LABORATORY PROCEDURE: **IMMUNOPHENOTYPING**: Lymphocyte Staining for FACS Analysis

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PURPOSE: To delineate the subsets of human lymphocytes based on the expression profiles of different phenotypic markers by FACS analysis

SCOPE: This procedure applies to all normal and autoimmune blood specimens processed in the Anolik laboratory.

PRINCIPLE: Lymphocytes are stained with various fluorescently labeled antibody markers that can be analyzed with an analytical flow cytometer to determine a subject's cell subset distribution.

SAFETY PRECAUTIONS: All work should be performed under the biological safety cabinet observing safety regulations. Personal protective equipment such as: lab coat, gloves and glasses, should be used during the procedure. Specimens should be handles as if capable of transmitting infection. All contaminated supplies should be properly disposed of in biohazard or sharps containers and liquid waste should be decontaminated with bleach for 20min before being poured down the drain.

NOTE: Pay particular attention to **"^HOT SPOT**" steps. These are crucial to optimize cell yield and viability. Please contact the representative if you have any SOP related questions

MATERIALS AND REAGENTS:

Supplies+Equipment

Foil
Centrifuge
5ml FACS tubes *(Falcon 352054)*
Tube rack
Refrigerator 4°C or Ice/bucket
10 ml l pipet *(VWR 89130-898)*
P-20, 200,1000 + Tips
15 ml conical *(Falcon 352097)*
50 ml conical (*Falcon 352070)*
Vacuum aspirator
Pipet aid
Vortex

Reagents

Primary antibody
Secondary antibody if using biotin
1% formaldehyde
FACS buffer
Fc receptor *(Biolegend 422302)*
Live/Dead aqua *(Invitrogen L34957*
1X PBS *(Cellgro 21-040-CV)*
ArC Aqua beads *(Invitrogen A10346)*
Comp Beads *(Bangs, 556)*
10% Formaldehyde ultrapure *(Polyscience, 04018)*
dH2O *(cellgro 46-000-CM)*
1% BSA
Albumin from Bovine (*Sigma, A-9647)*

REAGENT PREPARATION: a. 1% Formaldehyde: In a 50ml conical add 45ml of 1X PBS and add 5ml of 10% formaldehyde.

b. FACS buffer: In a 50ml conical place 45 ml of 1X PBS and add 5ml of 10% BSA

c. 1% BSA: In a 50 ml conical weigh 0.5 grams of power albumin and dissolve it in 50ml of 1X PBS

REAGENT STORAGE: **Room Temperature:** 1X PBS, 10% Formaldehyde, dH2O **4C:** Primary antibody, Secondary antibody, 1% Formaldehyde, FACS buffer, Fc receptor, 1% BSA, Albumin, AcR beads **-20C:** Live/Dead aqua

SPECIMAN STORAGE: The cells should be on ice while doing the procedure. When the cells are stained and fixed they can be stored at 4C overnight.

QUALITY CONTROL: Ensure that all reagents are prior to expiration date.

PROCEDURE:

To stain compensation controls

- For each florochrome + 1, place a 5ml FACS tube in a rack. Set aside one tube of beads as the unstained control. To the remaining tubes add 2ul of each antibody to each tube as single-stain control.
- Dispense 2 drops of Simply Cellular Compensation Standard beads to each tube **^HOT SPOT** and vortex or shake well
- Incubate on ice for 30 min in the dark
- Wash the beads with 1ml of 1X PBS and centrifuge at 350 RCF/g for 5 min 4C.
- Aspirate supernant and resuspend the beads in 200 µl of 1% Formaldehyde * with the **exception** of the biotin tube. (Proceed to Biotin step if you are using a Biotinylated conjugate)
- ***If using Biotin***: : (Skip if there is no Biotin in your panel)
  - Make a 1:500 dilution of streptavidin antibody in 1X PBS
  - Resuspend biotin labelled beads in 100ul.
  - Incubate for 10 min on ice in the dark
  - Wash cells with 1 ml 1XPBS and centrifuge at 350 xg for 5 mins 4C
  - Aspirate supernatant and resuspend the beads in 200 µl of 1% Formaldehyde

For Aqua compensation control

- Use ArC beads for Aqua compensation control. Gently vortex ArC bead components. Add 1 drop of Component A (reactive beads) to a 5ml FACS tube. Add 1 uL of live/dead Aqua directly to the droplet of the reactive beads and incubate on ice for 15 mins in the dark.

