MICHIGAN MEDICINE

Human Organoid Core (formerly Translational Tissue Modeling Laboratory)

PASSAGING COLONOID/ENTEROID CULTURES

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Adapted from: Dame et al; *Lab Invest*, 2014; PMC4108175

A. *Cold Mechanical Dissociation Method*

MATERIALS

- Insulated ice tray: spray with 70% EtOH-wipe and add fresh ice with each split
- Cell lifter (Corning 3008)
- BSA-coated plasticware: 0.1% BSA in PBS ((BSA: Sigma A8806; sterile-filtered) with 100 µg/mL Primocin (InvivoGen #ant-pm-2); store at 4°C for up to 6 months. Coat at room temp the below times or overnight at 4°C. Store coated plasticware for up to 30 days at 4°C, but do not let it dry.

  Note: Recommended to minimize loss of precious tissue/crypts/enteroids due to adherence to surfaces.

  Polypropylene, coat 20 minutes: 1 mL tip, 5 mL tube and 15 mL tube

  Polystyrene, coat 3 minutes: 5 mL serological pipette
- Centrifuge with 5 mL & 15 mL tube buckets pre-cooled to 4°C
- Warm packs (Fisher #0353153)
- Humidified 37°C, 5% CO_2 incubator
- 6-well culture dishes (USA Scientific CytoOne CC7682): pre-warm in incubator; Matrigel droplets must bead up; recommend comparing brands for other multi-well plates.

REAGENTS

- Colonoids: approximately 3000 colonoids per well of 6-well dish (dense)
- Human Colonoid Medium with 2.5 µM CHIR99021, 10 µM Y27632 and 100 µg/ml Primocin

  Alternate Media:
  - IntestiCult-human (StemCell Technologies 06010): alternate to HCM for normal cultures; add to 10µM-Y27632 and 100 µg/ml Primocin
  - YEP (for select neoplastic cultures), Gastroid Medium, KGMG (Lonza 00192060)
- 10 µM Y-27632 (Tocris; 125410): 2.5 mM stock in H_2O (presumed sterile). Serum-free cultures can use 5 µM.
- 2.5 µM CHIR99021 (Tocris-Fisher 4423): 10 mM stock in DMSO (4000X)

  Note: dilute 1:100 in warm AdvDMEM, then 1:40 in *Human Colonoid Medium*
- *DPBSpg*: DPBS (no Ca, Mg; Gibco 14190250) with 100 µg/mL Primocin and 50 µg/mL gentamicin (Invitrogen 15750060)

- Matrigel (BD Biosciences; #354234; 9 mg/mL or above; less than 1.5 EU/mL): example stock concentration 10.3 mg/mL
- *Matrigel Medium ("mini-tube")*: 65 µL including volume of cell pellet*
    - Human Colonoid Medium: 59 µL HCM (with already added Y27632 and CHIR99021) + 0.84 µL 2.5mM-Y27632 + 5.5 µL 100 µM-CHIR99021

      or
    - IntestiCult (with already added Y27632 and CHIR99021): same
- *Matrigel-medium-mix*: Made to 8 mg/mL Matrigel with *Matrigel Medium*

  Example: 220 µL Matrigel stock (10.3 mg/mL) + 65 µL *Matrigel Medium*, including pellet volume*
  = 285 µL plating *Matrigel-medium-mix* (8 mg/mL)

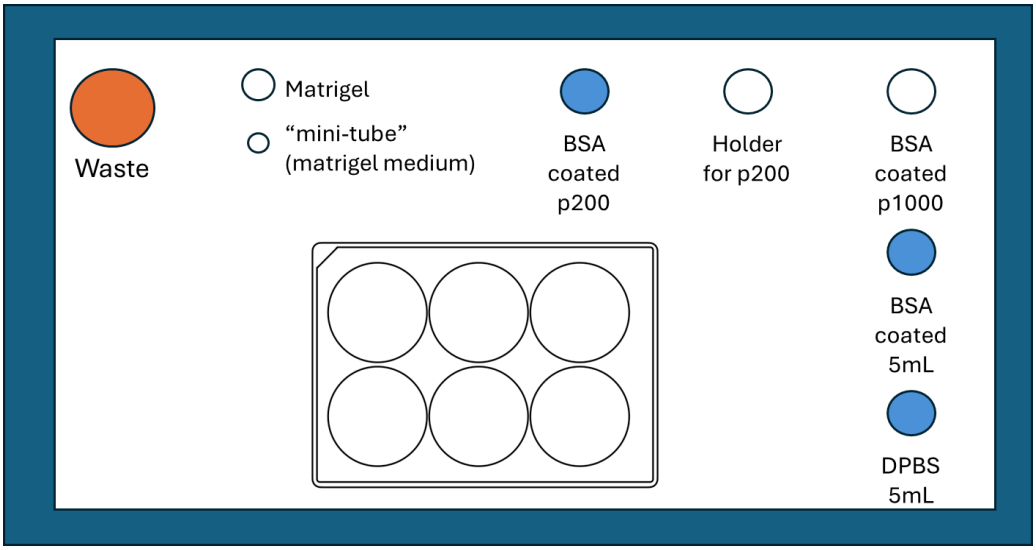
*Volumes are dependent on starting stock concentration of Matrigel in order to formulate 8 mg/mL

See Matrigel Dilution Calculator

PROCEDURE

Note: Below volumes for 1 well of 6-well plate

1) Place the cell culture medium and DPBSpg in the 37C water bath
2) Place the following tubes on ice: 50mL waste tube, BSA coated 200uL and 1000uL tips, 15mL tube to hold pipet and 200uL BSA coated tip, BSA coated 5mL tube(s), 5mL DPBS tube(s), Matrigel aliquot, Matrigel medium "mini-tube".



