FLOW CYTOMETRY SURFACE STAIN FOR MONOCYTES

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BACKGROUND

This surface stain is for the detection of monocyte populations in mouse blood and bone marrow. It includes antibodies for detection of surface markers **CD45** (leukocyte common antigen), **CD11b** (neutrophils & monocytes), **Ly6C** (monocyte), and **F4/80** (macrophage). This stain also includes on a single fluorophore surface markers **CD19** (B-cell), **CD3** (T-cell), **NK1.1** (natural killer cell), and **Ly6G** (neutrophils), to facilitate their gating from monocyte populations.

Ly6C^{hi} monocytes exit the bone marrow in a CC-chemokine receptor 2 (**CCR2**)-dependent manner. CCR2^{+} monocytes recently left the bone marrow. CCR2^{-} monocytes are located in the periphery.

Recruitment of monocytes to inflamed tissues leads to their differentiation into TNF- and iNOS-producing dendritic cells (TIP DCs), inflammatory macrophages or inflammatory DCs, some of which can subsequently migrate to draining lymph nodes. **Ly6C^{low}** monocytes patrol the blood vessel lumen by associating with the vascular endothelium. **Ly6C^{low}** monocytes are also recruited to sites of inflammation and may contribute to wound healing by differentiating into alternatively activated macrophages.

MATERIALS

- FACS Wash Buffer (0.5% (w/v) BSA, 5mM EDTA (pH 7.4-7.6), 2mM NaN_3)
  - For 500mL FACS Wash, mix 495 mL MilliQH_2O, 2.5g BSA, 5mL 0.5M EDTA
- 1-step Fix/Lyse Solution (10X) (eBioscience Cat. No. 00-5333)
  - Dilute to 1x in MilliQH_2O
- 1x PBS (1.8 mM KH_2PO_4, 2.7 mM KCl, 10 mM Na_2HPO_4, 137 mM NaCl)
- Fluorophore-conjugated antibodies (see protocol)

PROTOCOL

1. Prepare the stain mix by diluting the antibodies below in FACs Wash Buffer (50 µL total/sample).

Surface Stain

Marker	Conjugated Fluorophore	Company	Catalogue No. and Clone	Volume (µL)
CD45	eFluor 450	eBioscience	48-0451 (30-F11)	0.34
CD11b	PE-Cy7	eBioscience	25-0112 (M1/70)	0.125
Ly6C	AF 488	BioLegend	128022 (HK1.4)	0.125
CCR2	PE	R&D Systems	FAB5538P (475301)	0.4
F4/80	APC	eBioscience	17-4801 (BM8)	0.1
CD3	AF 700	eBioscience	56-0032 (17A2)	0.2
CD19	AF700	eBioscience	56-0193 (eBio1D3)	0.2
NK1.1	AF 700	eBioscience	56-5941 (PK136)	0.1
Ly6G	AF 700	eBioscience	5-5931 (RB6-8C5)	0.1
CX3CR1	BV650	BioLegend	194033 (SA011F11)	0.1
				to 50 µL with FACs Wash Buffer

N.B. CCR2, F4/80 and CX3CR1 are assessed as geometric mean expression on the surface of monocytes with this stain. They may be considered to be optional depending on experimental goals. Inclusion of Ly6G provides a better approach to identify neutrophils than SSC/Ly6C alone.

2. Prepare the isotype control stain mix by diluting the isotype controls below in FACs Wash Buffer.

Surface Stain Isotype Control

Marker	Conjugated Fluorophore	Isotype	Company	Catalogue No. and Clone	Volume (µL)
CD45	eFluor 450	Rat IgG2b, κ	eBioscience	48-4031 (eB149/10H5)	0.34
CD11b	PE-Cy7	Rat IgG2b, κ	eBioscience	48-4031 (eB149/10H5)	0.125
Ly6C	AF 488	Rat IgG2c, κ	BioLegend	400715 (RTK4174)	0.125
CCR2	PE	Rat IgG2b, κ	R&D Systems	IC013P (141945)	0.4
F4/80	APC	Rat IgG2a, κ	BioLegend	400511 (RTK2758)	0.1
CD3	AF 700	Rat IgG2b, κ	eBioscience	56-0032 (17A2)	0.2
CD19	AF700	Rat IgG2a, κ	BioLegend	400528 (RTK2758)	0.2
NK1.1	AF 700	Mouse IgG2a, κ	BioLegend	400247 (MOPC-173)	0.1
Ly6G	AF 700	Rat IgG2a, κ	BioLegend	400528 (RTK2758)	0.1
CX3CR1	BV650	Mouse IgG2a, κ	BioLegend	400265 (MOPC-173)	0.1
					to 50 µL with FACs Wash Buffer

3. Collect blood (100 µL/stain) from mice. Blood is usually collected reto-orbitally by heparinzed capillary tube under isoflurane anaesthesia. Unstained and isotype controls (minimum of one set per stain experiment) also require aliquots of blood.