- Wash with 1 mL of 1X PBS and centrifuge at 350 RCF/g for 5 min 4C.
- Aspirate the supernant and resuspend in 200 uL of FACS buffer.
- Add in 1 drop of ArC (negative beads) to the tube and vortex.

To stain the samples

- Transfer up to 10x10E^6 of PBMC into a 1.5ml microfuge.
- Centrifuge at 350 RCF/g for 5 mins
- Aspirate the supernant and resuspend the cells in 5ul of Fc Block and incubate at room temp for 5 minutes
- Add 100 µl of the antibody cocktail (Appendix A) and transfer to a 5ml FACS tube. **^HOT SPOT** vortex or shake well to ensure proper mixing
- Incubate on ice for 30 min in the dark.
- Wash cells with 3 ml 1X PBS and centrifuge at 350 RCF/g for 5 mins 4C
- Aspirate supernant (Proceed to Biotin step if you are using a Biotinylated conjugate)
- ***If using Biotin***: (Skip if there is no Biotin in your panel)
  - Make a 1:500 dilution of streptavidin in 1X PBS
  - Resuspend cells in 100ul
  - Incubate tubes on ice for 10 min in the dark
  - Wash cells with 3 ml 1XPBS and centrifuge at 350 RCF/g for 5 mins 4C
  - Aspirate supernatant
- Make a dilution of the Live/Dead Aqua in a 15ml conical by adding 500ul of 1X PBS + 1ul of aqua for each tube. Incubate on ice for 15min in the dark.
- Wash cells with 3 ml 1XPBS and centrifuge at 350 RCF/g for 5 mins 4C.
- Aspirate the supernant and resuspend cells in 200ul of 1% formaldehyde.
- Run samples on the LSR2 the either the same day or the second day (store at 4C overnight)

LIMITATIONS: Stain from 1x10^6 up to 10X10^6 cells per tube.

CALCULATONS: When making the master mix, allow for 1 to 2 extra samples to account for volume loss. So if you have 5 patient samples and 1 internal control, N=6, use N=7 multiplied by the volume listed in the appendix.

INTERPRETATION: Data is collected and auto-compensated using FACS Diva software on the BD Fortessa. The FCS files are transferred onto the AIR server. Files are analyzed using Flowjo v10 software program.

RESULTS REPORTING: The results are reported in Excel with Sample ID, Date, visit number (if applicable) on X and the populations on the Y. The % parent is inputted on worksheet 1 and % B cell or T cell on worksheet 2.

TRAINING: Personnel will be trained by staff. Up to one time visual shadowing of staff member AND up to one time hands on training with staff member AND one or more times independent performance with successful completion of SOP.

APPENDIX A:

MEMORY PANEL:

Florochrome	Antibody	Volume (ul)
FITC	IgD	10
PE	CXCR3	10
PE-A610	CD24	2.5
PE-Cy5	CD21	10
PerCP-Cy5.5	CD38	2.5
BV 421	CD3	5
APC	CD95	10
APC-Cy7	CD19	5
Alexa 700	CD20	2.5
BL 605	CD27	2.5

Tcombo PANEL:

Alx 647	CCR4	5
PEcy7	CCR7	5
BV 650	CD25	5
PE	CXCR3	10
BV 421	CCR6	5
BV 786	CD127	5
FITC	CD3	20
APC-Cy7	CD4	5
PERCP5.5	CD45RA	5
PE-A610	CXCR5	5
BV 605	PD1	5
BT-SA-PEcy5	ICOS	1