Protocol: Microwell Staining for Multiparameter Flow Cytometry

Notes:[1]

- All steps are performed in a clean, biological safety cabinet.
- Once the Vital Stain is introduced into the samples, the lights within the biological safety cabinet are turned off whenever the samples are present.
- All centrifugation is done with the temperature set at 4 C.

Supplies

1. 200 mcL round-bottom, 96-well plate (round bottom preferred), hard bottom for vortex
2. PBS
    1. Made as a 25X stock solution and diluted to 1X prior to use
        1. 175.33g NaCl
        2. 5.03g KCl
        3. 3.88g NaH_2PO_4
        4. 31.05g Na_2HPO_4
        5. Dissolve into 1 liter deionized water.
3. Wash Buffer
    1. PBS + 4% fetal calf serum (FCS) + 0.1% Na Azide (or alternative such as Kathon)
4. Paraformaldehyde (PFA) 1% (PFA is poisonous)
    1. Created by diluting 20% stock 1:10 into PBS
5. R10, warm
    1. Created by adding 10% FCS by volume to RPMI
6. Fluorescent labelled antibodies and Live/Dead Blue
7. BD FoxP3 Fixation Buffer
8. BD FoxP3 Permeabilization Buffer
9. 1.5 mL microcentrifuge tubes to mix antibodies
10. flow tubes with lids
11. Cotton gauze either 2x2 or 4x4 inch size (to process fresh BALF)
12. 50 mL conical tubes (to process fresh BALF)

[1] Version 2.4 (2011-06-21)

13. 15 mL conical tubes (to thaw cryopreserved cells)
14. Ammonium chloride lysing solution
    1. 10x concentrate for 1 liter
        1. Ammonium chloride 82.9 g (15 4mmol/L)
        2. Potassium bicarbonate 10.0 g (10 mmol/L)
        3. EDTA disodium salt 0.37 g (0.1 mmmol/L)
        4. Distilled or DI H2O 1 liter

Process Fresh BALF If only previously cryopreserved cells are to be used, then omit this step and proceed to the next step.

1. Filter BALF through 2x2 or 4x4 gauze to remove mucous and other large non-cellular debris such as clots.
    1. Place the gauze on top of a 50 mL conical tube, making a small dimple in the center.
    2. Pre-soak the gauze with PBS to minimize loss of the sample.
    3. Carefully pour the entire sample through the gauze filter.
    4. Rinse the gauze with PBS to a maximum volume fo 45 mL per 50 mL conical tube. This may aid in recovering cells trapped in the gauze.
2. Pellet cells with centrifugation at 1200 rpm for 8 minutes. There is usually about 40 mL of PBS in each 50 mL conical tube.
3. Remove supernatant the supernatant by your prefered method, being careful not to disturb the cell pellet.
    1. If more than one collection tube was obtained for the same sample, the cell pellets can be combined into one tube at this time.
4. Resuspend the cell pellet in 1 mL of PBS for Vital stain as below.

Thaw Cryopreserved Cells If cryopreserved cells are not to be used then this step is omitted.

1. Prepare sufficient warm R10 and sufficient number of 15 mL conical tubes. You will need one tube per cryopreserved sample.
2. Add 2 mL warm R10 to each 15 mL conical tube.
3. Carefully thaw cryopreserved cells in a warm water bath until the solution is the consistency of slush.
4. Wash the outside of each cryotube quickly with 70% ethanol.
5. Place thawed cells (slush) into the 15 mL tube.
6. Sit at room temperature for 5 minutes.
7. Slowly add 8 mL warm R10 to each 15 mL tube. Ensure that the cells mix thoroughly as you add the R10 dropwise to avoid clumping the cells.

8. Centrifuge at 1200 rpm x 2 minutes to pellet the cells.

Vital Stain This is performed for all samples, fresh or frozen.

1. Resuspend cell pellet in 1 mL PBS or serum free tissue culture media (e.g., RPMI). The presence of serum protein interferes with vital stain labeling. Labeling is typically more intense in PBS than in serum free media.
2. Add 1 mcL Live/Dead Fixable Dead Cell Stain Kit Blue (Invitrogen). This is previously reconsituted per the manufacturers directions with 50 mcL of DMSO. The reconstituted stock is kept in the dark at 4 C.
3. Vortex gently.
4. *Incubate at 37 C x 20 minutes* Longer incubation is of no benefit.
5. Add 9 mL warm R10 to quinch the reaction.
6. Centrifuge at 1200 rpm x 2 minutes to pellet cells.
7. Resuspend each cell pellet in 180 mL R10 for transfer to 96-well plate for staining.

Surface Staining This step can be completed in about 60 minutes depending upon the number of samples. Make sure that the biosafety cabinet hood lights are off.