3) Place the plate on ice.
4) Aspirate and discard medium.
5) Empty the BSA coated 5mL tube(s)
6) Add 2 mL** cold DPBS***, lift pads with a cell lifter and add to the BSA-coated 5 mL tube on ice.

   **_1.0 mL per well_ for 2-3 wells/tube

   0.5 mL per well for 3-4 wells/tube

   1.5 mL per well for 4-6 wells/tube, using a 5 mL BSA-coated pipette into a 15 mL BSA-coated tube.

   ***Alternatively, use growth medium when the pellet is predicted to be small and loose.

7) Triturate 30X with 1 mL$^{\Delta}$ BSA-coated tip and bring up to 5 mL of cold DPBS.

   $^{\Delta}$4-6 wells of 6-well plate, triturate 15X at the bottom of the tube, bring up to 15 mL DPBS.

8) Spin at 300 x g for 3 minutes at 4°C.
9) Keeping on ice, swiftly aspirate off supernatant, finishing with a 200 µL tip to get as much of the remaining supernatant.

   Note: If culture contains many dead structures, we do a "clean-up split" and follow Step-6 by resuspending in DPBS, with a second spin at 100 x g for 3 minutes at 4°C to remove debris and detached single cells. To assist in recovery of cultures that have differentiated we supplement HCM with 10-50% IntestiCult. After the culture has recovered we usually switch it back to HCM.

10) Add *Matrigel Medium* to the pellet for a final volume including pellet of *65 µL* (volume dependent on Matrigel stock concentration)
11) Resuspend and break up enteroids in the *Matrigel Medium* by triturating 30X with a 200 µL pipet tip (avoid making air bubbles)

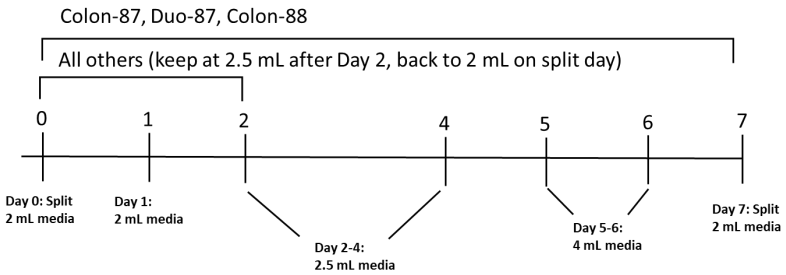
    Note: Resuspension occurs in the Matrigel medium, not the Matrigel. Once the cells are added to the Matrigel, they are VERY HARD to break up.

12) Add to Matrigel for final 8 mg/mL. For large volumes (>2mL), transfer to a cold 5 mL tube.
13) Triturate 15X in the *Matrigel-medium-mix* with a cold 200 µl tip (avoid making air bubbles).
14) Using 20 µL pipet tip, dispense 25- 10 µl *Matrigel-medium-mix* raised drops/well of a warmed 6 well plate on a warm pack. Every 15 drops, triturate 5X.
15) Place in a humidified 37°C, 5% CO_2 incubator.
16) After 10 minutes add 2 mL warm medium.
17) To empty wells, add 2.5mL warm *DPBSpg* to maintain humidity and reduce "edge effect".
18) Look at your cells under a microscope to ensure proper density and degree of trituration.

19) UV the cell culture hood after each split.

20) Full media changes every day for optimal growth (recent finding support this regime; Shin et al. iScience 2020, PMC7398973)

21) For robust cultures (e.g. Colon-87, Duo-87, Colon-88) in HCM, when day-old medium appears bright yellow in color, increase medium volume to 2.5 mL on day-3, and 4 mL on day-5. See example images of Colon-87 across 1-6 days post passaging.



B. EDTA Dissociation Method: if removing residual Matrigel is necessary

Note: As of 11/4/19 we no longer use EDTA for routine passaging. Useful for single cell preparation, and transductions, and cellular protein preps.

ADDITIONAL MATERIALS & REAGENTS

- 2 mM EDTA in DPBS (cold) diluted from 0.5M EDTA stock (Lonza 51201)
- Tube rotator at 4°C

PROCEDURE

1) Place the plate on ice.
2) Aspirate and save used medium on ice.
3) Add 2 mL cold DPBS and discard.
4) Add 2 mL cold 2 mM EDTA.
5) With a cell lifter remove Matrigel pad in 2 mM EDTA and transfer to 5 mL or 15 mL tube. Triturate 30X with 1 mL tip or 5 mL pipet. Fill tube with 2 mM EDTA.
6) Rotate at 4°C for 15 minutes.
   Note: Add 3 minutes for every additional day beyond 7 days in Matrigel culture.
7) Centrifuge at 250 x g for 3 minutes at 4°C
   Note: 100 x g to eliminate a high percentage of excess dead single cells.

8) Wash #1: resuspend in cold DPBS and triturate 10X. Spin at 250 x g for 3 minutes at 4°C.
9) Wash #2: resuspend in cold DPBS. Spin at 250 x g for 3 minutes at 4°C.
10) Wash #3: resuspend in saved growth medium collected in step 2. Spin at 250 x g for 3 minutes at 4°C.
11) Steps A7-A16 of Cold Mechanical Dissociation Method.