Flow Cytometry SOP for Influenza U01

Reagents

RPMI Thawing media

- 439.5ml RPMI + L-glutamine (remove 60.5ml from bottle)
- 50ml heat inactivated FBS
- 5ml Penicillin-Streptomycin
- 5ml Sodium Pyruvate
- 0.5ml DNAse

Flow buffer

- 1,000ml PBS
- 25ml FBS
- 100mg Sodium Azide

Fluorochrome labeled antibodies

Cytoperm Wash Buffer (for T cell activation panel **only**)

- 50ml Perm/Wash Buffer (BD catalog #554723)
- 950ml dH_2O

4% paraformaldehyde

- 980ml PBS
- 40g paraformaldehyde
- 100ul 10N NaOH
- Heat/stir until dissolved. Cool to room temp and filter. Aliquot and store at -20C

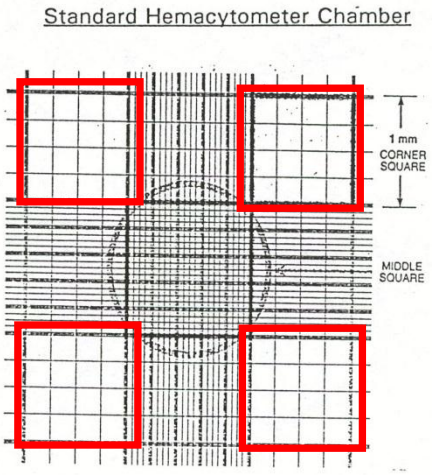
Overview

1. Thaw and count cells.
2. Aliquot into conical tubes.
3. Wash, stain, and fix cells.
4. Run samples on flow cytometer.
5. Analyze data.

Thaw and count cells

1. Check out 1 cryovial of PBMC (1ml) (2 – 1ml cryovials for T cell activation panel) for each of 12 subjects from VATS according to VATS checkout protocol.
2. Place PBMC cryovials in -80°C freezer until you are ready to begin the thawing process.
3. Place RPMI thawing media containing DNase in 37°C water bath for 15 minutes to warm up (this is essential to recover maximal cells). Wipe the bottle with 70% ethanol before placing in tissue culture hood.
4. Label one 15ml conical tube with the subject ID on both the side and the cap.
    a. NOTE: Ensure that the caps are kept in the same order as the tubes
5. Add 100 µl of RPMI thawing media containing DNase/10% FCS to each 15ml conical tube using a p200 pipette.
6. Remove PBMC cryovials from -80°C freezer and place cryovials in styrofoam rack. Place sytrofoam rack containing PBMC in 37°C water bath in tissue culture room. Rapidly shake the rack (manually) so thawing can occur. Do this until a small chunk of ice remains.
7. Quickly wipe the PBMC vial with 70% ethanol and place the thawed PBMC in a blue cryotube rack (in the SAME order as the 15ml conical tubes) and place in tissue culture hood.

