PROCESSING AND STAINING OF MOUSE LUNGS

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BACKGROUND

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NOTES

- The flow panel can be run on the Fortessa flow cytometers. Compensations are crucial for data analysis – some markers stain very brightly whereas others are quite dim even after titration.

EQUIPMENT

Tissue Isolation:

- Scissors, tweezers, and other usual mouse sac instruments and material (70% ethanol, absorbent pads)
- 1.5 mL tubes
- 5 mL syringes and needles (perfusion)
- 1x DPBS (+$MgCl_2$ and $CaCl_2$) (Gibco #14080-055)
  - Dilute to 1x DPBS with sterile H_2O
  - Store at 4 °C
- RPMI (without FBS, L-glutamine or Pen/Strep)

Tissue Processing

- Heat block
- 50 ml conical tubes
- 70 μm filters (Falcon Cell Strainers - 40 μm mesh; Fisher Scientific 08-771-2)
- 1 ml syringes
- 10 ml pipettes
- Electronic pipettor
- Hemocytometer

- 1x PBS
- RPMI (without FBS, L-glutamine or Pen/Strep)
- 10x HBSS (Gibco #14065-56)
- HEPES (1 M)
- FBS
- Trypan Blue
- DNAse I stock solution 10 mg/mL (Sigma Aldrich 10104159001)
  - Prepare stock solution 10 mg/ml in 1x PBS; freeze aliquots
  - One sample requires 0.1 mg/mL
- Collagenase A (Sigma Aldrich 10103586001)

    - 1.5 mg/mL
- Sterile MilliQ H_2O
- 10 mL Digestion solution (for 1 sample 1.6 mL needed)
    - 1 mL HBSS (10x)
    - 0.1 mL HEPES (1 M)
    - 0.5 mL FBS
    - 0.1 mL DNase stock solution (10 mg/mL)
    - 15 mg Collagenase
    - MilliQ H_2O

Flow Cytometry

- U-bottom plates
- Facs Wash
    - 500 mL PBS
    - 0.5 g BSA (kept in the fridge)
    - 5 mL 0.5 M EDTA
    - dissolve and leave over night in the fridge
    - filter through 0.2 µM filter
    - store at 4 °C
- Antibodies (see table later in protocol)
- 1-Step Fix/Lyse solution (10x) (Invitrogen #00-5333-57)
    - Prepare 1x solution with sterile H_2O
    - Store at 4 °C
- CountBright Absolute counting beads (Invitrogen, sold by ThermoFisher Scientifc #C36950)
- OneComp eBeads Compensation Beads (Invitrogen, sold by ThermoFisher Scientifc #01-1111-41)

PROTOCOL

Before Tissue Collection:

- Prepare 5 mL syringes with 5mL DPBS (1x)
- Prepare 1.5 mL tubes with 1 mL RPMI and put on ice
- Take DNase and FBS out of the freezer and let it thaw
- Set heat block to 37 °C

Tissue Collection:

- Bleed and perfuse mice with 5 mL DPBS through the right ventricle
- Collect ¼ - ½ of whole lung (large right lung lobe) in 1 mL RPMI and store on ice
- prepare HBSS with 1.5 mg/mL Collagenase A and 0.1 mg/mL DNase. Store at 2–8 °C. (1.6 mL per mouse)

Tissue Processing:

- prepare digestion solution (1.6 mL per mouse)
- Transfer the lung of one mouse into a 2 mL tube containing 1.6 mL digestion solution
- Incubate the tubes at 37°C for 45 min, vortex every 15 min for 15 sec
- *Prepare cell staining*
- Apply the digestion solution and the lung tissue to a 70 µm sieve placed on a 50 mL tube
- Mash the lung tissue with the plunger of a 1 mL syringe through the sieve
- Wash the 70 µm sieve once with 10 mL ice-cold RPMI1640.
- Discard the sieve and centrifuge cell suspension at 400×g for 5 min at 4°C
- Aspirate supernatant completely by pouring

