Cytotrophoblast Isolation from Term Placenta

References:

- CY Kuo, et al. Placental Basement Membrane Proteins are Required for Effective Cytotrophoblast Invasion in a 3D Bioprinted Placenta Model. Journal of Biomedical Materials Research, Part A. (2018) (Pubmed)

Day 1 – materials/reagent prep

Autoclave the following items

- Surgical tools
- Beakers (250 mL, 500mL, 1L)
- Glass petri dish
- Cell dissociation sieve

Prepare the following

1. DNase (30,000 U DNase/mL)
    a. If using DNase from Sigma (Sigma, D-5025-150KU) add 5 mL sterile 0.9% NaCl just before use
    b. If using DNase from Worthington Biochem (Fisher Scientific, NC9709009), add 6.67 mL sterile 0.9% NaCl just before use
    c. Filter sterilize and keep on ice until use
    d. Store at -20 ^{o}C for later use.
2. 1 L 0.9% NaCl
    a. 9g NaCl + 1L DI water
    b. Filter sterilized
3. 10x Hank's balanced salt solution, 1L
    a. 4 g of KCl (5.36 mM)
    b. 0.6 g KH2PO4 (4.4mM)
    c. 80g NaCl 1.37M
    d. 0.4788g Na2HPO4 (3.37mM)
    e. 10g D-glucose (55.5 mM)
    f. All in 1 L DI water
    g. Filter sterilize
4. 1x Ca/Mg-free HBSS (CMF-Hank's)
    a. 100 mL 10x HBSS from (2)
    b. 25 mL 1M HEPES (Sigma, H-0887)
    c. 875 mL DI water
    d. Adjust the pH to 7.4
    e. Filter Sterilize
5. Enzyme digestion buffer
    a. 35 mL 10x HBSS
    b. 4.65 mL 7.5% Sodium Bicarbonate
    c. 8.75 mL 1-M HEPES
    d. 266.1 DI Water
    e. Filter sterilize
    f. Distribute into three bottles containing
        i. 133.5 mL
        ii. 89 mL
        iii. 66.8 mL

g. Store at 4C

6. 2.5 % Trypsin: 10x concentration (Thermo Fisher Scientific, 15090-046)
    a. Thaw a 100-mL bottle of trypsin and distribute 33 mL over three sterile tubes. Store at -20 C and thaw just prior to use.
7. Cytotrophoblast cell culture medium
    a. IMDM + 10% heat inactivated FBS (56ºC for 30 min) + 2 mM L-Glu + 1x Pen/Strep
8. Cell freezing medium: 10% DMSO in FBS
9. Percoll gradients
    a. Mix Percoll well, as Percoll undergoes spontaneous formation of gradients.
    b. Prepare 90% Percoll stock - 117 mL Percoll (Sigma, cat. No. P-4937) + 13 mL sterile 10X HBSS
    c. In 50 mL tubes, prepare 14 dilutions of the Percoll using the 90% stock solution with sterile CMF-Hank's, referring to table below for appropriate volumes
    d. Starting with the 70% solution of Percoll, slowly layer 3 mL of each concentration into the 50 mL Falcon centrifuge tubes. Store the gradients at room temperature away from disturbance.
        i. Tip - resting the tip of a 5-mL pipet on the side of the tube just above the liquid level and gently swinging the tip side-to-side against the tube to induce layering of a broad stream of liquid

Dilution Scheme for Preparation of Percoll Gradients

90% Percoll (mL)	CMF-Hanks (mL)	Final concentration
15.6	4.4	70%
14.4	5.6	65%
13.3	6.7	60%
12.2	7.8	55%
11.1	8.9	50%
10.0	10.0	45%
8.9	11.1	40%
7.8	12.2	35%
6.7	13.3	30%
5.6	14.4	25%
4.4	15.6	20%
3.3	16.7	15%
2.2	17.8	10%
1.1	18.9	5%

    e. 100 µm cell strainer

Day 2 – trophoblast isolation

1. Thaw 33mL of 10x trypsin in water bath
2. Thaw 50mL of FBS in water bath
3. Take out DNase out and equilibrate to RT
4. Make 3 aliquots of digestion buffer according to the table below and place in the water bath

