Preparation for Flow Cytometry:

**Objective**:

Harvest aorta (arch and carotids with bifurcations) from WD fed ApoE-/- mice

**Control**: 1 or 2 eYFP -/- mice pooled whole aorta on Western Diet

**Preparation:**

1. Make digestion buffer
2. 1X RBC lysis buffer
3. 1X HBSS (need a lot)
4. Make inactivation buffer
5. Make FACS buffer
   1. Everything in blue is on the shared drive in sheet labeled “Flow Digestion Buffer”
6. Set up surgery area:

**Blood and Bone Marrow**:

* 1. Needle, syringe, blood tubes
  2. Eppendorf tube for genotyping
  3. Eppendorf tube for blood counting (190mL Turk blood diluting fluid/tube)
  4. Eppendorf tube for bone marrow (1mL PBS/tube)
  5. 50mL tube for blood

**Perfuse and Harvest**:

* 1. 10mL sterile saline syringes + butterfly needle 23g
  2. 6 well plate with 2mL digestion buffer per aorta
  3. 50mL Falcon tube for aorta after digestion
  4. 70uM Falcon filters Nylon
  5. 3mL syringe 20g (yellow) needles

**Harvest:** Euthanize and weigh mouse

**Blood:**

1. Cardiac puncture with goal of 500-1000mL blood
2. Remove needle from syringe and put blood in 50mL tube
3. Place 10uL blood into Eppendorf tube with Turk 190uL on ice (to count)
4. Place ~500uL blood with 5mL of 1x RBC lysis buffer in 50mL tube on ice for 7 minutes, gently vortex every 2 min
5. Fill 50mL tube with 1x HBSS and spin for 10 min at 400g at 4°C
6. Remove supernatant and resuspend in 2mL 1X HBSS

**Bone Marrow:**

1. Cut off leg at hip joint
2. Cut away skin and muscle, pull off rest with gauze
3. Cut at knee
4. Place in Eppendorf tube with PBS

**Perfuse and Harvest Aorta:**

1. Prepare dish with PBS
2. ALWAYS START WITH YFP- MICE TO AVOID YFP+ contamination in your flow controls
3. Open thoracic cavity and perfuse with 10mL sterile saline or PBS
4. Remove organs except heart and kidneys from mouse
5. Clean aorta starting from the iliac bifurcation to the aortic arch
6. Keep top 1/3 of heart attached to aorta and dissect away heart tissue, leaving two valves
7. Place aorta in dish, clean, open aorta lengthwise like en face, mince
8. Place aorta in digestion buffer and put in 37°C incubator for 1.5 hours
9. After 1.5 hour incubation, inactivate digestion buffer with HBSS, homogenize aorta, and strain through 70uM filter
   1. Pipet 1mL HBSS through filter into 50mL Falcon tube
   2. Pipet 2mL HBSS into well with aorta
   3. Using 20g syringe, pipet up and down 10x and put through filter
   4. Repeat b-c with 3mL of HBSS 2x (grand total: 9mL HBSS/aorta)
10. Put on ice until all aortas are ready to be spun down

**Prepare Cells for Flow:** (Label tubes for staining)

1. Spin aortas in centrifuge for 5 minutes at 400g at 4°C
2. While this is spinning, fast cool the small centrifuge to 4°C for later experiments
3. Remove supernatant
4. Add 200uL 1x RBC lysis buffer, VORTEX, place on ice for 2 minutes
5. Add 2mL inactivation buffer
6. Spin aortas in centrifuge for 5 minutes at 400g at 4°C
7. Remove supernatant
8. Add FACs (200uL for sample, 100uL for pooled FMOs); resuspend; aliquot to specific eppendorf tubes
   1. This is where you figure out how much to dilute your sample.
   2. For experiments with SMC-YFP mice, you need
      1. A single stain YFP with 100uL of cells from YFP+ mouse
      2. An unstained sample with 100uL of cells from a YFP- mouse
      3. A FMO YFP with 100uL of cells from a YFP- mouse
   3. Single stains(SS) -> Need 100uL volume. Use compensation beads. 1 drop = 100uL. The exception is Live/dead stain which will need 100uL of pooled cells.
   4. EACH SAMPLE needs 200uL volume of cells from your specific sample
   5. EACH FMO needs 100uL of cells. You get this by pooling what is left over from your sample YFP+ mice and your YFP- mice and doling 100uL of that into each FMO.

Powerpoint slide for example experimental set up.

**Staining:**

**M1 vs M2 Panel**

1. Put 200uL of resuspended sample and 100uL of resuspended pooled cells in their proper tubes.
2. Stain with 1\*ab in correct dilutions and Fc block in FACS buffer for 30min at 4C.
3. Add 700uL of FACS and spin for 5min at 500g at 4C and remove supernatant
4. Stain L/D yellow for 20min at RT in dark for tubes that need it
   1. Dilution is 0.6ul L/D per 300uL PBS so make a mastermix and add 300uL per tube that needs live dead
   2. Live dead should be added to the following
      1. All samples
      2. All FMOs **EXCEPT** FMO live/dead
      3. SS Live/Dead
5. Wash by adding 700uL PBS per tube
6. Spin aortas in centrifuge for 5min at **500**g at 4°C and remove supernatant

**Conjugated Ab**

1. Add 500uL PBS for controls, 500uL PBS for samples, resuspend and VORTEX
2. Filter samples directly before adding to the machine (only tubes with cells need to be filtered through the blue caps, not the SS beads)

Counting Beads (Count Bright absolute counting beads, Invitrogen C36950)

1. Allow counting beads to come to room temperature.
2. Vortex and pipette to mix vigorously to resuspend beads in single cell
3. Add 10uL of counting beads per 500uL sample volume .
4. NOTE: Sample volume and 10uL counting bead volume must be AS PRECISE AS POSSIBLE. Counting beads working is absolutely dependent on these two things!!!

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| --- | --- | --- | --- | --- |
|  | **AORTA** |  |  |  |
|  | **M1 vs M2 panel- Cyan** | |  |  |
| **Marker (cell type)** | **Fluorophore** | **Channel** | **Dilutions** | **Clone/Company** |
| YFP (SMCs) | FITC | FL1 | 2uL/100uL | endogenous |
| ????????? | PE | FL2 |  | ??????? |
| CD45 (hematopoeitic) | PercP | FL4 | 0.5uL/100uL |  |
| CD11b (pan macrophage) | PE-Cy7 | FL5 | 1uL/100uL | Clone M1/70 Cat#101216 Biolegend |
| CD86 (M1 macrophage) | Pacific Blue | FL6 | 1uL/100uL | Cat #105022 Biolegend |
| Live/dead | Yellow | FL7 | 0.6uL/300uL |  |
| F4/80 (pan macrophage) | APC | FL8 | 0.5uL/100uL | Clone:BM8 Cat#17480180 eBioscience |
| CD11c (dendritic cells) | APC-eFluor780 | FL9 | 1uL/100uL | Clone N418 Cat#47011482 eBioscience |
| FC Block (CD16/CD32) | None | None | 1uL/100uL | Clone 93 Cat#14-016185 eBioscience |