8. Uncap the cryovial for each subject. Using a 2ml pipette, remove the cells from the cryovial. Be careful not to exceed the volume of the pipette when removing the cells. Transfer the PBMC with the 2ml pipette to the 15ml centrifuge tube slowly. **Do not pipette the cells up and down.**
9. Mix the cells with the media in the conical tube by shaking the tube gently.
10. **Set timer for 5 minutes.** Add 200 µl of RPMI thawing media with DNase/10%FCS to the cells SLOWLY, swirl the tube gently to mix the cells/media, wait one minute, then double the amount of media (0.5ml) and add slowly to cells, swirl the tube gently, wait one minute, add 1ml of media as described. Do this until cells are in a final volume of 10ml. You should be done when your timer goes off.
11. Cap each 15ml conical tube with the appropriately labeled cap. Invert the conical tube 5 times to mix the cells. Do not vortex the cells!
12. Centrifuge at 1200 RPM at room temperature for 7 minutes using a bench top centrifuge.
13. While cells are centrifuging, turn on the refrigerated centrifuge. Set temperature at 4°C so it is cool when you need to use it.
14. Remove the supernatant by vacuum aspiration, leaving only few hundred µl in the tube. DO NOT ASPIRATE CELL PELLET. Re-suspend cells in remaining media by swirling gently. DO NOT VORTEX CELLS.
15. Bring volume to 10ml with RPMI thawing media containing DNase/10% FCS.
16. Cap each 15ml conical tube with the appropriately labeled cap. Invert the conical tube 5 times to mix the cells. Do not vortex the cells!
17. Place conical in rack and incubate in 37°C water bath for 20 minutes with intermittent inverting of tubes (every 10 minutes).
    a. NOTE: Ensure that water in the water bath does NOT exceed the top of the tubes as contamination will occur. Cover the tubes with a weight if necessary to prevent tipping.
18. During incubation, prepare FACS tubes for counting. Label FACS tubes with sample ID and add 200 µl of 1X PBS and 37.5 µl of trypan blue
19. Cool cells on ice for 7 minutes.
20. Centrifuge at 1200 RPM at 4°C for 7 minutes using a refrigerated bench top centrifuge.
21. Carefully remove the supernatant by vacuum aspiration without disturbing cell pellet. Resuspend cells in each conical tube in 2ml RPMI with 5% FCS by swirling gently (MAKE SURE CELLS ARE RESUSPENDED WELL). Note: There may be dead cells and these will be filtered out in the next steps.
22. Label one 50ml conical for each subject with the subject ID.
23. Using a 2 or 5ml pipette, filter the cells from each subject through one BD Falcon cell strainer (catalog number 352350) into the pre-labeled conical tube for each subject.
24. Place the cells on ice.
25. Mix the conical tube containing the PBMCs by inverting 3 times. Using a p20 pipette, add 12.5 µl of PBMCs to the appropriate FACS tube immediately before counting. Only prepare cells for 1 subject at a time.
26. Mix the contents of the FACS tube by vortexing. Using a p10 pipette, add 10 µl of the cell suspension to the counting chamber of a hemacytometer. Do not flood the hemacytometer. Make sure there are no bubbles under the coverslip. If the hemacytometer is flooded, or if there are bubbles, clean the hemacytometer with 70% ethanol and again add 10 µl of the sample.
27. Place hemacytometer under microscope and count cells (live= clear, unstained) and dead (blue) in the outer 4 quadrants, as shown below.



Count cells in the quadrants outlined in red

28. Open the "U01 Flow counting template" located in the Influenza U01 2010 2010-2015/U01 Flow/Adaptive Flow subfolder. Copy the blank template into a new worksheet. Label the worksheet with the date and your initials. Record both live and dead cell count on the template. The spreadsheet automatically calculates cell concentration.

    Note: The spreadsheet calculates cells as follows:

    [number of live cells/4]*10,000* dilution factor of 20 *total volume (2ml) =total cells
29. Add the indicated amount of RPMI with 5% FCS (column L of template) to the appropriate conical tube containing PBMCs to adjust cell concentration to 1 x10^6 cells/ml. If the volume in column L is negative, spin sample down at 1200 RPM for 7 minutes at 4°C, aspirate/discard the media, and add the total amount of RPMI in column K.
30. Keep cells on ice until you begin the staining process**,** or proceed to "Infection" steps for T cell activation panel.

Infection (for T cell activation panel ONLY)

