**Blood Processing (All Patients)**

**Note: For blood draws use at least a 21G needle to minimize hemolysis. Also avoid aggressive suction during phlebotomy for this same reason.**

**PEth Card**

**Notes:**

* **DO NOT USE ETHANOL NEAR THE PEth CARDS.**
* **Use a purple top blood tube.**
* **When storing and shipping, do not use a plasticized envelope or airtight plastic specimen bag.**
1. Label a PEth card (USDTL) with the patient ID, the collection day (if applicable), and the date. Place a corresponding barcode label on a drying box.
2. Open the PEth card and lay on a new kimwipe.
3. PRIOR TO CENTRIFUGATION, load 0.5mL of whole blood from a purple top (EDTA) blood tube into a pipet tip.
4. Slowly pipet a large drop of blood at the end of the pipet tip. The drop should be big enough to fill a collection circle of the PEth card in a single attempt. Allow the drop of blood to come into contact with the center of a collection card and wick to fill the circle. DO NOT touch the card with the pipet tip or layer successive drops.
5. Repeat to fill the remaining collection circles.
6. Pipet any remaining whole blood back into the purple top tube and re-cap.
7. Close the PEth card to cover the blood spots and place the specimen in a barcode labeled drying box and close the ends.
8. Store at room temperature until shipment.

**Blood Draw for Serum, Plasma, and Buffy Coat Collection**

**Notes:**

* **Centrifuge tubes at 900X g for 10 min at room temperature.**
* **After collection, store all tubes at -80°C.**

**Green top (sodium heparin; AUDs, Controls, and Cannabis Users only)**(BD Vacutainer 4mL #367871, 6mL #367878) **-** aliquot 0.25-0.5mL aliquots of plasma off the top phase into 4 of the 1.8mL cryotubes (Nunc #375418) with green cap inserts (Nunc #355018), then collect red blood cells (RBCs) from the bottom phase and divide them into two 1.8mL cryotubes with yellow cap inserts (Nunc #355077). A 4mL green top will typically yield between 1 and 3mL of plasma.

**Red top** (BD Vacutainer 4ml #367812, 6mL #367815, 10mL #367820) **–** aliquot 0.25-0.5mL aliquots of serum off of the top phase into 8 of the 1.8mL cryotubes with red cap inserts (Nunc #354968). A 6mL red top will typically yield between 1 and 4mL of serum.

**Purple top (EDTA)**(BD Vacutainer 4mL #367861, 6mL #367863) **–** aliquot 0.25-0.5mL aliquots of plasma off the top phase into 8 of the 1.8mL cryotubes with purple cap inserts (Nunc #375922). A 4mL purple top will typically yield between 1 and 3mL of plasma. Then collect the buffy coat by pipetting the white layer of cells between the top phase and the red blood cell (RBC) bottom layer and pipetting it into a 1.8mL cryotube with a pink cap insert (Nunc #375884). Collect the RBC layer and pipette it into a 1.8mL cryotube with a grey cap insert (Nunc #375906).

**Blood draw for PBMC collection (AUDs, Controls, Cannabis Users)**

1. Centrifuge CPT tubes (BD #362761) at 1800X g for 30 min at room temperature.
2. Pipet off the top half of the supernatant. Collect the bottom half of the supernatant containing the PBMC layer and place into a 15mL conical tube. Bring the volume up to 10mL with 1XDPBS and centrifuge at 450 X g for 5 minutes.
3. Discard the liquid and resuspend the PBMC pellet in 5mL of 1XDPBS.
4. Count cells and check viability:
5. ***Manually***: Dilute 10μl of sample with 90μl trypan blue (VWR #45000-717) and applying 10μl of the dilution to a hemacytometer. Count all of the cells in the 4 quadrants then divide that number by 4. Multiply that number by 104 to calculate how many cells there are per mL. Multiply that number by the total volume (10mL) to calculate the total number of cells in the 10mL of cell suspension.
6. ***BioRad TC20 Automated Cell Counter:*** Dilute 10μl of sample with 10μl trypan blue (Bio-Rad #**1450021**), gently mix by pipetting up and down 10 times, and apply 10μl of the dilution to one side of a disposable cell counting slide (Bio-Rad #1450011). Insert the solution containing side into the cell counter for the automated cell count and viability. Print the count. Gate the cells at 6µm and 30µm. Print the count.
7. Divide up the cells according to assay need below.

**PBMCs for RNA:** Pipette 1x10 6 or 2x10 6 aliquots of cells into labelled microcentrifuge tubes. The number of aliquots will be determined by the total number of cells available. Centrifuge at 13000 X g for 2 minutes to pellet the cells. Aspirate off and discard the supernatant, and then immediately freeze and store the cell pellets at -80°C.

**PBMCs for Protein:** Pipette 1x10 6 or 2x10 6 aliquots of cells into labelled microcentrifuge tubes. The number of aliquots will be determined by the total number of cells available. Centrifuge at 13000 X g for 2 minutes to pellet the cells. Aspirate off and discard the supernatant, and then immediately freeze and store the cell pellets at -80°C.

