Preparation of Single Cell Suspensions of the Intra-Epithelial Layer and Lamina Propria from Human Intestinal Tissue

Steven B. Wells[1], Pranay Dogra[2], Josh Gray[2], Peter A. Szabo[2], Daniel Caron[2], Yoon Lee[2], Rory Morrison-Colvin[2]

[1]Department of Systems Biology, Columbia University Irving Medical Center, New York, NY 10032, USA
[2]Department of Microbiology and Immunology, Columbia University Irving Medical Center, New York, NY 10032, USA

Keywords

gut, intestine, jejunum, ileum, colon, gi, CD45, lymphocytes, myeloid, isolation, density gradient, ficoll, immune, 10x, scRNAseq, flow cytometry, leukocyte, single cell suspension, T cell, epithelium, lamina propria

1. Abstract

This protocol describes a method for the isolation of the immune cells, structural and epithelial cells, and progenitors from the epithelial layer and the lamina propria of human gut sections of about one gram of tissue. By providing defined media formulations, volumes at each step, and a defined dilution factor for density centrifugation, it yields consistent single-cell suspensions across samples. This protocol can be used for any section of the intestinal tract from duodenum to distal colon.

2. Materials

10mL Syringe (Fisher Scientific, Cat. No.: 14-955-459)
Benzonase Nuclease (Millipore Sigma, Cat. No.: E1014-5KU)
Dulbecco's Phosphate Buffered Saline (DPBS) (Fisher Scientific, Cat. No.: 14-190-144)
Penicillin-Streptomycin-Glutamine (100X) (Fisher Scientific, Cat. No.: 10-378-016)
50mL Centrifuge Tube (Fisher Scientific, Cat. No.: 12-565-271)
250mL Conical Centrifuge Tube (Fisher Scientific, Cat. No.: 12-566-441)
DTT (Cell Signaling Technology, Cat. No.: #7016)
Iscove's Modified Dulbecco's Medium (IMDM) (Fisher Scientific, Cat. No.: 12-440-053)
Fetal Bovine Serum (FBS) (Fisher Scientific, Cat. No.: 10-099-14)
EDTA 0.5M pH 8.0 (Fisher Scientific, Cat. No.: 15-575-020)
100µM cell strainer (Fisher Scientific, Cat. No.: 50-146-1428)
Ficoll-Paque™ PLUS Media (Fisher Scientific, Cat. No.: 45-001-749)

Collagenase D (2.5g) (Sigma, Cat. No.: 11088882001)

DNase (100mg) (Fisher Scientific, Cat. No.: NC9709009)

Mr. Frosty (Fisher Scientific, Cat. No.: 51000001)

Cryostor CS10 (Fisher Scientific, Cat. No.: NC9930384)

Cryogenic Vials (Fisher Scientific, Cat. No.: 09-761-71)

5mL Falcon™ Round-Bottom Polypropylene Tubes (Fisher Scientific, Cat. No.: 14-959-11A)

Solution 13 AO/DAPI (Chemometec, Cat. No.: 910-3013)

NC-Slide A8 (Chemometec, Cat. No.: 942-0003)

3. Equipment

Multi-Axle-Rotating Mixer

Centrifuge

Cell Counter - NC-3000

Surgical scissors

Scale

4. Protocol

4.1. Preparing Mediums and Buffers

4.1.1. Create the following **IMDM-FBS-PSQ Media** in a 500mL bottle of IMDM by using the table below:

Component	*Volume (mL)*	*Starting Conc.*	*Final Conc.*
IMDM	500		-
Penicillin-Streptomycin-Glutamine	5	100X	1X
FBS	50	100%	10%

Table 1.

4.1.2. Create the following **DPBS-FBS Solution** in a bottle of DPBS by using the table below:

Component	*Volume (mL)*	*Starting Conc.*	*Final Conc.*
DPBS	500	-	-
FBS	25	100%	5%

Table 2.