Batch	**I**	**II**	**III**
Digestion buffer	133.5 mL	89 mL	66.8 mL
10X trypsin	15 mL	10 mL	7.5 mL
DNase	1.5 mL	1 mL	0.75 mL
Total volume	*150 mL*	*100 mL*	*75.05 mL*

Just prior to each of the three digestion stages, add the appropriate volume of trypsin and DNase to prewarmed enzyme digestion buffer. The final concetrations will be 1X HBSS, 25mM HEPES, 0.25% Trypsin, and approx 300 U/mL DNase

5. Obtain a human placenta and process as soon as possible after delivery
6. Place the placenta in a tray in a sterile field within a biosafety cabinet (BSC) with the maternal side facing up.
    a. Note – all of the following steps should be done in BSC
7. Prepare histological, RNA or protein sample if desired
8. Using a sharp, fine-point scissors and blunt forceps, dissect one cotyledon at a time
9. Remove the overlaying basal plate tissue, about 3mm from the surface
    b. Avoid the chorionic plate
10. Collect 40-50g of villous tissue into the preweighed 250mL beaker
    c. Do not collect more than 50g of tissue, which would result in poor yield
11. Rinse the tissue several times with 0.9% NaCl by swiring with forceps, using 1L beaker for liquid waste
12. Transfer all of the tissue to a 150mm Petri dish and minc finely with scissors
13. Transfer half of the tissue to the cell dissociation sieve and rinse with 0.9% NaCl extensively until the eluate become clear
14. Transfer the minced tissue to a 500-mL sterile Erlenmeyer flask
15. Repeat with the second half of the tissue
16. The dissociation is performed in three stages. To prewarmed enzyme dilution buffer (labeled batch I), add DNase and trypsin as indicated below. Add the mixture to the Erlenmeyer flask containing the tissue, and incubate for 20 min at 37 ºC in a rotating water-bath shaker (150 rpm)
    d. During this incubation, add 5 mL FBS to 7 50mL centrifuge tubes

• **Batch I**	
• Digestion buffer	• 133.5 mL
• 10X trypsin	• 15 mL
• DNase	• 1.5 mL
• *Total volume*	• *150 mL*

17. After the batch I digestion, se the digestion flask at a tilt until tissue settles. Remove about 13.5 mL of supernatant, taking care not to collect undissociated tissue
18. Slowly layer the suspension over the 1.5mL serum in 15-mL conical centrifuge tubes.
    a. Repeat for seven additional tubes or until most of the digestion supernantant is transferred. Centrifuge the tubes at 1000g for 15min at RT
19. While batch I is in the centrifuge, to pre-warmed enzyme dilution buffer (labeled batch II), add DNase and trypsin as indicated below. Add the mixture to the Erlenmeyer flask containing the tissue, and incubate for 20 min at 37 ºC in a rotating water-bath shaker (150 rpm)

• **Batch II**	
• Digestion buffer	• 89 mL
• 10X trypsin	• 10 mL
• DNase	• 1 mL
• *Total volume*	• *100 mL*

20. Slowly layer the suspension over the 1.5mL serum in 15-mL conical centrifuge tubes.

a. Repeat for additional tubes until most of the digestion supernantant is transferred. Centrifuge the tubes at 1000g for 15min at RT

21. While batch II is in the centrifuge, to prewarmed enzyme dilution buffer (labeled batch III), add DNase and trypsin as indicated below. Add the mixture to the Erlenmeyer flask containing the tissue, and incubate for 20 min at 37 ºC in a rotating water-bath shaker (150 rpm)

Batch III	
Digestion buffer	66.8 mL
10X trypsin	7.5 mL
DNase	0.75 mL
Total volume	*75.05 mL*