**Freezing PBMCs for Flow Cytometry:**

1. After cells have been counted, make an aliquot of 2x106 - 4x106.
2. Pellet cells at 400 X g for 5 minutes and remove supernatant.
3. Resuspend in freezing media at a final concentration of 10 x 106 cells/mL (Freezing Media: 10% DMSO - Sigma #D2650 + 20% RPMI - Cellgro #10-040-CV + 70% FBS - Atlanta Biologicals #S11550H, filter sterilized -Corning #430320). For 2 x 106 cells, resuspend in 200ul of freezing media, for 4 x 106 cells, resuspend in 400ul of freezing media.
4. Place them in a freezing container (Nalgene Cryo 1°C Freezing Container or Biocision Cool Cell) and store at -80°C for 24 hours. Store the cells in a liquid nitrogen storage tank long term.

**PBMCs for Seahorse (Set up for experiment the next day):**

1. Plate 300,000 cells in 80µl of RPMI (Cellgro, #10-040-CV) plus 10% FBS (Atlanta Biologicals, #S11550H) and antibiotics (Cellgro, #MT-30-002-CI) per well in a Seahorse XFe96 Cell Culture Microplate (Agilent, #101085-004).The total number of wells will depend on the total number of cells obtained. **The corner wells must remain empty!**
2. Incubate at 37℃ and 5% CO2 overnight.
3. Hydrate the Seahorse XFe96 Extracellular Flux Assay Kit (Agilent, #102416-100). Open the Flux Assay Kit pack and carefully remove the green sensor plate and cover and place cover side down on the bench. Do not touch the sensors! Apply 200µl of Seahorse XF Calibrant (Agilent, #100840-000) to each well (for all 96 wells) of the Flux Assay utility plate. Reassemble, wrap the cartridge in parafilm, and place into the incubator overnight.

**Bronchial Brushings Processing (AUDs, Controls, Cannabis Users)**

**Cytology Brush - Buffer RLT**

1. One cytology (ConMed, #129R) brush will arrive in the lab in a labeled clear sterile 2mL microcentrifuge tube containing 0.7mL 0.7mL DNA/RNA Shield (Zymo Research R1100-50).
2. One cytology brush will arrive in the lab in a labeled clear sterile 2mL microcentrifuge tube containing 1mL 1XDPBS (Cellgro #21-031-CV).
3. Vortex the tubes and store the cytology brushes at -80°C.

**Cytology Brush – Cytospins**

1. One cytology brush will arrive in the lab in a labeled clear sterile 2mL microcentrifuge tube containing 1mL 1XDPBS (Cellgro #21-031-CV).
2. Vortex the cytology brush in 1XDPBS. Using a 1 mL pipet tip, loosen the cells left on the brush by washing the brush with the 1XDPBS in the tube. Cap the microcentrifuge tube and vortex the brush again. Discard the brush.
3. Count cells and check viability:
4. ***Manually***: Dilute 10μl of sample with 90μl trypan blue (VWR #45000-717) and applying 10μl of the dilution to a hemacytometer. Count all of the cells in the 4 quadrants then divide that number by 4. Multiply that number by 104 to calculate how many cells there are per mL. Multiply that number by the total volume (10mL) to calculate the total number of cells in the 10mL of cell suspension.
5. ***BioRad TC20 Automated Cell Counter:*** Dilute 10μl of sample with 10μl trypan blue (Bio-Rad #**1450021**), gently mix by pipetting up and down 10 times, and apply 10μl of the dilution to one side of a disposable cell counting slide (Bio-Rad #1450011). Insert the solution containing side into the cell counter for the automated cell count and viability. Print the count. Gate the cells at 6µm and 30µm. Print the count.
6. Create 4 cytospins using 200ul of the cells in 1XDPBS for each slide.
7. Fix 2 of the cytospins in 4% paraformaldehyde for 15 minutes at room temperature.
8. Fix 2 of the cytospins in methanol for 2 minutes at room temperature.
9. Store the cytospins in 1XDPBS at 4°C.

**Nasal Brushings Processing (AUDs, Controls, Cannabis Users)**

**Nasal Cytology Brush - Buffer RLT**

1. One nasal cytology brush (Medical Packaging Corporation, #CYB-1) will arrive in the lab in a labeled clear sterile 2mL microcentrifuge tube containing 0.7mL DNA/RNA Shield (Zymo Research R1100-50).
2. Store the cytology brush at -80°C.

**Nasal Cytology Brush – Cytospins**

1. One cytology brush will arrive in the lab in a labeled clear sterile 2mL microcentrifuge tube containing 1mL 1XDPBS (Cellgro #21-031-CV).
2. Vortex the cytology brush in 1XDPBS. Cut the tip off a 1mL pipet tip and cut it down to form approximately a 0.5 inch tube. Using tweezers to place and hold the 0.5 inch tube in the microcentrifuge tube and tweezers to hold the top of the cytology brush, run the brush through the tube several times to loosen the cells left on the brush. Remove the 0.5 inch tube and discard. Recap the microcentrifuge tube and vortex the brush again. Remove and discard the brush.
3. Count cells and check viability:
4. ***Manually***: Dilute 10μl of sample with 90μl trypan blue (VWR #45000-717) and applying 10μl of the dilution to a hemacytometer. Count all of the cells in the 4 quadrants then divide that number by 4. Multiply that number by 104 to calculate how many cells there are per mL. Multiply that number by the total volume (10mL) to calculate the total number of cells in the 10mL of cell suspension.
5. ***BioRad TC20 Automated Cell Counter:*** Dilute 10μl of sample with 10μl trypan blue (Bio-Rad #**1450021**), gently mix by pipetting up and down 10 times, and apply 10μl of the dilution to one side of a disposable cell counting slide (Bio-Rad #1450011). Insert the solution containing side into the cell counter for the automated cell count and viability. Print the count. Gate the cells at 6µm and 30µm. Print the count.
6. Create 4 cytospins using 200ul of the cells in 1XDPBS for each slide.
7. Fix 2 of the cytospins in 4% paraformaldehyde for 15 minutes at room temperature.
8. Fix 2 of the cytospins in methanol for 2 minutes at room temperature.
9. Store the cytospins in 1XDPBS at 4°C.

