Standard Operating Procedure for the DK U54 Cooperative Center of Excellence in Hematology (CCEH) Hypoxia Core at the Indiana University School of Medicine

NOTE: The below methodology may require some changes in the future as we learn more from our work and the work of others.

Staining for Flow Cytometry in Hypoxic Chamber

Materials:

NOTE: Our chambers are set to 3% O_2 unless otherwise directed. It is important to always include extra supplies other than just what you calculated needing. Additional material cannot be added into chamber at time of experiment as it will not have been acclimated to the lower O_2 levels. All hardware and reagents (e.g. culture media, antibodies, etc.) must be pre-equilibrated to hypoxia chamber at least 18 hours before the chamber is to be used for experiments. Be advised that very small volumes left to equilibrate overnight might result in loss of volume due to the evaporation caused by the moving gasses.

Materials

- Cell samples
- Flow cytometry tubes (filter capped preferred if primary tissue that is known to be 'clumpy')
- Eppendorf tubes
- 15 mL tubes, 50 mL or specimen jars (1X Red Blood Cell Lysis Buffer if applicable, 1X D-PBS)
- Sharpie
- Liquid waste container
- Pipette tips waste container
- Aluminum foil
- Parafilm

Reagents

- 10X Red Blood Cell Lysis Buffer if needed
- ddH_2O to dilute 10X Red Blood Cell Lysis Buffer if blood samples
- Antibodies made into antibody cocktail
- F_c block (if using, and do NOT use if staining for myeloid progenitors if FcγR is part of staining panel)
- 16% Formaldehyde
- 1X D-PBS

Procedure

Figure 1: Modified Coy Lab Products chamber.

NOTE: Prepare all reagents/master mixes/etc. in the hypoxia chamber and then transfer only the portion that will be used in ambient air out of the hypoxia chamber. When placing materials in the hypoxia chamber, unlock the airlock, place all materials in the airlock, and relock the airlock. Allow time for oxygen to be flushed from airlock chamber. If using the smaller Coy Lab Products hypoxia chamber (Fig. 1), press the "Purge Airlock" button. After the timer has completed, use the hypoxia chamber gloves to open the airlock from inside the chamber. Move the materials to the chamber and close the airlock. If using the larger BioSpherix hypoxia chamber (Fig. 2), wait ~5 minutes prior to removing the material from the airlock to give time for the system to automatically recalibrate to the set O_2 concentration. Then move the materials to the chamber as described above. Make sure that all glove sleeves are airtight when using so that there is no large leakage of air into/out of the chambers. This is crucial. Also, for safety make sure hands fit well into the gloves.

1. <u>At least 18 hours in advance, ensure all necessary materials are available within the hypoxia chamber so they can acclimate to the hypoxic environment.</u>
    a. Items that are individually wrapped for sterility should be left slightly open to allow them to fully acclimate.
    b. Materials may include pipettes, pipette tips, microcentrifuge tubes, 15 mL conical centrifuge tubes, 50 mL conical centrifuge tubes, tube racks, needles, syringes, 1X D-PBS, etc.
    c. Caps on fluids should be left loose. Vortex fluids in chamber to ensure fluids have been exposed to air with lower O_2.
2. Cells after isolation in hypoxia chamber should be split in half with half removed to air to be acclimated to ~21% O_2 for at least 1-4 hours. All the steps from here on out will have to be performed in both ambient air and hypoxia.
3. Label Eppendorf tubes for all unknown samples. (Flow staining

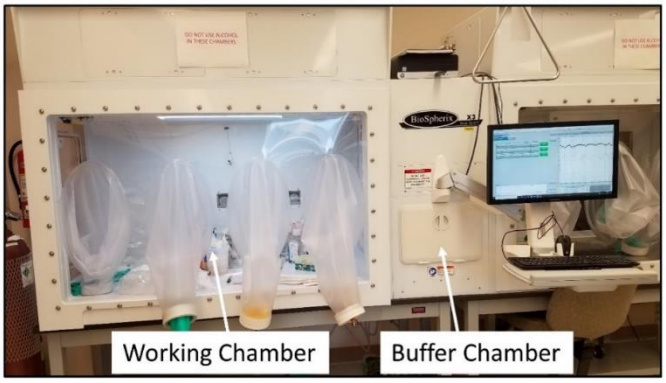

Figure 2: Modified BioSperix chamber.

for hypoxia should be done in Eppendorf tubes as provides tighter seal for minimizing exposure to ambient air.)

