Flow Cytometry SOP: Monocytes from Frozen Cells

Purpose

This SOP standardizes the procedure for measuring immune cells using flow cytometry in ACTG Immunology Laboratories.

Materials

1. 12x75mm flow tubes
2. 1X PBS without Ca2+/Mg2+ (Fisher, Catalog #21-031-CM)
3. BSA (Sigma, Catalog #A9418)
4. Flow Buffer
    a. 1X PBS + 1% BSA
5. CompBead, Anti-mouse and Anti-Rat Ig (BD, Catalog #552843, 552844)
6. Molecular Probes ArC Amine Reactive Compensation Bead Kit (Catalog #A10346)
7. 1% Formaldehyde
8. Staining Antibodies for CD3, CD4, CD8, HLA-DR, CD38, CD19, CD14, CD16, CD56, CCR2, and CX3CR1. Alternative antibodies to use may include CCR5 , CXCR4, CD11b, CD142, PDL-1, CD120a, CD120b, CD163, and CD62P.
9. BV Stain buffer (BD, Catalog #563794)
10. Live/Dead Fixable Aqua Dead Cell Stain Kit for 405 Excitation (Molecular Probes, Catalog #L34957)
11. Spherotech 7-peak beads (Fisher, Catalog #RCP-30-5A-7)

1. Sample Requirement
    a. Blood should be collected using heparin or EDTA as an anticoagulant.
    b. All testing will be done on frozen samples.
        i. Whole blood should be processed within 6 hours and cryopreserved at clinical site to avoid being shipped.
        ii. The samples are to be cryopreserved using the ACTG Cryopreservation SOP (Cross-Network PBMC Processing SOP. HANC-LAB-P0001v4).
        iii. Once samples are frozen, they will then be shipped to BRI. BRI will send batched samples to ISL for flow cytometry analysis.
            1. One ISL will be responsible for all flow cytometry analysis for a particular study protocol. Samples are not to be split between sites.
2. Validation
    a. Reagent Validation
        i. FMO (fluorescence minus one) tubes for HLA-DR, CD38, CCR2, CX3CR1, CD14, CD16, and CD56 should be run only during initial study setup on HIV+ donors. It is not necessary to run FMO tubes daily with patient samples.
            1. Set the gates based on the negative population in the FMO plot and these gates should be carried over for use with study samples.
    b. Instrument Validation
        i. Instrument should be standardized daily using 7-peak beads to maximize instrument performance and minimize inter-assay variability.
            1. If using a BD machine, CST must be run daily to ensure cytometer performance.
        ii. When setting up the experiment for the first time, choose your voltages to minimize bleed over between fluorochromes and then run Spherotech 7-peak beads to establish *baseline* MFI value for each fluorochrome.
            1. Draw a gate around the 7-peak beads in a FSC/SSC plot.
            2. In histogram view, draw a gate around the peak for each fluorochrome. This should be gated off the prior bead gate.
            3. Use the statistics view to determine the MFIs.
            4. Record these values on a worksheet (example shown in Appendix B).
3. Thawing Samples
    a. All labs must participate in a cross lab validation of thawing that will be established by the IQA.
    b. Thaw using the IQA SOP.
    c. Wash the cells 2 times with 1X PBS to remove FBS. After wash decant liquid. Do not add additional volume to the tube. Proceed with staining the cells.
4. Staining
    a. Stain for viability with Molecular Probes Live/Dead® Fixable Aqua Dead Cell Stain Kit.
        i. Resuspend cell pellets in 12x75mm flow tube. Stain at a concentration of 1µL per sample (1uL Live/Dead stain plus 49uL 1X PBS per sample).
        ii. Also stain Live/Dead compensation tube at this time by placing 3µL of Live/Dead stain and 2 drops of positive ArC Amine beads in a flow tube.
        iii. Incubate at room temperature for 15-30 minutes in the dark. Protect samples from light from this step forward.
    b. Wash cells once with 2mL Stain buffer to remove Live/Dead stain. After wash decant liquid. Do not add additional volume to the tube.
        i. Add 2 drops of negative ArC Amine beads to the Live/Dead compensation tube and fix in 1% formaldehyde.
    c. The following antibodies may be used as the Monocyte panel (substitute fluorochromes as appropriate). Alternative antibodies in red may be considered for further investigation depending on the study goals: CCR5, CXCR4, CD11b, CD142, PDL-1, CD120a, CD120b, CD163, and CD62P.