**BAL Processing (AUDs, Controls, Cannabis Users)**

**Notes:**

* **In general, 150-200mL sterile isotonic saline will be used for bronchoalveolar lavage, instilled in 50mL aliquots. We have used hand aspiration to more carefully control suction amount in the lung.**
* **The first 50mL aliquot instilled/aspirated is reflective of the airways, and is kept separate from the subsequent aliquots and should be marked ‘Upper’. The volume aspirated from this first aliquot (assuming 50mL instilled) is generally only 10-20mL.**
* **The second, third, and any subsequent aliquots reflect distal airway/alveolar sampling and should be marked ‘Lower’.**
* **Aliquot cell free BAL in a biosafety cabinet (hood).**
1. Record volume received for the Upper BAL and the Lower BAL.
2. Centrifuge at 1000 X g for 10 minutes at room temperature to pellet the cells.
3. Aliquot 100ul of Upper BAL and place into a 2mL microcentrifuge tube. Repeat with the Lower BAL. Store at -80°C until ready to measure total protein.
4. Aliquot the Upper BAL into 4mL cryotubes (Simport #T310-4A).
5. Aliquot 3.5mL of the Lower BAL into 4mL cryotubes.
6. Store Upper and Lower BAL aliquots at -80°C

**BAL Cell Processing (AUDs, Controls, Cannabis Users)**

1. In a biosafety cabinet (hood), resuspend the cells from the above aliquots (‘Upper’ and ‘Lower’) in a total of 10mL 1XDPBS. Place 2-3mL of 1XDPBS in each 50mL conical tube, gently resuspend the cells, and then combine all of the resuspended cells into one tube.
2. Count cells and check viability:
3. ***Manually***: Dilute 10μl of sample with 90μl trypan blue (VWR #45000-717) and applying 10μl of the dilution to a hemacytometer. Count all of the cells in the 4 quadrants then divide that number by 4. Multiply that number by 104 to calculate how many cells there are per mL. Multiply that number by the total volume (10mL) to calculate the total number of cells in the 10mL of cell suspension.
4. ***BioRad TC20 Automated Cell Counter:*** Dilute 10μl of sample with 10μl trypan blue (Bio-Rad #**1450021**), gently mix by pipetting up and down 10 times, and apply 10μl of the dilution to one side of a disposable cell counting slide (Bio-Rad #1450011). Insert the solution containing side into the cell counter for the automated cell count and viability. Print the count. Gate the cells at 6µm and 30µm. Print the count.
5. Divide up the cells according to assay need below.

**Cytospins:** Resuspend 240,000 macrophages in 1.6ml of 1XDPBS (Cellgro #21-031-CV). Cytospin 4 slides (Fisher Scientific #12-544-7) at 30,000 cells per slide, following the equipment’s instructions. Immediately after cytospin, fix the slides in the methanol fixing solution from the Protocol Hema 3 kit (Fisher Scientific #22-122-911) for 10 minutes, remove and store 3 slides in 1XDPBS at 4°C. The remaining slide is stained using the remaining solutions from the Protocol Hema 3 kit, dried, and stored in a slide box at room temperature for subsequent tabulation of differentials. Cytospin the remaining slides and fix them in 4% paraformaldehyde (16% paraformaldehyde diluted with DI water, Electron Microscopy Sciences #15710) for 15 minutes. Wash them briefly in EtOH and store in 1XDPBS at 4°C.

**Cells for RNA:** Pipette 1x10 6 or 2x10 6 aliquots of cells into labelled microcentrifuge tubes. The number of aliquots will be determined by the total number of cells available. Centrifuge at 13000 X g for 2 minutes to pellet the cells. Aspirate off and discard the supernatant, and then immediately freeze and store the cell pellets at -80°C.

**Cells for Protein:** Pipette 1x10 6 or 2x10 6 aliquots of cells into labelled microcentrifuge tubes. The number of aliquots will be determined by the total number of cells available. Centrifuge at 13000 X g for 2 minutes to pellet the cells. Aspirate off and discard the supernatant, and then immediately freeze and store the cell pellets at -80°C.

**Freezing AMs for Flow Cytometry:**

1. After cells have been counted, make an aliquot of 2x106 - 4x106.
2. Pellet cells at 400 X g for 5 minutes and remove supernatant.
3. Resuspend in freezing media at a final concentration of 10 x 106 cells/mL (Freezing Media: 10% DMSO - Sigma #D2650 + 20% RPMI - Cellgro #10-040-CV + 70% FBS - Atlanta Biologicals #S11550H, filter sterilized -Corning #430320). For 2 x 106 cells, resuspend in 200ul of freezing media, for 4 x 106 cells, resuspend in 400ul of freezing media.
4. Place them in a freezing container (Nalgene Cryo 1°C Freezing Container or Biocision Cool Cell) and store at -80°C for 24 hours. Store the cells in a liquid nitrogen storage tank long term.