4. Label Eppendorf tubes for all control samples (unstained, single stained, fluorescence-minus-one stained if necessary).
5. If red blood cell lysis is not needed, skip to step 15.
6. Dilute 10X Red Blood Cell (RBC) Lysis Buffer to 1X with ddH_2O (can be left at room temperature).
    a. If this is needed, do at least 18 hours before and place into hypoxia chamber after dilution to acclimate to the lower O_2 levels.
7. Vortex sample, then add the predetermined volume of each sample to its corresponding Eppendorf tube.
8. If samples were diluted, centrifuge all samples at 1600 rpm, 5 min, 4°C, with the brake on, before performing lysis step. **NOTE:** If hypoxic samples need to be removed to air, use parafilm to wrap around lid of Eppendorf tube and minimize the time out of the chamber.
9. Gently pipet supernatant off and place into liquid waste container.
10. Briefly vortex each tube so the pellet is broken up.
11. Add 1 mL 1X RBC Lysis Buffer to each tube.
12. Incubate 5 minutes at RT.
13. Centrifuge all samples at 1600 rpm, 5 min, 4°C, with the brake on.
14. Remove supernatant and add 1 mL of PBS and centrifuge as in step 13 to wash lysis buffer out.
15. If one wishes, you can use F_c block. Prepare the block while the samples are centrifuging.
    a. Dilute the F_c block in 1X D-PBS (use the PBS in the hypoxia chamber. Once mixture is made take out what is needed for your samples in ambient air).
    b. Vortex to mix.
16. Prepare the antibody cocktail while samples are centrifuging if time allows.
    a. If making an antibody cocktail, try to make a volume that will mixed with PBS acclimated to hypoxic conditions. Then split the mixture in two and bring half out into the ambient air group. This ensures that the same antibody cocktail is used in each setting and minimizes the exposure of your cells to an antibody cocktail that is not acclimated to lower O_2.
    b. Recap the tube and vortex.
    c. Cover with aluminum foil until use (if it will be a long time, keep at 4°C until use by placing in ice).
17. After samples have been spun down (step 14), gently remove supernatant.
18. Vortex samples well to break up the pellet.
19. Vortex the F_c block again and add appropriate volume to every sample tube.
20. Add the appropriate volume of the single antibody to its corresponding single-stain tube.

21. Vortex all control tubes.
22. Add 50 µL of antibody cocktail to all unknown sample tubes.
23. Vortex all unknown sample tubes.
24. Lightly shake the tube rack and incubate in the dark at room temperature for 15 minutes.
25. Add 1.5mL 1X D-PBS to each tube and centrifuge all samples at 1600 rpm, 5 min, 4°C, with the brake on.
26. Prepare the fixative while the samples are centrifuging.
    a. Dilute 16% Formaldehyde stock to 1.5% with 1X D-PBS. Use fixative and PBS that has been acclimated to lower O_2. Remove the needed amount of fixative to ambient air for the ambient air samples.
27. Remove supernatant after the samples have finished spinning.
28. Resuspend the cells in an acceptable volume of 1.5% fixative for flow cytometric analysis, depending on the general size of the pellets and when flow cytometry will be performed.
    a. We usually will use the range of 300-500 µL of PFA for 1-3 million bone marrow cells.
29. Incubate the cells in hypoxia with fixative for at least 15 minutes. Once this is done, the samples can be removed from the hypoxia chamber.
    a. If flow cytometry will not be performed same day or next day, wash off the fixative (1.5mL 1X D-PBS to each tube and centrifuge all samples at 1600 rpm, 5 min, 4°C, with the brake on) then resuspend in 1X D-PBS.
30. Once the cells are fixed, transfer the resuspended, stained cells to a flow tube (filtered through filter cap if bone marrow samples or if samples appear clumpy).
31. Recap all tubes. Wrap tube rack in aluminum foil.
32. Store at 4°C, covered in aluminum foil, until flow cytometry.

References

Aljoufi A, Cooper S, Broxmeyer HE. Collection and Processing of Mobilized Mouse Peripheral Blood at Lowered Oxygen Tension Yields Enhanced Numbers of Hematopoietic Stem Cells. Stem Cell Rev Rep. 2020 Oct;16(5):946-953. PMCID: PMC7484397 (available on 2021-10-01)

Broxmeyer HE, Capitano ML, Cooper S, Potchanant ES, Clapp DW. Numbers of long-term hematopoietic stem cells from bone marrow of fanca and fancc knockout mice can be greatly enhanced by their collection and processing in physioxia conditions. Blood Cells Mol Dis. 2021 Feb;86:102492. PMID: 32896825; PMCID: PMC7686233.

Capitano ML, Mohamad SF, Cooper S, Guo B, Huang X, Gunawan AM, Sampson C, Ropa J, Srour EF, Orschell CM, Broxmeyer HE. Mitigating oxygen stress enhances aged mouse hematopoietic stem cell numbers and function. J Clin Invest. 2021 Jan 4;131(1):e140177. PMID: 33393491; PMCID: PMC7773345.

Mantel CR, O'Leary HA, Chitteti BR, Huang X, Cooper S, Hangoc G, Brustovetsky N, Srour EF, Lee MR, Messina-Graham S, Haas DM, Falah N, Kapur R, Pelus LM, Bardeesy N, Fitamant J, Ivan M, Kim KS, Broxmeyer HE. Enhancing Hematopoietic Stem Cell Transplantation Efficacy by Mitigating Oxygen Shock. Cell. 2015 Jun 18;161(7):1553-65. PMID: 26073944; PMCID: PMC4480616.