4.1.3. Create the following **IMDM-FBS-PSQ-EDTA-DTT Media** in a 500mL bottle of IMDM by using the table below:

Component	*Volume (mL)*	*Starting Conc.*	*Final Conc.*
IMDM	500	-	-
FBS	50	100%	10%

Penicillin-Streptomycin-Glutamine	5	100X	1X
EDTA	10	0.5M	10mM
DTT	1	1M	2mM

Table 2.

4.1.4. Create the following **DPBS-FBS-EDTA Solution** in a bottle of DPBS by using the table below:

Component	*Volume (mL)*	*Starting Conc.*	*Final Conc.*
DPBS	500	-	-
FBS	25	100%	5%
EDTA	1	0.5M	1mM

Table 2.

4.2. **Tissue Preparation**

4.2.1. Use a surgical scissors to remove about 7-8cm of intestinal tissue section from the mysentary. Remove any remaining mysentary from the intestinal tissue.

4.2.2. Gently massage the chyme or fecal matter out of the tissue over a bucket.

4.2.3. Cut open the tissue on a tray containing cold DPBS-FBS Solution and add the tissue to a 250mL conical with 100mL of cold DPBS-FBS Solution and using a forceps gently agitate the tissue to remove yellow/brown chyme, fecal matter and/or mucus.

4.2.4. Discard the DPBS-FBS solution into a bucket, replace with 100mL of cold DPBS-FBS Solution and continue to wash the tissue until the DPBS-FBS Solution is no longer brown – when it has been successfully cleaned the DPBS-FBS Solution it should appear cloudy yellow/white. Depending upon how clean the tissue is this may take numerous washes (anywhere between 3-10, perhaps more).

4.3. **Tissue Dissociation – Epithelial Stripping (IE Fraction)**

4.3.1. Add 1±0.2 grams of the cleaned intestinal tissue to a 50mL centrifuge tube and record the weight below:

Total weight. _____g.

NOTE: Going beyond the 1±0.2 grams of tissue without concomitantly increasing the number of tubes reduces the efficacy of the eventual enzymatic digest and lowers yields.

4.3.2. Add 20mL of room temperature IMDM-FBS-PSQ-EDTA-DTT Media to the tissue-containing 50mL tube. Incubate on a shaker for 30 minutes at 37°C.

4.3.3. Filter the cell suspension through a 100µM filter into a 50mL conical, rinse the tissue and the filter with 20mL of DPBS-FBS Solution. Set the cell suspension aside at 4°C. Place the remaining tissue into a back into its original 50mL conical.

4.3.4. Re-add 20mL IMDM-FBS-PSQ-EDTA-DTT Media to the tissue and incubate on a shaker for 30 minutes at 37°C.

4.3.5. Filter the cell suspension through a 100µM filter into a 50mL conical, rinse the tissue and the filter with 20mL of DPBS-FBS Solution. Set the cell suspension aside at 4°C. Place the remaining tissue into a back into its original 50mL conical.

4.4. Tissue Dissociation – Lamina Propria Digestion (LP Fraction)

4.4.1. Add 5mL of room temperature IMDM **(NO ADDITIVES! Just the base media formulation)** to the tube and use a scissors to chop the tissue into a fine "mash".

4.4.2. Add 40mL of room temperature **IMDM (NO ADDITIVES)** and spike in 0.400mL of Collagenase D, and 0.400mL of DNAse to the tube to begin the enzymatic digestion. Place on a shaker for 30 minutes at 37°C.

4.4.3. After digestion, add 0.500mL of EDTA 0.5M pH 8.0 to the digested cell suspensions and incubate for 2 minutes at 20°C.

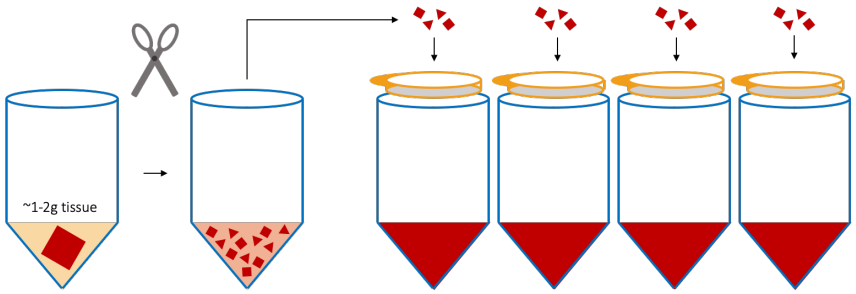

Figure 1. Steps 4.4.4 through 4.4.5.