Marker	Clone	Fluorochrome	Manufacturer	Catalog Number
CD3	UCHT1	PE-Cy7	BD Pharm	563423
CD4	RPA-T4	BB515	BD Horizon	564419
CD8	SK1	APC-H7	BD Pharm	560179
HLA-DR	G46-6	BV605	BD Horizon	562845
CD38	HIT2	APC	BD Horizon	555462
CCR2	48607	BV421	BD Horizon	564067
CX3CR1	2A9-1	PE	BD Pharm	565796
CD14	M5E2	BV786	BD Horizon	563698
CD16	3G8	PE-CF594	BD Horizon	562293
CD56	B159	AF700	BD Pharm	557919
CD19	HIB19	AF700	BD Pharm	557921
CCR5	2D7/CCR5		BD	
CXCR4	12G5		BD	
CD11b	ICRF44		BD	
CD142	323519		R&D	
PDL-1	MIH1		eBioscience	
CD120a	16803		R&D	
CD120b	22235		R&D	
CD163	GHI/61		BD	
CD62P	AK-4		BD	

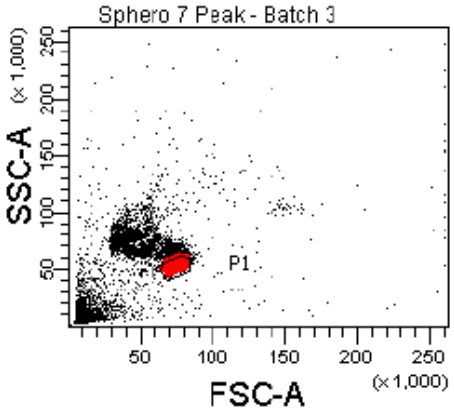
i. Prepare antibody cocktail for appropriate number of samples + 1 extra. Use BV Stain Buffer at 50uL/test.
ii. Prepare compensation tubes at this time using BD CompBeads.
    1. Add one test worth of antibody to the specific compensation tube. Add 1 drop each of the positive and negative CompBeads.
iii. Add 120µL (50uL BV stain buffer + 1 test per antibody) of the antibody cocktail to each sample tube and vortex. Incubate at room temperature for 30 minutes in the dark.

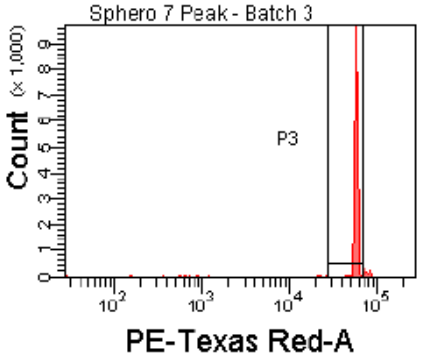
d. Wash the cells with 2mL Flow Buffer.
e. Fix cells in 1% formaldehyde and run on a flow cytometer within 48 hours.

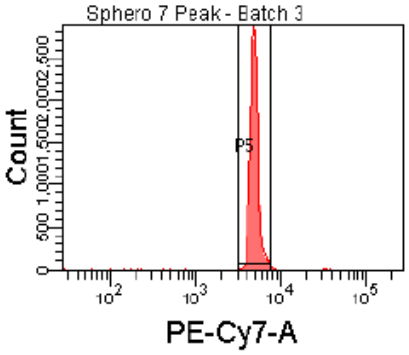
5. Sample Collection
    a. Acquire/analyze samples on BD LSR-Fortessa flow cytometer.
    b. Run Spherotech 7-peak beads first.
        i. Prepare Spherotech 7-peak beads to standardize the instrument by putting 2 drops of beads in 1mL PBS. Protect the beads from light and store at 2-8 °C. The beads are good for one week.
        ii. Run at an event rate of 150-500 events/second. Adjust PMT voltages to match the baseline MFI values acquired at study set up (see section 2.b.ii).
        iii. Record at least 10,000 events and track the daily MFI and PMT voltage values on a worksheet.
    c. Transfer 7-peak PMT Voltage values to compensation tubes in new experiment in FacsDiva. Run compensation tubes and collect at least 25,000 events per tube.
    d. Calculate and apply compensation.
    e. Run sample and aim to collect $1x10^6$ events.
        i. Be sure to collect FSC-A, FSC-H, and SSC-A, SSC-H parameters for samples.
6. Analysis

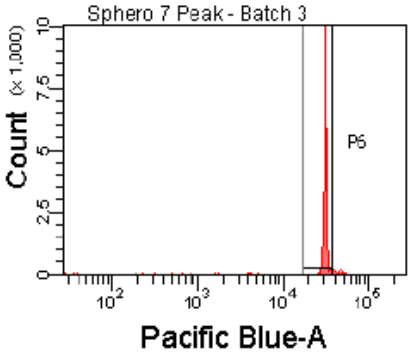
a. All analysis should be performed by using FlowJo, or other appropriate software.
b. Only samples with viability ≥50% will be reported. Viability is determined by gating on Live/Dead negative lymphocytes. See gating strategy in Appendix C.
c. Due to the standardization of the machine with Spherotech beads daily, the fluorescence gates should not change from day to day. The scatter gates may change slightly.
d. Use the gating strategy in Appendix C to analyze T lymphocytes and Monocytes.
e. The following parameters should be reported for all studies using this staining protocol.
    i. Immune activation (CD38/HLA-DR reported as % of CD3+CD4+ and as % of CD3+CD8+).
    ii.
    iii. Consult with study group for additional analyses as appropriate.

