THE UNIVERSITY OF TEXAS
MD Anderson Cancer Center
Making Cancer History®

Establishing Enteroids from Human Biopsies

Reagents

5X ICS (500ml Incomplete chelating solution, vacuum filter):

- 500 ml MilliQ H2O
- 2.49 g Na_2HPO_4-$2H_2O$
- 2.7 g KH_2PO_4
- 14 g NaCl
- 0.3 g KCl
- 37.5 g Sucrose
- 25 g D-Sorbitol

1xCCS (50ml Complete Chelating solution) 'cold'

- 10 ml incomplete chelation solution 5x
- 40 ml sterile MilliQ H2O
- 26ul 1M DTT

Note: Need to add DTT fresh each time.
1M DTT (5g DTT dissolved in 32ml H_2O, vacuum filter)

Enteroid Medium

CMGF- (complete media without growth factor): Stored for a maximum of 4 weeks at 4^0C

- 500 ml Advanced DMEM/F12 (contains albumin, and ITS)
- 5 ml Glutamax 100x
- 5 ml HEPES 1M
- 5 ml Pen/Strep

CMGF+ (complete media with growth factor): Stored for a maximum of 2 weeks at 4^0C

- 1.5 ml CMGF-
- 5 ml Wnt 3 conditioned media (ATCC L-Wnt3a cell line Cat #CRL-2647)
- 200 ul B27 (50x)
- 100 ul N2 (100x)
- 20 ul n-acetylcysteine (500mM)
- 2 ml Rspo-1 conditioned medium (obtained cell line from Dr. Calvin Kuo, Stanford University)
- 1 ml Noggin (obtained cell line from Dr. Muncan V. Van den Brink GR)
- 10 ul EGF (1000x final conc. 50 ng/ml)
- 10 ul Gastrin (1000x final conc. 10 nMI)
- 100 ul Nicotinamide (final conc. 10 mM)
- 10 ul A83 (TGFb type I receptor inhibitor) (1000x final conc. 500 nM)
- 10 ul SB202190 (P38 inhibitor) (1000x final conc. 10uM)

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Equipment

24 well Nunclon delta surface tissue culture dish	Thermo Scientific 142475
Refrigerated centrifuge with swing rotor	
Sterilized filter pipette tips	
Corning 15ml tubes	
Sterile scissor and tweezer	
Serological disposal pipette 5ml, 10ml and 25ml	

Procedure

All steps performed on ice, in the hood
With the exception of CMGF+, all reagents should be cold
Matrigel thawed overnight night before use at 4°

1) Collect biopsies in ice cold CMGF- or PBS. Store on ice for as short a time as possible
    a. Biopsies can be collected in a 15 ml tube or any collection container with a lid.
    b. Fill entire collection container with CMGF- or PBS
    c. Ideally, you want a fresh tissue biopsy, however, you can start this protocol 4-5 hrs after biopsy was collected
    d. If absolutely unavoidable, biopsies can be left overnight at 4° in CMGF- (this is not recommended – most likely will result in a low yield of crypt cells)
2) Transfer the biopsy sample to a puddle of CMGF- (or PBS) in a 10 cm dish
3) Using sterile scissors and tweezers, cut the tissue into small pieces keeping the tissue in the liquid while you are cutting/mincing
4) Pipet entire volume of media containing minced tissue into a 15 ml tube. Add 5 ml CCS and pipet up and down 8-10 times. Try to prevent bubbles
5) Allow biopsies to settle down to the bottom of the tube, and then remove supernatant carefully using a pipet
    a. You do not need to completely remove all the supernatant. It is more important to not disturb/lose your biopsy sample

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6) Add 5 ml CCS to the biopsy pellet
7) Pipet up and down 8-10 times. Allow biopsies to settle down to the bottom and remove most of the supernatant, but leave ~1 ml. Transfer sample to new 15 ml tube and add 10 ml CCS

8) Repeat step 7: 4-5 times or until the supernatant is clear moving the samples to a new tube with every wash
9) Transfer biopsies to one well of a 6-well plate. Add 3 ml CCS and 120 µl of EDTA(0.5M Stock, final 20mM, for ileal use 180ul for a final 30mM))
10) Place the 6-well plate on a rotary (orbital) shaker at 4° (~250-300 rpm)
    a. Mouse small intestine: 30'
    b. Mouse colon: 60'
    c. Human colon: 60'
    d. Human small intestine: varies between samples, check every 10'
    e. **THESE ARE GENERAL GUIDELINES FOR EDTA INCUBATION**
    f. Look for the **absence** of crypts under a microscope; 'holes' in the tissue, floating crypts resemble dense sausages
11) Transfer biopsies + supernatant to a 15 ml tube. Add 5 ml CCS and pipet up and down 8-10 times. Add 2 ml FBS, then pipet up and down again. Allow tissue to settle (this is your biopsy sample that you no longer need, unless you are repeating the EDTA step)
12) Transfer supernatant (this should contain your crypt cells!) to a 15 ml tube. If necessary, reuse biopsies for another EDTA step (repeat steps 9-11)
    a. If repeating EDTA step, remember to keep each fraction (supernatant) on ice!
    b. If you are not repeating the EDTA step, you are now finished with the biopsy samples
13) Spin down supernatant(s): 150g (1,158rpm), 5 minutes at 4°. Faint pellet (crypt cells!) should be visible.
14) Remove supernatant and suspend the crypts in 2 ml CMGF-. When removing supernatant, be very careful so that you do not disturb the pellet. It is OK to leave a small amount of the sup (less than 1 ml) rather than risk losing your pellet. Add an additional 10 ml CMGF-. Pipet up and down 6-7 times
15) Spin down: 70g (791rpm), 5 minutes at 4°
16) Remove entire supernatant using P200 tips being careful not to disturb the pellet.
17) Add thawed Matrigel to your pellet (in the 15 ml tube). Gently pipet Matrigel up and down 8-10 times while preventing bubbles
    a. 30 µl Matrigel per well of a 24-well plate. Therefore, adjust according to how many wells you will be using (i.e. 150 µl Matrigel for 5 wells)
    b. Use **cold** P200 tips for pipetting Matrigel and keep sample on ice
    c. In general: three small biopsies will yield ~4 wells (in a 24 well plate) with approx. 10-20 crypts per well

d. Add 30 µl suspensions (crypts + Matrigel) to one well (remember 30 µl per well for as many wells as you need) of a 24-well plate.

18) Incubate the plate at 37° for 5-10'

19) Add 500 µl of **room temperature** CMGF+ per well (of a 24-well plate) and place back into 37° incubator
    a. Check for budding crypts each day (starting with day one)
    b. Add fresh **room temperature** CMGF+ every 2 days (500 µl)
    c. Enteroids should be split every 7 days (possibly earlier for human small intestine; therefore careful monitoring of cultures is necessary)