1. Make a 1:100 dilution of trypsin-TPCK in serum-free media (5ul trypsin-TPCK + 495ul serum-free RPMI media).
2. Make a 1:5 dilution of influenza virus in serum-free media (300ul influenza virus + 1.2ml serum-free RPMI media).
3. Add 15ul of trypsin-TPCK working solution to the virus and incubate at 37°C for 30 minutes prior to infection.
4. Make PMA working solution: 1.5ul PMA + 1.5ml media.
5. Add 2x10^6 cells in 100ul of media (RPMI) per well of 96-well round-bottom plate.
6. Designate wells for PMA stimulation, influenza ("FLU") stimulation, and no stimulation ("UNSTIM") and add the following to the respective wells.
    a. PMA wells: 100ul of PMA working solution (from step #4)
    b. FLU wells: 100ul of virus working solution (result of steps #2 and 3)
    c. UNSTIM wells: 100ul serum-free media (RPMI)
7. Incubate plate at 37°C for 2 hours.
8. Prepare master mix of 12ul BFA + 9ul MON + 579ul serum-free media.
9. Add 10ul master mix per well.
10. Incubate plate at 37°C for 18 hours.

Aliquot cells

1. Open the "Flow Worksheet Template" located in the Influenza U01 2010 2010-2015/U01 Flow/Adaptive Flow subfolder. Copy the template worksheet to a new worksheet. Update the date and subject IDs. Print worksheet and save file.
2. Set up the required number of FACS tubes and label them numerically.
3. For the T reg., B cell, and innate cell panels, place 2.0ml of cells in each FACS tube.
4. For the T cell function panel, transfer cells to appropriate FACS tube (1 well of cells per FACS tube). Keep the conditions in mind (PMA, FLU, UNSTIM) to make sure cells are aliquotted appropriately.
5. Combine cells from multiple subjects and place 1ml in each control tube.

Wash, stain, and fix cells

Protocol for T reg., B cell, and innate panels ONLY

1. Add~4ml of flow buffer to each FACS tube
2. Spin for 5 minutes at 1200 RPM.
3. Decant liquid into sink and gently resuspend cells.
4. Prepare antibody cocktail mixture (see appropriate Antibody Labeling Worksheet).
    a. Add required volume of antibody mixture to each sample tube and place tube on ice.
    b. Add required antibodies to each control tube and place on ice.
5. Incubate tubes at 4°C **in the dark** (lights off and cover with foil) for 30 minutes.
6. Add~4ml of flow buffer to each tube
7. Spin for 5 minutes at 1200 RPM.
8. Decant liquid into sink and gently resuspend cells.
9. Add~4ml of flow buffer to each tube
10. Spin for 5 minutes at 1200 RPM.
11. Decant liquid into sink and gently resuspend cells.
12. Add 200ul of flow buffer to each tube.
13. Spin for 5 minutes at 1200 RPM.
14. Decant liquid into sink and gently resuspend cells.
15. Add 200ul of 4% paraformaldehyde solution to each tube.
16. Cap each tube tightly and cover completely with aluminum foil.
17. Place samples in 4°C cold room until ready to be run on flow cytometer.

Protocol for T cell panel ONLY

1. Add~4ml of flow buffer to each FACS tube
2. Spin for 5 minutes at 1200 RPM.
3. Decant liquid into sink and gently resuspend cells.
4. Prepare *surface* antibody cocktail mixture (see appropriate Antibody Labeling Worksheet).
    a. Add required volume of antibody mixture to each sample tube and place tube on ice.
    b. Add required antibodies to each control tube and place on ice.
5. Incubate tubes at 4°C **in the dark** (lights off and cover with foil) for 20 minutes.
6. Add~4ml of flow buffer to each tube
7. Spin for 5 minutes at 1200 RPM.
8. Decant liquid into sink and gently resuspend cells.
9. Add~4ml of flow buffer to each tube.
10. Spin for 5 minutes at 1200 RPM.
11. Decant liquid into sink and gently resuspend cells.