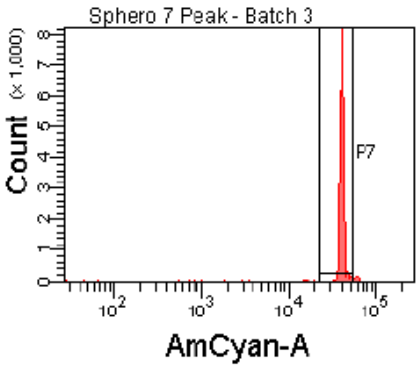
Appendix A: Spherotech 7-Peak Bead Acquisition

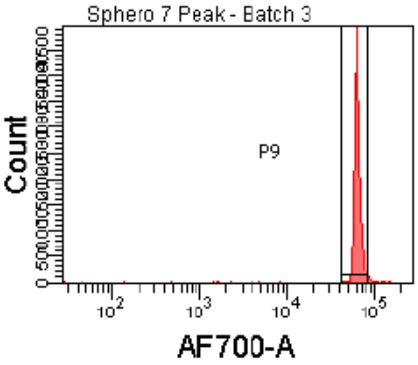


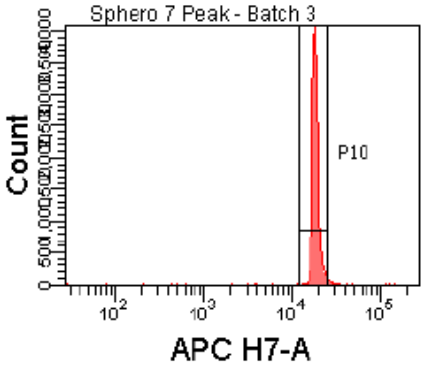












Experiment Name: 7 Peak Beads
Record Date: Feb 24, 2012 11:00:26 AM

Population	PE-Texas R... Median	PE-Cy7-A Median	Pacific Blue... Median	AmCyan-A Median	AF700-A Median	APC H7-A Median
All Events	57,250	4,902	31,606	40,867	62,389	18,529
P1	57,117	4,856	31,527	40,742	62,076	18,412
P3	57,003	4,819	31,453	40,623	61,800	18,313
P5	57,106	4,851	31,521	40,730	62,044	18,401
P6	57,056	4,834	31,479	40,663	61,891	18,346
P7	57,080	4,844	31,502	40,703	61,984	18,380
P9	57,082	4,845	31,505	40,706	61,986	18,381
P10	57,092	4,848	31,511	40,717	62,011	18,391