**16 Well Culture Slide (Set up for experiment the next day):**

1. Take an aliquot of 800,000 cells and bring to a final volume of 2mL in media.
2. Pipet 200µL each well of the cell suspension to 10 wells of a 16 well culture slide (Fisher Scientific, #12565110N).
3. Place slide into a 37°C incubator with 5% CO2 and incubate for 22 hours.



**16 Well Culture Slide (Experiment Day):**

1. Create DCFH-DA (ThermoFisher Scientific, #D399), MitoTracker Red CMXRos (Cell Signaling Technology, #9082), and MitoSOX (Invitrogen Molecular Probes, #M36008) stock solutions and store at -20°C for future use. Add 13µl DMSO to 50µg containing MitoSOX vial to make a 5mM stock solution. Add 94.1µl DMSO to 50µg containing MitoTracker Red CMXRos vial to make a 1mM stock solution. Add 100uL DMSO to DCFH-DA powder to make a 1mM stock solution.
2. After incubating for 22 hours, transfer 10µl of fluorescent pHrodo-labeled S. aureus (Thermo Fisher #A10010) to 2 of the wells (see culture slide map above).
3. Incubate for another hour.
4. At the end of the 1 hour incubation, remove media from Amplex Red (Molecular Probes/Life Technologies, #A22188) reserved wells, combine, and store at -80°C in a microcentrifuge tube labeled Amplex Red Media.
5. Add 25uL of KRPG buffer (see protocol below, will be provided by the Yeligar Lab and stored at -20°C) to each of the 2 wells.
6. Incubate for 1 hour.
7. Collect the KRPG buffer from the Amplex Red reserved wells, combine in a microcentrifuge tube lebeled Amplex Red KRPG Buffer, and store at -80°C.
8. Lyse the cells with 25µl of RIPA buffer (Pierce, #89900) in each well. Pipette off the RIPA buffer, combine, and store at -80°C in a microcentrifuge tube labeled Amplex Red Protein.
9. Return to incubator for the remainder of the 2nd hour.
10. During the last 30 min of incubation, add 0.25µL of DCFH-DA stock solution into 2 labeled wells.
11. During the last 30 min of incubation, add 0.25µL of MitoTrackerCMXRos stock solution into 2 labeled wells.
12. During the last 10 min of incubation, add 0.25µL of MitoSOX stock solution into 2 labeled wells.
13. Remove all media from the remaining chamber slide wells. Do not remove the chamber.
14. Fix all well-cultured cells with 4% paraformaldehyde for 20 min at room temperature.
15. Aspirate paraformaldehyde from wells and wash cells once with PBS.
16. Aspirate the PBS wash and remove the chamber. Immediately store in a slide mailer with PBS to keep the cells hydrated during storage. Seal the slide mailer with Parafilm to keep the PBS from evaporating. Up to 5 slides can be stored in each slide mailer. Note the contents on the side of the slide mailer. Store fixed slides in the dark at RT or at 4°C.

**AMs for Seahorse (Set up for experiment the next day):**

1. Plate 80,000 cells in 40µl of RPMI (Cellgro, #10-040-CV) plus antibiotics (Cellgro, #MT-30-002-CI) per well in a Seahorse XFe96 Cell Culture Microplate (Agilent, #101085-004).The total number of wells will depend on the total number of cells obtained. **The corner wells must remain empty!**
2. For half of the wells, add an additional 40µl of RPMI with antibiotics.
3. For the other half of the wells, add 40 µl of RPMI with antibiotics and 200ng/mL LPS (Sigma #L-6529) to one well (final concentration of 100ng/mL LPS).
4. Incubate at 37℃ and 5% CO2 overnight.
5. Hydrate the Seahorse XFe96 Extracellular Flux Assay Kit (Agilent, #102416-100). Open the Flux Assay Kit pack and carefully remove the green sensor plate and cover and place cover side down on the bench. Do not touch the sensors! Apply 200µl of Seahorse XF Calibrant (Agilent, #100840-000) to each well (for all 96 wells) of the Flux Assay utility plate. Reassemble, wrap the cartridge in parafilm, and place into the incubator overnight.