12. Add 250ul Cytofix/Cytoperm to each FACS tube.
13. Incubate tubes at 4°C **in the dark** (lights off and cover with foil) for 30 minutes.
14. Add 1ml Cytoperm Wash Buffer to each FACS tube.
15. Spin for 5 minutes at 1200 RPM.
16. Decant liquid into sink and gently resuspend cells.
17. Add 1ml Cytoperm Wash Buffer to each FACS tube.
18. Spin for 5 minutes at 1200 RPM.
19. Decant liquid into sink and gently resuspend cells.
20. Prepare intracellular antibody cocktail mixture (see appropriate Antibody Labeling Worksheet)
    a. Add required volume of antibody mixture to each sample tube and place tubes on ice.
    b. Add required volume of antibodies to each control tube and place on ice
21. Incubate tubes at 4°C **in the dark** (lights off and cover with foil) for 30 minutes.
22. Add 1ml Cytoperm Wash Buffer to each FACS tube.
23. Spin for 5 minutes at 1200 RPM.
24. Decant liquid into sink and gently resuspend cells.
25. Add 1ml Cytoperm Wash Buffer to each FACS tube.
26. Spin for 5 minutes at 1200 RPM.
27. Decant liquid into sink and gently resuspend cells.
28. Add 200ul of flow buffer to each tube.
29. Spin for 5 minutes at 1200 RPM.
30. Decant liquid into sink and gently resuspend cells.
31. Add 200ul of 4% paraformaldehyde solution to each tube.
32. Cap each tube tightly and cover completely with aluminum foil.
33. Place samples in 4°C cold room until ready to be run on flow cytometer.
    a. Note: Due to the addition of virus, cells must be fixed with 4% PFA for at least 24 hours before running on flow cytometer.

Run samples on flow cytometer

1. Flow core facility is located on Gugg 14. We will be using the LSR II instrument.
2. Fill out a sample registration form for Core Facility.
3. Make the appropriate changes, as listed below, to the default LSR II instrument laser configuration (default laser configuration is in Table 1 below).
    a. For the innate panel:
        i. 488 laser, detector A: Filter: 695/40 BP; DC: 635 LP
    b. For the B cell panel:
        i. 407 laser, detector A: Filter: 610/20; DC: 595 LP
        ii. 407 laser, detector F: Filter: 444/24; DC: blank
    c. For T reg panel:
        i. 407 laser, detector A: Filter: 610/20; DC: 595 LP
        ii. 407 laser, detector F: Filter: 444/24; DC: blank
        iii. 488 laser, detector A: Filter: 695/40; DC: 635 LP
    d. For T cell panel:
        i. 407 laser, detector A: Filter: 710/50BP; DC: 685LP
        ii. 407 laser, detector B: Filter: 605/12; DC: 595LP

Table 1: Default LSRII Laser Configuration

Laser Line	Detector	Dichroic	Filter	Wavelength
355 nm (UV)	A	450 LP	450/65	450-483
	B	Blank	450/50	425-450
407 nm (Violet)	A	595 LP	605/12	599-611
	B	570 LP	585/42	570-595
	C	550 LP	560/20	550-570
	D	535 LP	525/50 532 nm RB	535-550
	E	505 LP	515/20	505-525
	F	Blank	450/50	425-475
488 nm (Blue)	A	635 LP	670/14	663-677
	B	505 LP	530/30 532 nm RB	515-545
	C	Scatter	488/10	
532 nm (Green)	A	755 LP	780/60	755-810
	B	685 LP	710/50	685-735
	C	635 LP	660/40	640-680
	D	600 LP	610/20	600-620
	E	Blank	575/26	562-588
635 nm (Red)	A	755 LP	780/60	755-810
	B	710 LP	730/45	710-753
	C	Blank	660/20	650-670

4. FACSDiva software should be up and running, if not, ask techs for help.
5. Open the latest experiment and copy without data. Rename newly created experiment and tubes. Delete old experiment. Delete all tubes except #1 for the new experiment.
6. Run the unstained and each single color control tube on the cytometer for 3-4 seconds. Remove tube from the cytometer and check all voltages and compensations. Tweak as necessary.
7. Count and save 5,000 events for the unstained and single color control tubes.
8. Count and save 200,000 events for the experimental tubes.
9. Export/save the experiment and the sample files to the hard drive.
10. Transfer sample files to the server.
11. Print off the cytometer status report.
12. Close the experiment.
13. Follow the clean-up procedure for the cytometer.