WHOLE BLOOD REGULATORY T CELL STAIN – FLOW CYTOMETRY

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Materials and Equipment

- Antibodies (See Page 5)
- FACs Wash (0.5% (w/v) BSA, 5 mM EDTA (pH 7.4-7.6), for 500mL 2.5g BSA, 5mL of 0.5M EDTA)
- 2mL microcentrifuge tubes
- eBioscience FoxP3 Fixation/Permeabilization Concentrate and Diluent (00-5523-00)
- eBioscience FoxP3 Permeabilization Buffer (00-5523-00)
- eBioscience RBC Lysis Buffer (420301)
- eBioscience OneComp eBeads (01-1111-41)
- FlowJo software

Protocol

Buffer and solution preparation

1. Prepare fresh Foxp3 Fixation/Permeabilization working solution by mixing 1 part of Foxp3 Fixation/Permeabilization Concentrate with 3 parts of Foxp3 Fixation/Permeabilization Diluent.

2. Prepare a 1X working solution of Permeabilization Buffer by mixing 1 part of 10X Permeabilization Buffer with 9 parts of distilled water.

3. Prepare a 1X working solution of RBC Lysis by mixing 1 part of 10X RBC Lysis with 9 parts of distilled water.

Note: Similar FoxP3 Buffers are available from BioLegend. They do not work as well for this protocol.

Cell Staining

1. Prepare cell surface marker stain in a 2mL microcentrifuge tube. Stain for 1 sample is made up to 50 µL in FACs Wash (8 µL antibody + 42 µL FACs Wash) and is 3X working concentration. *Concentrations shown below are for 50 µL final volume.*

Cell Surface Marker	**Fluorophore**	**Antibody Concentration**	**Volume (µL)**
CD3	AF700	1/33	1.5
CD4	PerCPCy5.5	1/100	0.5
CD8	PECy7	1/100	0.5
CD25	PE	1/17	3.0
CD127	APC	1/20	2.5
FACS Wash			42.0

Note: For each sample, three stains are required. One unstained control (50µL of FACs wash). One intracellular stain ISOtype, and one complete stain. The intracellular stain ISOtype control has an identical extracellular stain composition as the complete stain.

2. Add 100µL of whole blood that was collected in a heparinized coated collection tube. Incubate for 30 minutes at room temperature away from light.

3. Top up 2mL microcentrifuge tube containing stain and blood with 1X RBC Lyse solution Incubate for 10 minutes at room temperature in the dark with intermittent inversion.

4. Spin down at 2000rpm for 5 minutes at 4˚C

5. Aspirate supernatant. Wash pellet with 2mL FACs Wash. Spin down at 2000rpm for 5 minutes at 4˚C

6. Aspirate supernatant and resuspend in 1mL of FOXP3 Fix/Perm working solution. Incubate for 45 mins at room temperature in the dark.

7. Spin down at 2000rpm for 5 mins at 4˚C.

8. Aspirate supernatant and resuspend in 1mL of 1X Perm Buffer. Spin down at 2000rpm for 5 minutes at 4˚C

9. Aspirate supernatant and resuspend cells in 100µL FOXP3 Perm buffer. Add the amount of intracellular antibody detailed below. Incubate for 30 minutes at room temperature in the dark.

Cell Surface Marker	Fluorophore	Volume (μL)
FoxP3	FITC	10.0
ISOtype	FITC	2.5
Unstained	N/A	0

