TISSUE COLLECTION

COLON

Specimens will be collected for the following assays:

· **Histopathology**: samples from each region collected in histocassettes and fixed in formalin
· **RNA:** samples collected in 2 ml cryovials with 200 µL RNA later
· **LPL isolation**: Sample collected in 50 mL tube with 7.5 mL CMF/FBS buffer

SPLEEN

· **Spleenocytes isolation**: Sample collected in 15 mL tube with 10 mL 1X PBS 5% FBS

BUFFERS

- 1 L PBS for the necropsy
- CMF/FBS for colon collection
- RPMI-collagenase for LP isolation form the colon
- 1x PBS + 5% FBS for spleen collection

CELL ISOLATION

SOP 123b LPL Isolation

Isolation of Mouse/pig Stomach Lamina Propria Leucocytes (lymphocytes, macrophages, dendritic cells, neutrophils, eosinophils)

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Materials

- Magentic stir bars
- 50 ml tubes
- 100um cell strainer
- 15 ml tubes
- 10ml Pipets

Solutions and Chemicals

- 10x HBSS (Sigma)

- Fetal bovine serum (FBS)
- Acetylcysteine (Sigma)
- Hepes
- Gentamicin
- RPMI 1640 (Mediatech)
- Collagenase (Sigma, C2139)
- DNAse (Sigma, DN1)
- Percoll (GE Healthcare)
- ddH2O

Reagents

10% Acetylcysteine (200ml)

20g Acetylcysteine

200ml CMF/FBS

Adjust pH to 7-7.4 with NaOH (~8M)

Filter if needed sterile!

CMF/FBS (recipe for 1L)

10x HBSS	100ml
FBS	100ml
Hepes	25ml
2xGentamicin	2ml (only add if working sterile)
dH2O	773ml

Add Acetylcysteine (10%) at a dilution of 1:2 to CMF/FBS directly before incubation.

RPMI/FBS (recipe for 1L)

RPMI 1640	873ml
FBS	100ml
Hepes	25ml
2x Gentamicin	2ml (only add if working sterile)

Add Collagenase C6885, Sigma (16000U/ml) at a dilution of 1:53.3 to RPMI/FBS (final conc. 300U/ml Collagenase) directly before incubation

Add DNAse I DN1, Sigma (15000U/ml) at a dilution of 1:300 to RPMI/FBS (final con. 50U/ml DNAse directly before incubation

Procedure

- Cut stomach open longitudinally, Rinse stomach 2 times in a 15 ml tube of PBS. Remove remaining fat and connective tissue.
- Cut stomach in small pieces and collect in 50ml tubes containing 7.5ml CMF/10%FBS

Pre-treatment of tissue:

- Treat tissue with 5% Acetylcysteine to dissolve the gastric mucus (7.5ml of 10% Acetylcysteine to 7.5ml CMF/FBS) and incubate for 10 minutes at room temperature
- Invert tube several times every 3-4 minutes and vortex at the end of the incubation time

LPL Isolation:

- Remove supernatant from pre-treatment step
- To wash the tissue, add PBS (10ml minimum) to 50ml tube containing tissue
- Remove PBS from tube by either pipetting or careful pouring (make sure not to loose tissue!!!)
- Add 15ml RPMI/FBS+300 U/ml Collagenase+50 U/ml DNAse (Collagenase Stock 15000-16000 U/ml, DNAse Stock 12000-15000 U/ml)
- Incubate for 90min at 37^C while stirring
- Filter cell containing media into new tube using a 100um cell strainer
- Filter cell containing media another time using a 40um cell strainer
- Count LPL and note the volume, take a small aliquot (200ul) for flow if necessary!
- Centrifuge cell suspension at 1500 rpm, 10 min, 4^C
- Pour supernatant off, break pellet, resuspend cells in max. 1ml CMF/10%FBS
- take out aliquot for counting (40ul) and Flow (1 Panel, 100ul)
- Purify rest of cell suspension using a Percoll gradient (67%/44%).

PERCOLL GRADIENT:

- All solutions should be at RT
- Resuspend cell solution in 44% Percoll (100% Percoll diluted with CMF/10%FBS)
- carefully overlay on top of 5 ml 67% Percoll (100% Percoll diluted with CMF/10%FBS) in a 15 ml tube
- Centrifuge for 20 min at 2000 rpm and RT with deceleration set to 1.
- Aspirate solution until 2cm above the interphase
- Carefully collect the cells in the interphase

- dilute with the 10fold volume of 1xPBS/5% FBS
- centrifuge at 1400 rpm for 10 min at 4^C
- Remove supernatant and resuspend cells in 1 ml buffer
- Count and adjust to 2-3x10^6c/ml.

SOP#015 Spleenocytes isolation

Lymphocyte isolation from spleen

1. Place spleens in a large Petri dish with 3 ml of complete RPMI-1640 and crash them between the frosted ends of two microscope slides (sterile if cells will be cultured). FACS buffer can be used instead of the media listed above when the only downstream application is flow cytometry.
2. With a Pasteur pipet transfer cells to a 15 ml polypropylene conical tube
3. Let cells sit for 5 min. Debris will deposit at the bottom. Pipet out the supernatant (with cells) and transfer it to a new 15 ml tube.
   Spin cells at 1200 rpm for 5 min.

4. Eliminate the supernatant, brake the pellet and lyse red blood cells by adding 2 ml of RBC lysis buffer for 2 min, and then 9 ml of 1x PBS (if cell membranes become visible, eliminate them with a 1 ml pipette). Spin cells at 1200 rpm for 12 min.
5. Resuspend cells (after breaking the pellet) in 5 ml of complete RPMI and determine cellular concentration in the Coulter Counter
   - dispense 10 ml of diluent in a vial
   - pipet 40 ml of sample
   - add 3 drops of Zap-oglobin
   - dispense another 10 ml of diluent
   - count

7. FOR MOST FUNCTIONAL AND FLOW CYTOMETRIC ASSAYS: Calculate ml of cell suspension required for $2x10^6$ cells/ml, and make 2 ml of this concentration (the volume will depend on the number of functional assays included in each experiment).