- **Optional RBC lysis** (depends on how well perfusion went)
    - Dilute 10x RBC lysis buffer in deionized water
    - Add 5 mL of 1xRBC buffer to lung cells
    - Invert the tube and incubate protected from light at RT for 10 – 15 min
    - Centrifuge 350x g for 5 min
    - aspirate supernatant without disturbing cell pellet by pouring
- Resuspend cells with 5 mL cold (1x) PBS
- centrifuge cell suspension at 400×g for 5 min at 4°C
- Aspirate supernatant completely by pipetting
- Resuspend cells with 0.5 mL cold PBS and store on ice
- **Count the live cells**
    - Mix 10 µL resuspended cells with 10 µL Trypan blue
    - Use 10 µL of this solution to count the live cells the hemocytometer
    - 16 quadrants x 2 x 10^4= cells/mL

Cell Staining*:*

2 Mio medium-size cells should be stained in this protocol.

Number	Cell marker	Fluorophore	1x Reaction	MM Samples (9x)	MM FMOs (5x)	
1	Live/Dead	Near-IR	0.1	0.90	0.50	
2	CD45	FITC	0.125	1.13	0.63	
3	Sig-F	PE	0.125	1.13	0.63	**I**
4	CD11b	PE-Cy7	0.075	0.68	0.38	
5	CD11c	PerCP-Cy5.5	0.125	1.13	0.63	
6	MHCII	AF700	0.125	1.13	0.63	
7	CD24	BV711	0.1	0.90	0.50	
8	CD64	PE-Dazzle594	0.15	1.35	0.75	**II**
9	Ly6C	BV510	0.125	1.13	0.63	
10	Ly6G	BV421	0.2	1.80	1.00	
	FacsWash		**49**	**441.00**	**each 245**	

- Perform staining in 96 well U-bottom
- Add 2 Mio cells per well (1 well each per sample and one well each for each FMO, 1 well for an unstained sample)
- Spin plate at 1800 rpm for 2 min at 4°C to pellet cells (centrifuge in bacteria room)
- Aspirate supernatants by dumping
- Resuspend cells with 50 µL Staining Mix, add Facs wash to the unstained sample
- Cover plate and incubate at room temperature for 30 min
- *Prepare compensation*
- Spin plate at 1800 rpm for 2 min at 4°C to pellet cells
- Aspirate supernatants by dumping
- Fix the cells by adding 250µl 1x 1-Step Fix/lysis buffer to wells (pipet up and down)
- Cover plate and incubate at room temperature for 10 min
- Spin plate at 1800 rpm for 2 minutes at 4°C to pellet cells
- Aspirate supernatants by dumping

- Resuspend cells in 200µl 1x PBS
- Spin plate at 1800 rpm for 2 minutes at 4°C to pellet cells
- Aspirate supernatants by dumping
- Resuspend cells in 200µl FacsWash
- Store plate wrapped in foil until running on flow cytometer
- Transfer the FMO samples onto the mesh (40 µm) of flow tubes
- Using **count beads** for absolute quantification of cells in samples:
    - vortex count beads for 15 sec
    - add 5 µL of beads per sample onto mesh of flow tubes
- add the entire sample on top of the count beads to the mesh
- spin tubes at 1500 rpm for 2 min at 4°C

Prepare compensation

- Mix 20 µL of Arc(+) beads with 0.2 µL Live/Dead stain
- Mix well by pipetting
- Incubate for 30 min at room temperature
- Add 200 µL PBS
- Spin down at 1500 rpm for 2min
- Remove the supernatant by pipetting
- Add 20 µL Arc(-) beads
- Add 200 µL PBS
- Protect from light

- Vortex "**OneCompsbeads**" for 15 sec
- Add 3 drops to 4 mL of PBS and mix by pipetting
- Prepare 1 flow tube (without lid for each antibody)
- Add 300 - 350 µL of this mix to each of these tube (have 1 to 2 reactions extra)
- Directly before running the compensation, add 0.5 µL of antibodies to the appropriate tube (each tube contains only one antibody)
- Arc(+)/Arc(-) + LiveDead is used for the Compensation of the LiveDead staining
- Ly6G should be added at the very last

RUN FLOW CYTOMETRY!!!

ANALYSIS/SOFTWARE

- FlowJo Software (TreeStar)

LINKS AND REFERENCES

- Misharin et al., Am J Respir Cell Mol Biol. 2013 Oct; 49(4): 503–510. doi: [10.1165/rcmb.2013-0086MA]
- Yu et al., PLoS One. 2016 Mar 3;11(3):e0150606. doi: 10.1371/journal.pone.0150606. eCollection 2016.