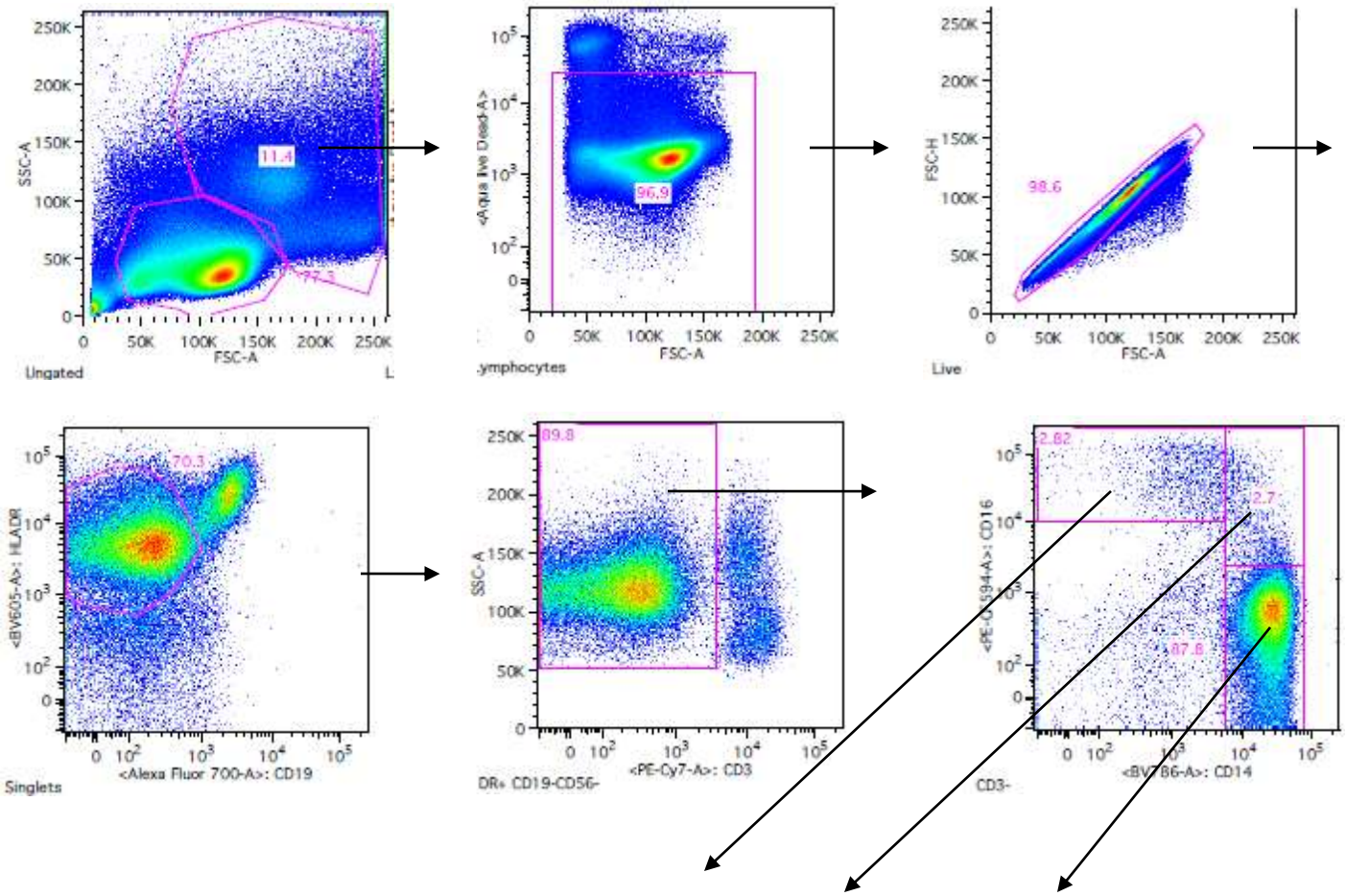
Appendix B: Example of Spherotech 7-Peak Bead Worksheet for MFIs

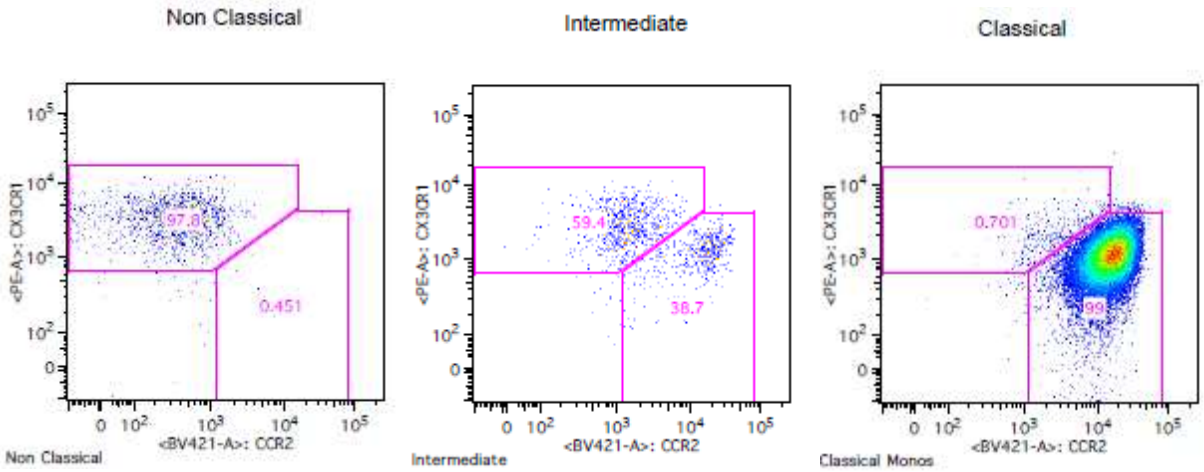
Date	BB515	APC	AF700	APCH7	BV421	L/D	BV605	BV786	PE	PE-CF594	PECy7
Baseline	38,276	91,626	12,333	8,644	56,295	83,634	28,436	2,807	46,903	57,617	6,071

Voltages

	Baseline	
AF488	480	
PerCPCy5.5	595	
APC	565	
AF 700	530	
APC H7	575	
BV 421	475	
Aqual L/D	390	
BV 605	550	
BV650	625	
BV711	575	
BV786	580	
PE	485	
PE-CF594	510	
PECy7	545	

Appendix C: Gating Strategy




Non Classical
Intermediate
Classical
<PE-A>: CX3CR1
<BV421-A>: CCR2
97.8
0.451
59.4
38.7
0.701
99
Non Classical
Intermediate
Classical Monos