**Seahorse (Day of Experiment):**

1. Prepare Assay Medium
	1. Aliquot 35mL of XF Base Medium into a 50mL conical tube.
	2. To the media, add:
		* 350μl of 100mM Sodium pyruvate (final concentration of 1mM)
		* 0.063g Glucose powder (10mM final concentration)
		* 350μl of 20mM L-Glutamine (2mM final concentration)
	3. Warm the solution to 37°C.
	4. Calibrate the pH meter and adjust the pH to 7.4+/- 0.05 at 37°C.
* pH the media while it is warmed to 37 degrees.
* The media is unbuffered, so it will not require much base to adjust the pH. Use 10-20μl at a time of 0.1M NaOH.
1. Dissolve Mitochondrial Stress Test Kit components:
	1. Remove one pouch from the freezer.
	2. Let pouch come to room temperature (about 15 min).
	3. Into the **oligomycin** tube, add **630μl** of assay media (to make a 100 μM stock solution).
	4. Into the **FCCP** tube, add **720μl** of assay media (to make a 100 μM stock solution).
	5. Into the **Rotenone/Antimycin A** tube, add **540μl** of assay media (to make a 50 μM stock solution).
	6. For each compound, pipette up and down 10 times to solubilize the compounds.
2. Prepare Working Concentrations for the Mitochondrial Stress Test:
	1. For **Oligomycin**, mix **2,520μl of Complete Media** with **480μl of 100µM Oligomycin**. This makes 16µM.
	2. For **FCCP**, mix **2,865μl of Complete Media** with **135μl of 100µM FCCP**. This makes 4.5µM.
	3. For **R/A**, mix **2,700μl of Complete Media** with **300μl of 50µM R/A**. This makes 5µM.
3. Check the Seahorse Cell Culture plate under a microscope and ensure that spheroids are healthy and are generally centered within the bottom of the wells with no gaps present. Aspirate out the media (from one row at a time) and apply 175μl of the media with additives.
4. Transfer the cell culture plate with adherent cells to the Seahorse Core’s incubator and let equilibrate for at least 30 min. This will bubble out residual CO2 from the cells/media.
5. Gently lift the green port-containing plate slightly out of the Calibrant and replace back into it. This will remove any residual air bubbles that may be trapped between the green plate and the wells of the utility plate.
6. Load ports with 25μl of each stock compound using the guide plate and then insert Flux Pak cartridge into the Seahorse machine. Load ALL PORTS (including corners and empty wells!) If a port is empty, all the air in the system will run out that well and none of the ports will get injected.
	1. Final concentration of 2 μM oligomycin (25 μl of 16 μM oligomycin stock into 175 μl well volume = 2 μM final)
	2. Final concentration of 0.5 μM FCCP (25 μl of 4.5 μM FCCP stock into 200 μl well volume = 0.5 μM final)
	3. Final concentration of 0.5 μM R/A (25 μl of 5 μM R/A stock into 225 μl well volume = 0.5 μM final)

Port A = oligomycin

Port B = FCCP

Port C = rotenone/antimycin

Note: The addition of each compound will increase the total volume by 25 μl = **250 μl total final volume**

1. Set up experiment on Seahorse workstation.
2. After the Seahorse run is complete, hold the top Flux Pak cartridge (green) into the light and look for incompletely injected ports (will have pink solution left in the port). On a 96-well template page, write down every incorrectly injected well and indicate which port was affected. Later, you can exclude these wells from the analysis.
3. Take the cell plate to the microscope and look at your hAM. If the hAM are still alive, you can collect protein from the plate for normalization.
4. Wash cells once with PBS, then add 20 μl SESSA buffer + protease inhibitor/phosphatase inhibitor. Later perform a protein assay.

To download the Seahorse software onto a computer, go to:

<http://www.seahorsebio.com/support/software/update-xfe.php>

**BAL, Mini-BAL, and BAL Cell Processing (Burn and Respiratory Failure Patients)**

**Notes:**

* **BAL or Mini-BAL will be immediately transported on ice to the lab.**
* **Aliquot BAL in a biosafety cabinet (hood).**
1. Record volume received.
2. Filter the raw sample through a BD Falcon 100 micron cell strainer (BD #352360) into a 50mL conical tube (BD Falcon #352098). The sample only needs to be filtered once, but more than 1 filter may be necessary due to the particulate matter or mucous in the sample.
3. Centrifuge at 900 X g for 10 minutes.
4. Aliquot supernatant in 4mL cryotubes (Simport #T310-4A) and store BAL at -80°C.
5. Resuspend pellet in 5mL ACK RBC lysis buffer (Gibco #A10492).
6. Incubate at room temperature for 2 minutes then add 10mL of 1XDPBS.
7. Centrifuge at 900 X g for 10 minutes.
8. If needed, repeat the RBC lysis, steps 5-7.
9. Resuspend the cells in 1-3mL of 1XDPBS, depending on size of cell pellet.
10. Count cells and check viability:
11. ***Manually***: Dilute 10μl of sample with 90μl trypan blue (VWR #45000-717) and applying 10μl of the dilution to a hemacytometer. Count all of the cells in the 4 quadrants then divide that number by 4. Multiply that number by 104 to calculate how many cells there are per mL. Multiply that number by the total volume (10mL) to calculate the total number of cells in the 10mL of cell suspension.
12. ***BioRad TC20 Automated Cell Counter:*** Dilute 10μl of sample with 10μl trypan blue (Bio-Rad #**1450021**), gently mix by pipetting up and down 10 times, and apply 10μl of the dilution to one side of a disposable cell counting slide (Bio-Rad #1450011). Insert the solution containing side into the cell counter for the automated cell count and viability. Print the count. Gate the cells at 6µm and 30µm. Print the count.
13. Create 4 cytospins using 100,000 cells per slide, following the equipment manufacturer’s instructions. Fix the slides in the methanol fixing solution from the Protocol Hema 3 kit (Fisher Scientific #22-122-911) for 10 minutes, remove and store 3 slides in 1XDPBS at 4°C. The remaining slide is stained using Protocol Hema 3 (Fisher Scientific #22-122-911), dried, and stored in a slide box at room temperature for subsequent tabulation of differentials.
14. Aliquot the remaining cells into labelled microcentrifuge tubes, preferably in 1 million or 2 million cell aliquots, then centrifuge at 13000 X g for 2 minutes to pellet the cells. The media is aspirated off and discarded, and then the cells are immediately frozen and stored at -80°C.