22. For each batch, following centrifugation, aspirate the supernantant without disburbing the pellot.
    a. The trophoblast cells are predominately in the white portion of the pellet, overlaying the red blood cells.
23. Resuspend the cell pellet in each tube in 1 mL culture medium, and combine the resuspended pellets for each digestion group
24. Hold the cells at RT
25. Filter the suspension using a 100 πm nylon cell strainer inserted in the top of a sterile 50mL conical centrifuge tube
    b. If the filtration of the cell suspension slows, lift upward on the filter to draw a vaccum within the tube
26. Centrifuge at 1000g for 10 min, and resuspend in 6 mL CMF-Hank's.
27. The total volume of the resuspended cells should be approx. 8 mL
28. Carefully layer half of the cells onto each of two preformed Percoll gradients
29. Centrifuge the gradients at 1200g for 20min at room temperature in a swinging bucket rotor without a brake
30. Aspirate the upper diffuse "band" down to 30mL
31. Use a Pasteur pipet fitted with a bulb to manually collect the cells that fractionate near center of the tube into each of the two sterile 50mL tubes (collect the fraction between 30 to 12mL)
32. Dilute the cells fractions four fold with culture medium and centrifuge at 1000g for 5min
33. R-esuspend cells in each tube in 10mL culture medium, combine, and determine yield by counting.
    c. Typical yields are between 1.5 - 3 x 10^8 cells per approx. 40g tissue
34. If not proceeding immediately with immunopurification or cell culture, centrifuge remaining cells at 250g for 10 min and resuspend in freezing medium at 1 x 10^7
35. Aliquot 1mL cells per cryovial, place vials in a room temperature cell freezing unit and store at -80 ºC overnight. Transfer cryovials to a liquid nitrogen freezer the next day.

Day 3 – MACS purification

Make Cell separation buffer (CSB, 30mL)

1. Make Cell Separation Buffer
    a. PBS without Ca^{2+} or Mg^{2+} (Sigma D-8537)
    b. 0.15g of bovine serum albumin (BSA, Sigma A-9056, final concentration 0.5 %)
    c. 0.018g of EDTA (Invtrogen 15575-038, final concertation 2mM).
    d. Sterile filter
2. Thaw frozen stock of villous mononuclear cells quickly in 37 ºC water bath

3. Transfer cells into a 50mL tubes and resuspend slowly in about 20 mL cool media per 5 x 10^7 cells
4. If cell clumping is evident, treat the cells with DNase
    a. Centrifuge at 200g for 10 min at 4 ºC
    b. Resuspend in 0 mL of cool DPBS containing 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5 % BSA, and 100 U/mL DNase.
    c. Incubate for 45 min at room temperature or 15 min at 37 ºC in a shaking water bath
    d. Centrifuge at 200g for 10 min at 4ºC
    e. Carefully remove the supernatant
    f. Resuspend in CSB and check for clumping
    g. Repeat until no clumping is observed
    h. Count the cells
5. Transfer cells to sterile 15mL tubes (no more than 5x10^7 cells / 15mL tube)
6. Label the cells with MACS anti-human CD45 by adding 100 µL (per 5x10^7 cells) to the 400 µL cells and incubate 15 min in the refrigerator
7. Wash the cells once by adding 3 mL CSB and centrifugate at 300g for 10 min
8. Resuspend cells in 0.5 mL CSB per < 5x10^7 cells
9. Keep on ice
10. Prepare the MACS separation system
    a. Attach the MiniMACS magnet(s) to the MACS multiStand and place an MS+ separation column in the MiniMACS magnet
    b. To the column, attach a 23-27 gauge needle to act as a flow resistor
    c. Authors used 25 gauges x five-eighths-inch needles
    d. Prepare column by applying 500 µL ice-cold CSB on top of the column
    e. Let the buffer flow through and discard the effluent
    f. Prewet the pre-separation filter by vigorously pipetting 0.5 mL ice-cold CSB onto the membrane
    g. Place the filter atop the separation column.
    h. Place a clean 15mL centrifuge tube under the column to collect trophoblast cells
11. Separate the immunomagnetic bead-labeled cells in the prepared MiniMACS column by applying 500 µL cell suspension to the pre-separation filter
12. Allow the suspension to run through the pre-filter and column, collecting effluent in a sterile tube.
    a. If columns run slowly:
        i. A micropipette fitted with a yellow tip can be used to disrupt any cells that may have settled on top of the column
        ii. Without introducing bubbles into the cell suspension over the column, pipet up and down to dislodge settles cells
        iii. Use the plunger supplied with the column to introduce light pressure
        iv. Place the plunger over the head of the column and gently press down
13. Rinse the tube that contained the cells with 500 µL ice-cold CSB and apply to column.
14. Wash the column 3 times with 500 µL ice-cold CSB, each time collecting in same effluent tube.
15. Centrifuge the cells at 400g and re-suspend in cytotrophoblast culture medium
    a. Typical yield is 60-70% of the starting population
16. Analyze purity of cells by FACS
    a. Positive for Cytokeratin-7 and negative for CD-14, β-hCG and CD9