13. Top with 2mL FoxP3 Perm Buffer. Spin down at 2000rpm for 5 minutes at 4˚C.

14. Repeat step 13 for a second wash.

15. Aspirate supernatant and resuspend in 240 µL FACS wash.

16. Filter cells by through 0.45 µm mesh to ensure single cell suspension. Analyze samples by flow cytometry.

Compensation Controls

Note: Controls should be made right before running flow cytometry

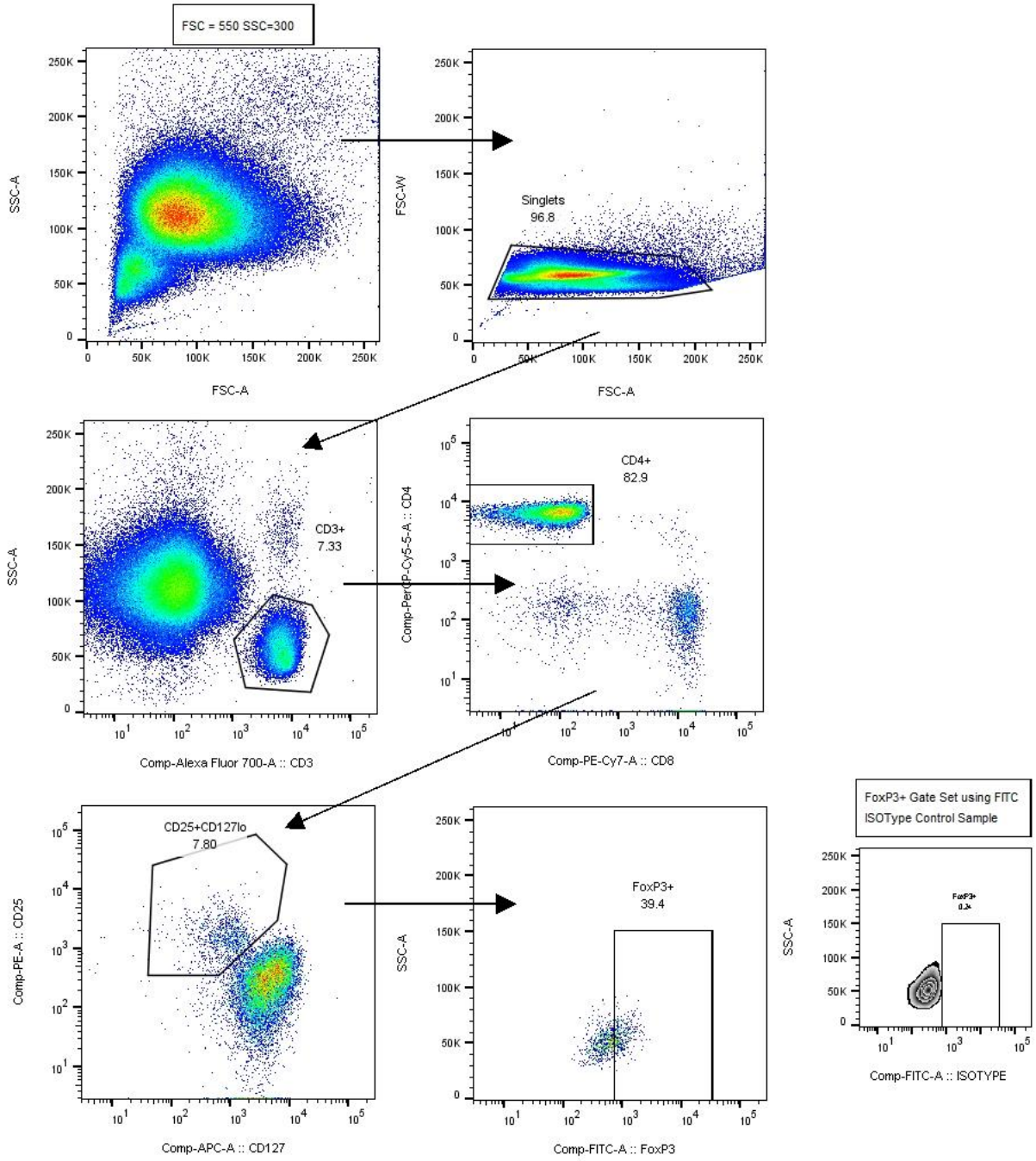
1. In a 5 mL polystyrene tube, dilute 2 drop of One Comp eBeads to 1.68 mL with FACs Wash and vortex. Be sure to vortex OneComp eBeads before use.

2. Aliquot 240uL of diluted eBeads into 7 polystyrene tubes.

3. Add 0.5uL of each antibody used in the stain into one tube. (i.e. 0.5 uL of CD3 antibody into tube 1, 0.5 uL of CD4 antibody into tube 2, etc) leaving one tube unstained. Vortex tubes.

4. Keep tubes away from light until needed.

Regulatory T cell Gating Strategy



Antibody List

Antibody	Fluorophore	Company	Catalog #
CD3	AF700	BD	557943
CD4	PerCPCy5.5	eBioscience	45-0048-42
CD8	PE-Cy7	BD	557746
CD25	PE	eBioscience	12-0259-42
CD127	APC	eBioscience	17-1278-42
FoxP3	FITC	eBioscience	11-4777-42
IgG1K (FoxP3 ISOtype)	FITC	eBioscience	11-4714-42