**Tracheal Aspirate Processing (Burn and Respiratory Failure Patients)**

**UCSF Tracheal Aspirate Protocol:**

1. Collect sample on ice.
2. Thaw freezing media (10% DMSO, 10% FBS, 80% DMEM; frozen in aliquots and stored at -20℃)
3. Take aliquot destined for supernatant fraction. If the same is thick/mucous-y, do a DTT (Sputolysin) incubation as follows, otherwise, proceed without DTT incubation.
	1. In a biosafety cabinet, add one volume of Sputolysin (Millipore Sigma #560000-10ML; frozen in aliquots and stored at -80℃) to one volume of sample, mix with a pipette 10 times, and then incubate at room temperature for 8-10 minutes.
4. Centrifuge at 400 x g for 10 minutes.
5. Remove supernatant and create 250μl aliquots in labelled snaptop microcentrifuge tubes. Store at -80°C.
6. Resuspend pellet in 1mL cold 1 x PBS.
7. Count cells and check viability:
8. ***Manually***: Dilute 10μl of sample with 90μl trypan blue (VWR #45000-717) and applying 10μl of the dilution to a hemacytometer. Count all of the cells in the 4 quadrants then divide that number by 4. Multiply that number by 104 to calculate how many cells there are per mL. Multiply that number by the total volume (10mL) to calculate the total number of cells in the 10mL of cell suspension.
9. ***BioRad TC20 Automated Cell Counter:*** Dilute 10μl of sample with 10μl trypan blue (Bio-Rad #**1450021**), gently mix by pipetting up and down 10 times, and apply 10μl of the dilution to one side of a disposable cell counting slide (Bio-Rad #1450011). Insert the solution containing side into the cell counter for the automated cell count and viability. Print the count. Gate the cells at 6µm and 30µm. Print the count.
10. Create 4 cytospins using 40,000 cells per slide, following the equipment manufacturer’s instructions. Fix one slide in the methanol fixing solution from the Protocol Hema 3 kit (Fisher Scientific #22-122-911) for 10 minutes, stain using Protocol Hema 3 (Fisher Scientific #22-122-911), dry, and store in a slide box at room temperature for subsequent tabulation of differentials. Fix the remaining 3 slides in 4% paraformaldehyde for 15 minutes, remove and store slide in 1XDPBS at 4°C.
11. Aliquot cells, preferably in 1 million or 2 million cell aliquots, into labelled microcentrifuge tubes then centrifuge at 13000 X g for 2 minutes to pellet the cells. The media is aspirated off and discarded, and then the cells are immediately frozen and stored at -80°C.
12. Centrifuge again at 400 x g for 5 min.
13. Remove supernatant and discard.
14. Re-suspend pellet at a final concentration of 5x106 cells/mL of freezing media, aliquotting 1mL of re-suspended cells per cryovial with a grey cap insert (Nunc #375906). Label the cap with subject ID.
15. Place in a controlled cooling container (Nalgene Cryo 1°C Freezing Container or Biocision Cool Cell) in a -80℃ freezer. The next day store the sample in liquid nitrogen.

**Aschner Tracheal Aspirate Cell Culture Protocol:**

1. Record total volume of tracheal aspirate.
2. Bring volume of tracheal aspirate up to 10mL in 1XDPBS.
3. Filter through sterile gauze that has been pre-wet with 1XDPBS into a 50mL conical tube.
4. Centrifuge at 1000 x g for 10 minutes.
5. Resuspend cell pellet in 2mL of RPMI (Cellgro, #10-040-CV) plus 10% FBS (Atlanta Biologicals, #S11550H), antibiotics (Cellgro, #MT-30-002-CI), 2.5 ug/mL amphotericin, 100 ug/mL gentamycin
6. Count cells and check viability:
	1. **Manually:** Dilute 10μl of sample with 90μl trypan blue (VWR #45000-717) and applying 10μl of the dilution to a hemacytometer. Count all of the cells in the 4 quadrants then divide that number by 4. Multiply that number by 104 to calculate how many cells there are per mL. Multiply that number by the total volume (10mL) to calculate the total number of cells in the 10mL of cell suspension.
	2. **BioRad TC20 Automated Cell Counter:** Dilute 10μl of sample with 10μl trypan blue (Bio-Rad #1450021), gently mix by pipetting up and down 10 times, and apply 10μl of the dilution to one side of a disposable cell counting slide (Bio-Rad #1450011). Insert the solution containing side into the cell counter for the automated cell count and viability. Print the count. Gate the cells at 6µm and 30µm. Print the count.
7. Bring volume up to 10mL in RPMI + 10% FBS + antibiotics and plate in a T25 cell culture dish.
8. Change media every 3 days until there is a confluent monolayer.