4.4.4. Distribute and filter the mash of tissue over 100µM cell strainers above 50mL tubes (about 4 filters/gram of tissue).

NOTE: Cell yields and ease of pushing through the filter are increased by using multiple filters/gram of tissue, default to using more filters to decrease processing time, and increase yields.

4.4.5. Apply pressure with the black rubber bottom or the plastic end of a 10mL syringe plunger to any remaining, partially digested tissue on the cell strainers, and intermittently wash through with DPBS-FBS-EDTA Solution from a transfer pipet. When finished, combine the tubes of cell suspension and proceed to the next section.

4.5. Ficoll-Paque

4.5.1. Centrifuge the cell suspensions (EL and LP fractions) for 10 minutes at 400 × g at 20°C.

4.5.2. Remove the EL and LP supernatants and combine the cell pellets down to a single 50mL, 1g/tube, keep the fractions distinct, add 10mL with room temperature IMDM (NO ADDITIVES).

4.5.3. Add 10µL of benzonase/1 gram of tissue to the EL and LP fractions and incubate at 37°C for 30 minutes.

4.5.4. Add 15mL of IMDM (NO ADDITIVES) to the cell suspension, spike in 0.250mL of EDTA 0.5M pH 8.0 to all tubes.

4.5.5. Filter both the EL and LP cell suspensions through a 100µM cell strainer.

4.5.6. Layer 25mL of cell suspension (both IE and LP fractions) on top of 15mL of Ficoll-Paque Media PLUS.

4.5.7. Spin for 20 minutes, 1200 x g at 20°C with 4 acceleration and 0 brake, evenly distribute the tubes across the entire rotor to prevent wobbling (use all four buckets if possible as opposed to just two).

4.5.8. For both fractions, remove the mononuclear cell layer with a transfer pipet and transfer to a separate 50mL tubes. Add cold DPBS-FBS-EDTA Solution to a final volume of 50mL and centrifuge the cell suspensions for 10 minutes at 400 × g, 4°C.

4.5.9. Remove the supernatant and re-suspend the cell pellet in 50mL cold DPBS-FBS-EDTA Solution and centrifuge the cell suspension for 10 minutes at 120 × g, 4°C.

4.5.10. Remove the supernatant and re-suspend the cell pellet in cold 10mL IMDM-FBS-PSQ Media.

4.6. Cell Count

4.6.1. IE Fraction - Count cells, and viability by using the NC-3000 cell counter. Calculate total viable cells and record below:

cell number: _____cells/mL,_____% viable

final volume: _____mL

$$cell\ number\left(\frac{cells}{mL}\right) * viability(\%) * final\ volume(mL) = total\ viable\ cells$$

Total Viable Cells: _____

4.6.2. LP Fraction - Count cells, and viability by using the NC-3000 cell counter. Calculate total viable cells and record below:

cell number: _____cells/mL,_____% viable

final volume: _____mL

$$cell\ number\left(\frac{cells}{mL}\right) * viability(\%) * final\ volume(mL) = total\ viable\ cells$$

Total Viable Cells: _____

4.7. Freeze-down

4.7.1. **(Optional QC)** Aliquot 2 x 10^6 cells to a 5mL Falcon tube and place on ice for subsequent flow cytometric analysis.

4.7.2. Aliquot cells for analysis or experimentation, and then freeze down cells in up to 5 x 10^6 aliquots using Cryostor CS10 Medium, a Mr. Frosty, and a -80°C freezer (1-1.5mL aliquots, round down to the nearest 5 million cells and discard/freeze/use any left over cells). Record the number of vials frozen: _____.