1. Get each sample into wells on the 96 well plate. Each well will become a flow tube.
    1. Place 180 mcL of sample into each well, i.e., the entire sample.
    2. Centrifuge plate at 1200 rpm x 2 minutes.
    3. flip drip dry
    4. Wipe off the top of the plate.
    5. Agitate the plate via a quick vortex.
2. Stain the cells.
    1. Add the ster x of surface mAb sufficient quantity to each well.
        1. If the total staining volume per well is not >=24 mcL, add sufficient Wash Buffer to make each well's total volume >=24 mcL.
    2. x each well with 10 mcL pipet, up and down 5–10 times.
    3. *Incubate at 4 C for 30 minutes* in a cold, dark refrigerator.
3. Wash excess antibody off of the cells.
    1. Add 180 mcL of washing solution to each well.
    2. Centrifuge the plate at 1200 rpm x 2 minutes.
    3. flip drip dry
    4. Wipe off the top of the plate.
    5. Agitate the plate via quick vortex.

Intracellular Staining This step generally takes over two hours to complete depending upon the number of samples. Again, the light within the biosafety cabinet should be turned off when working with the cells.

1. Fix cells
    1. Add 180 mcL Fixation Buffer to each well.
    2. x well with the pipet time. The cells will likely clump together.
    3. *Incubate 60 minutes at 4 C*
2. Wash off Fixation Buffer.
    1. Centrifuge the plate at 1200 rpm x 2 minutes.
    2. flip drip dry
    3. Wipe off the top of the plate.
    4. Agitate the plate via quick vortex.
3. Wash cells in Permeabilization Buffer.
    1. Add 180 mcL Permeabilization Buffer to each well.
    2. Centrifuge the plate at 1200 rpm x 2 minutes.
    3. flip drip dry
    4. Wipe off the top of the plate.
    5. Agitate the plate via quick vortex.
4. Add the fluorescent labelled antibodies.
    1. Add Permeabilization Buffer to each well such that total staining volume per well equals 12 mcL.
    2. Add intracellular stains for the day. The stains used will vary depending upon the experiment. Examples and volumes are given below.
        a. 4 mcL FoxP3-V450
        b. 2 mcL Ki67-FITC
        c. 2 mcL each IL-17 A & F Alexa488
        d. 2 mcL PE-ψ 5 IFN-γ
    3. *Incubate 30 minutes at 4 C*
5. Wash off excess antibody in Permeabilization Buffer.
    1. Add 180 mcL Permeabilization Buffer to each well.
    2. Centrifuge the plate at 1200 rpm x 2 minutes.
    3. flip drip dry
    4. Wipe off the top of the plate.
    5. Agitate the plate via quick vortex.
6. Fix cells with paraformaldehyde.
    1. Add 150 mcL 1% PFA to each well.

2. Fill each flow tube with 150 mcL 1% PFA.
3. Add the contents of each well to one tube.
4. Vortex the tubes.
5. Cover the flow tube with a cap and keep in the dark at 4 C until ready acquire on a cytometer.

Acquisition and Analysis

1. Acquisition is performed using a LSR II (BD Biosciences) cytometer with the following configurations:
    1. Configuration New (used with Panel 2 and its variations)
        a. Blue laser (488nm) with the following filters 635LP (695/40), 505LP (530/30), 488/10;
        b. Red laser (640nm) with 755LP (780/60), 685LP (710/50), 670/30;
        c. Violet laser (405nm) with 750LP (780/60), 685LP (710/50), 635LP (660/40), 595LP (605/40), 505LP (525/50), 450/50;
        d. UV light (355nm) with 505LP (525/50) and 450/50;
        e. Yellow/Green (561nm) with 750LP (780/60), 630LP (670/30), 600LP (670/30), 582/15.
2. Compensation is performed using singly-stained tubes of each conjugated fluorochrome and BD CompBeads.
    1. Forward (FSC) and side scatter (SSC) is voltage set according to the characteristics of the cells to be characterized. For our BALF lymphocytes we have determined that a voltage such that the FSC is linear and the SSC is logicle transformed provides the best lymphocyte visualization. This voltage can be determined with unstained sample of BALF and optimizing the placement of the lymphocytes in the FSC and SSC. Alternatively, unstained peripheral blood mononuclear cells can be used as a first estimation of the FSC and SSC voltages prior to optimizing with a BALF sample as lymphocytes within the BALF samples can sometimes be very difficult to visualize depending upon the quality of the sample.
    2. Determine the ideal voltage for each PMT based on fluorescence of each fluorochrome in the set-up as per the flow cytometer's software requirements. We place the positive and negative beads in the same tube and collect them together.
    3. Once the PMT voltages are set, then compensation is estimated by the flow cytometer's software. Some of the PMT voltage settings may need adjustment based upon the results of the compensation calculations. Remember that small corrections in the compensation can be made in the analysis software.
    4. After PMT voltages are optimized and the compenstation matrix has been calculated by the flow cytometer's software, we acquire 10,000 events of Sphero

Rainbow Fluorescent Particles beads to generate median fluorescent voltages in each channel *without* compensation applied. This is saved as a FCS file and then the median fluorescence *without* compensation is each channel is noted. Prior to each subsequent acquisition of samples on the same cytometer and with the same lot of antibodies, a similar acquisition of Rainbow beads is performed without compensation applied. The PMT voltages are adjusted such that they are within +/-5% of the voltages obtained when the initial compensation was set. This allows us to avoid creating costly and time consuming new compensation controls for every acquisition.

3. Acquisition then proceeds as normally for the flow cytometer's operating instructions.