**Additional Protocols**

**Fresh PBMCs for Flow Cytometry:**

1. After cells have been counted, make an aliquot of 0.5x106 - 1x106.
2. Pellet cells at 400 X g for 8 min. Remove supernatant and resuspend cells in a total of 200ul of FACS buffer (calcium and magnesium free PBS (Fisher, #14190250) + 0.1% BSA (VWR, # 97063-624 ) + 2mM EDTA (Invitrogen, #AM9261). Regardless of how many cells you have per stain, the cells are resuspend in 100ul of FACS buffer per stain.
3. Split the resuspended cells into 2 tubes, 1 for each stain.
4. Add 1ul of each antibody in a stain to the tube (unless another volume is otherwise indicated on the antibody vial).
5. Incubate for 45min at 4℃.
6. Wash 2 times in FACS buffer, pelleting the cells at 400 X g for 8 min each time. After the second wash, resuspend the pellet in Stabilizing Fixative (BD Cat# 339860). If needed, 2% PFA can be used, but the Stabilization Fixative is better.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | FITC | PE | PERCP | PE-Cy7 | APC | APC-H7 | PacBlu | BV510 |
| **Stain 1** | CD4 | CD8 | CD19 | CD56 | CD25 | HLA-DR | CD3 | CD45 |
| **Stain 2** | CD80 | CD86 | CD206 | CD16 | CD163 | HLA-DR | CD14 | CD45 |

 \*The product info for antibodies is at the end of the protocol.

**Fresh AMs for Flow Cytometry:**

1. After cells have been counted, make an aliquot of 0.5x106 - 1x106.
2. Pellet cells at 400 X g for 8 min. Remove supernatant and resuspend cells in a total of 200ul of FACS buffer (calcium and magnesium free PBS (Fisher, #14190250) + 0.1% BSA (VWR, # 97063-624 ) + 2mM EDTA (Invitrogen, #AM9261). Regardless of how many cells you have per stain, the cells are resuspend in 100ul of FACS buffer per stain.
3. Split the resuspended cells into 2 tubes, 1 for each stain.
4. Add 1ul of each antibody in a stain to the tube (unless another volume is otherwise indicated on the antibody vial).
5. Incubate for 45min at 4℃.
6. Wash 2 times in FACS buffer, pelleting the cells at 400 X g for 8 min each time. After the second wash, resuspend the pellet in Stabilizing Fixative (BD Cat# 339860). If needed, 2% PFA can be used, but the Stabilization Fixative is better.

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| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | FITC | PE | PERCP | PE-Cy7 | APC | APC-H7 | PacBlu | BV510 |
| **Stain 1** | CD4 | CD8 | CD19 | CD56 | CD25 | HLA-DR | CD3 | CD45 |
| **Stain 2** | CD80 | CD86 | CD206 | CD16 | CD163 | HLA-DR | CD14 | CD45 |

 \*The product info for antibodies is at the end of the protocol.

**Thawing Frozen Cells for Flow Cytometry:**

1. Place tube in 37℃ water bath to thaw quickly. Once thawed, add to a 15ml conical tube along with 10 ml of RPMI (Cellgro #10-040-CV), plus 10% FBS (Atlanta Biologicals #S11550H), and antibiotics (Cellgro #MT-30-002-CI) that has been filter sterilized (Corning, #430320).
2. Wash cells 2 times in 10ml of media, pelleting the cells at 400 X g for 8 min each time.
3. After the final wash, resuspend the cells in 1 ml of media.
4. Count cells and check viability:
5. ***Manually***: Dilute 10μl of sample with 90μl trypan blue (VWR #45000-717) and applying 10μl of the dilution to a hemacytometer. Count all of the cells in the 4 quadrants then divide that number by 4. Multiply that number by 104 to calculate how many cells there are per mL. Multiply that number by the total volume (10mL) to calculate the total number of cells in the 10mL of cell suspension.
6. ***BioRad TC20 Automated Cell Counter:*** Dilute 10μl of sample with 10μl trypan blue (Bio-Rad #**1450021**), gently mix by pipetting up and down 10 times, and apply 10μl of the dilution to one side of a disposable cell counting slide (Bio-Rad #1450011). Insert the solution containing side into the cell counter for the automated cell count and viability. Print the count. Gate the cells at 6µm and 30µm. Print the count.

**Staining Cells for Flow Cytometry:**

1. After cells have been counted, make an aliquot of 0.5x106 - 1x106.
2. Pellet cells at 400 X g for 8 min. Remove supernatant and resuspend cells in a total of 200ul of FACS buffer (calcium and magnesium free PBS (Fisher, #14190250) + 0.1% BSA (VWR, # 97063-624 ) + 2mM EDTA (Invitrogen, #AM9261). Regardless of how many cells you have per stain, the cells are resuspend in 100ul of FACS buffer per stain.
3. Split the resuspended cells into 2 tubes, 1 for each stain.
4. Add 1ul of each antibody in a stain to the tube (unless another volume is otherwise indicated on the antibody vial).
5. Incubate for 45min at 4℃.
6. Wash 2 times in FACS buffer, pelleting the cells at 400 X g for 8 min each time. After the second wash, resuspend the pellet in Stabilizing Fixative (BD Cat# 339860). If needed, 2% PFA can be used, but the Stabilization Fixative is better.

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| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | FITC | PE | PERCP | PE-Cy7 | APC | APC-H7 | PacBlu | BV510 |
| **Stain 1** | CD4 | CD8 | CD19 | CD56 | CD25 | HLA-DR | CD3 | CD45 |
| **Stain 2** | CD80 | CD86 | CD206 | CD16 | CD163 | HLA-DR | CD14 | CD45 |

These panels identify/characterize T cells, monocytes, macrophages, NK, NKT and B cells.

