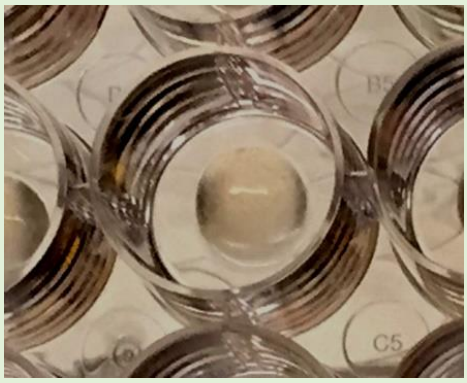

Image 1. Placement of Cultrex BME /organoid mixture in the center of the well of a 24 well plate.

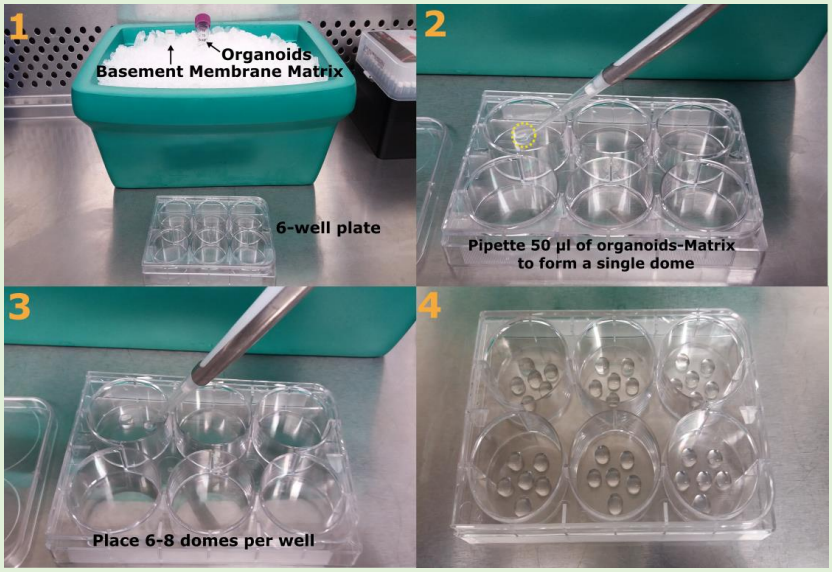

Image 2. Placement of Cultrex BME /organoid mixture in wells of a 6-well plate. 1) Cultrex BME and organoids are kept on ice, while the culture vessel is pre-warmed at 37 °C t o quickly induce polymerization of BME which prevents organoids from attaching to the bottom of the well. After harvest, the organoid pellet is resuspended in BME; 2) Domes are placed on each well by pipetting 50 µl of organoids-BME (yellow circle); 3) Six to eight domes fit in a well of a 6-well plate; 4) A 6-well plate with 6 domes in each well.

f. Incubate the plate in the cell culture incubator for 25 minutes to polymerize the Cultrex BME hydrogel.
g. Calculate the volume of Organoid Starting/Passaging Medium needed:
h. Prepare Organoid Starting/Passaging Medium by adding Y-27632 dihydrochloride (Rho Kinase Inhibitor) 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. **Note: Medium should be gently pipetted into the corner of the well away from the hydrogel to prevent disruption of the hydrogel.**
j. Return plate containing organoid cultures to the cell culture Incubator to promote organoid growth.

3. Organoid Culture Maintenance

The culture medium should be aspirated from each well and replaced with fresh Organoid Culture Medium every Monday, Wednesday, and Friday. Mouse enteric organoids can be cultured for up to two weeks before passaging (see below). **Note: Medium should be gently aspirated from and pipetted into the corner of the well away from the hydrogel to prevent disruption of the hydrogel.**

4. Passaging Organoids

    a. View organoids under the microscope. Each well should contain approximately 100 to 500 organoids for optimal growth. Organoids 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 Cultrex BME hydrogels containing 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 BME dome.
    d. Add 10 volumes of cold (4 °C) Organoid Harvesting Solution to each well to depolymerize the Cultrex BME hydrogel. Each well contained 50 µl of Cultrex BME, so 500 µl of Organoid Harvesting Solution will be needed per dome in the plate.
    e. Place the plate(s) in a 4 °C cooler with moderate shaking for one hour to depolymerize the Cultrex BME hydrogel. Note: Most of the Cultrex BME should be visibly depolymerized during this incubation; however, some small amount may remain.
    f. Pipet up and down three times with a serological pipet across the well to solubilize any remaining gel.
    g. Pass the organoid solution through a 20 gauge needle into a conical tube to fragment organoids.
    h. Centrifuge the tube at 500 x g at 4 °C for 5 minutes.
    i. Aspirate solution, but be careful not to disturb the organoid pellet.
    j. Resuspend pellet in 10 volumes of cold (4 °C) PBS.
    k. Centrifuge the tube at 500 x g at 4 °C for 5 minutes.
    l. Aspirate solution, but be careful not to disturb the organoid pellet.
    m. Repeat centrifugation and aspiration to remove all of the liquid to prevent dilution of the Cultrex BME.
    n. Resuspend segmented organoids in Cultrex BME, and dispense 50 µl of the Cultrex BME /organoid mixture to form a dome. Note: The hydrogel containing organoids should not touch the sides of the well.
    o. Incubate the plate in the Cell Culture Incubator for 25 minutes to polymerize Cultrex BME.
    p. Add 500 µl of Organoid Starting/Passaging Medium per dome. Note: Medium should be gently pipetted into the corner of the well away from the hydrogel to prevent disruption of the hydrogel.
    q. Return plate containing organoid cultures to the cell culture incubator to promote organoid growth.

5. Cryobanking Organoids

    a. View organoids under the microscope. Each well should contain approximately 100-500 organoids.
    b. Aspirate the medium without disturbing the Cultrex BME hydrogel containing 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 BME dome.
    d. Add 10-20 volumes of cold (4 °C) Organoid Harvesting Solution to each well to depolymerize the Cultrex BME hydrogel.
    e. Place the plate(s) in a 4 °C cooler with moderate shaking for one hour to depolymerize the Cultrex BME R1 hydrogel. Note: Most of the Cultrex BME should be visibly depolymerized during this incubation; however, some small amount may remain.
    f. Pipet up and down three times with a serological pipet across the well to solubilize any remaining gel.
    g. Pass the organoid solution through a 20 gauge needle into a conical tube to fragment organoids.
    h. Centrifuge the tube at 500 *x g* at 4 °C for 5 minutes.
    i. Aspirate solution, but be careful not to disturb the organoid pellet.
    j. Resuspend pellet in 10 volumes of cold (4 °C) PBS.
    k. Centrifuge the tube at 500 *x g* at 4 °C for 5 minutes.
    l. Aspirate solution, but be careful not to disturb the organoid pellet.
    m. Repeat centrifugation and aspiration to remove all of the liquid to prevent dilution of the Freezing Medium.
    n. Resuspend segmented organoids in 90% FBS, 10% DMSO, and 10 µM Y-27632, and dispense 500 µl of the organoid mixture into each labeled cryovial.

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o. Place 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.

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