**Stain 1:**

CD4-FITC (eBioscience; #11-0048-42)

CD8-PE (Biolegend; #344706)

CD19-PerCP-Cy5.5 (B cells; Thermo Fisher (Invitrogen); #45-0199-42)

CD56-PE-Cy7 (NK cells; Thermo Fisher (Invitrogen); #25-0567-42)

CD25-APC (eBioscience; #17-0259-42)

HLA-DR-APC-H7 (eBioscience; #47-9956-42)

CD3-PacBlu (eBioscience; #48-0037-42)

**Stain 2:**

CD80-FITC (eBioscience; #11-0809-42)

CD86-PE (eBioscience; #12-0869-42)

CD206-PerCP-Cy5.5 (BD; #550889)

CD16-PE-Cy7 (eBioscience; #25-0168-42)

CD163-APC (eBioscience; #17-1639-42)

HLA-DR-APC-H7 (eBioscience; #47-9956-42)

CD14-PacBlu (eBioscience; #48-0149-42)

CD45-BV510 (pulls out all hematopoietic cells and cleans up the data; BD Biosciences; #563204)

**Stain 2 (isotype control):**

Isotype Control-FITC

Isotype Control -PE

Isotype Control -PerCP-Cy5.5

CD16-PE-Cy7 (eBioscience; #25-0168-42)

Isotype Control -APC

HLA-DR-APC-H7 (eBioscience; #47-9956-42)

CD14-PacBlu (eBioscience; #48-0149-42)

CD45-BV510 (pulls out all hematopoietic cells and cleans up the data; BD Biosciences; #563204)

**Apoptosis slide staining protocol (TUNEL ASSAY):**

*Click-iT TUNEL Alexa Fluor Imaging Assay (Invitrogen C10245)*

Assay is performed per manufacturer protocol, beginning at step 1.4. The apoptosis slides are already fixed and stored in PBS, therefore they are already for the permeabilization at step 1.4. The optional positive control is not performed. After the permeabilization steps, the edges of the well boundaries are dried with a kim wipe and a pap pen is used to mark the boundaries around each of the wells containing the cultured cells. The pap pen marking is allowed to dry and the slides are placed in a humidified staining box. For the TdT and Click-iT reactions, a volume of 200µl of each reaction cocktail is used per slide, 50µl per well of the apoptosis slide. The wash buffer (3% BSA in PBS) is created by resuspending 4.5g BSA in 150mL 1XDPBS for each Coplin jar used for washing. The optional antibody detection is not performed. Instead of the DNA Staining (steps 6.1-6.3), Vectashield with DAPI is applied to each well of the culture slide, a coverslip is applied to the slide, and the coverslip is sealed with clear nail polish. The slides are stored at 4℃ and must be imaged within a week.

**RNA Extraction from cell pellets:** Frozen cell pellets are used for RNA extraction using the RNeasy Mini Kit (Qiagen #74104), following the standard protocol with optional QiaShredder columns (Qiagen #79654). RNA quantity and quality is determined using the NanoDrop Spectrophotometer (Thermo Scientific).

**RNA Extraction from bronchial brushings:** Frozen cell pellets are used for RNA extraction using the RNeasy Micro Kit (Qiagen #74004), following the standard protocol. RNA quantity and quality is determined using the NanoDrop Spectrophotometer (Thermo Scientific).

**DNA Extraction from bronchial brushings:**

Frozen cell pellets are used for DNA extraction using the QIAamp DNA Mini Kit (Qiagen #51304), following the standard protocol, as well as Appendix E and Appendix D instructions.

1. Centrifuge the sample for 10 minutes at 5000 x g (7500 rpm).
2. Resuspend the pellet in 600μl sorbitol buffer (Sigma #S6021).
3. Add 200 U lyticase (Sigma #L2524) and incubate in a heat block set at 30°C for 30 minutes.
4. Centrifuge for 10 minutes at 5000 x g (7500 rpm).
5. Resuspend the pellet in 180μl of 20 mg/ml lysozyme (Sigma #L7651); 20 mM Tris-HCl pH 8.0 (Invitrogen #15506-017); 2 mM EDTA (Sigma #E6758); 1.2% Triton (Fisher #327371).
6. Incubate at least 30 minutes at 37°C.
7. Add 20μl proteinase K and 200μl Buffer AL and mix by vortexing.
8. Incubate at 56°C for 30 minutes. Vortex every 10 minutes to increase yield.
9. Centrifuge for a few seconds.
10. Follow the “Protocol: DNA Purification from Tissues” from step 6 (page 34).

 DNA quantity and quality is determined using the NanoDrop Spectrophotometer (Thermo Scientific).

**Protein extraction:** Proteins from cultured and uncultured cells are extracted using the M-PER Mammalian Protein Extraction Reagent (Pierce #78503). Each mL of the M-PER is supplemented with 10μl of a 1:100 dilution of protease inhibitor (Sigma #P8340), 2μl of 100mM PMSF (Sigma #P7626), and 5μl of 100mM DTT (Sigma #D5545). Thaw and resuspend the frozen cell pellets in 20μl of supplemented M-PER per 1X106 cells. Rotate the resuspended cells end over end using a tabletop rotator for 15 minutes at room temperature. Centrifuge the samples at 13,000 X g for 15 minutes at 4°C to pellet the cell debris. Aliquot the supernatants containing the protein lysates in 20μl volumes in 0.5mL microcentrifuge tubes (Fisher Scientific #05-408-120) and stored at -80°C.

**BCA assay for protein measurements:** Total protein for the protein lysates and for the cell free BAL are measured using the Pierce BCA Protein Assay Kit (Pierce #23225).