Smaller volumes of blood can be stained if health of mice is of concern and experiment is not terminal, though the antibody quantities should be adjusted according to the blood volume to maintain the same concentrations.

4. For each sample, aliquot 100 µL of blood into a 2 mL microfuge tube. Add 50 µL of stain, or 50 µL isotype stain (isotype control), or 50 µL FACs Wash Buffer (unstained control), pipetting to mix. Incubate the samples in the dark (i.e. cover with aluminium foil or place in drawer) at room temperature for 30 minutes.

5. Add 1-step Fix/Lyse Solution (1X) to 2 mL and mix 5x by inversion. Incubate the samples in the dark at room temperature for 10 minutes.

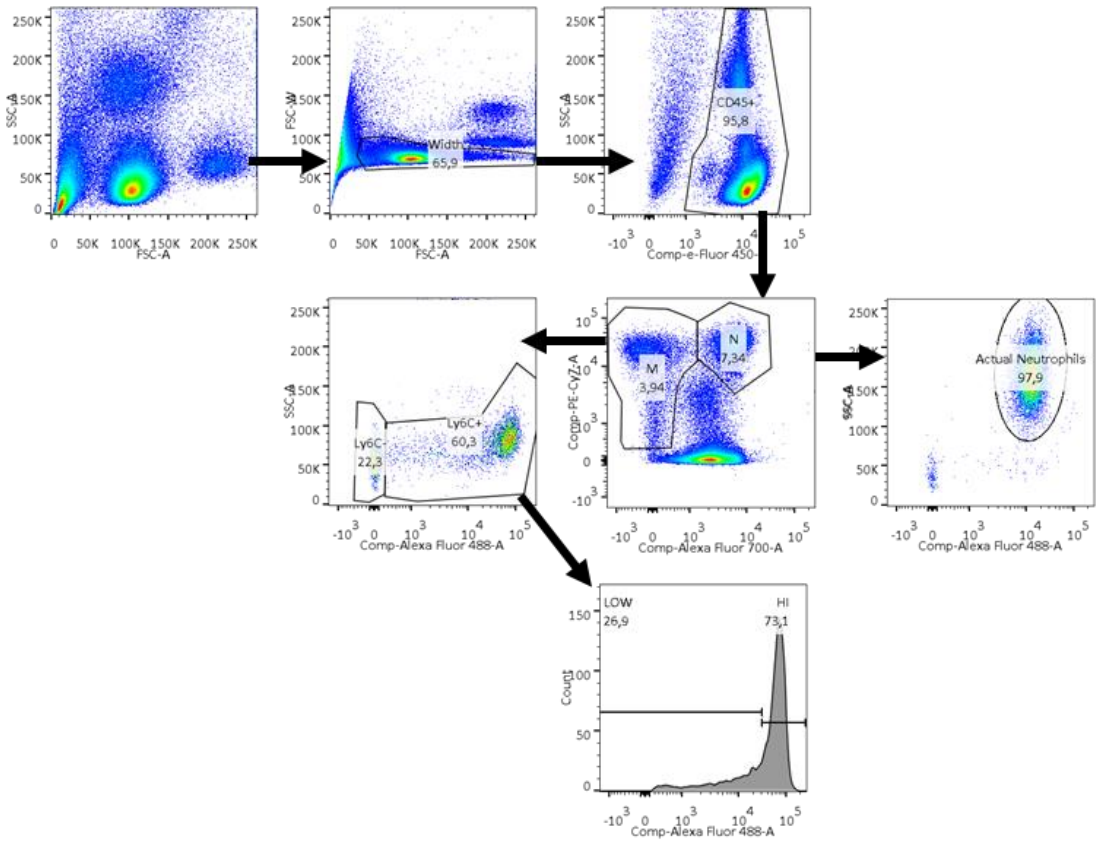
6. Centrifuge samples at 2000 rpm for 10 minutes. Remove the supernatant by carefully pipetting or by slow vacuum aspiration.

7. Add 1x PBS to 2 mL and mix 5x by inversion. Centrifuge samples at 2000 rpm for 10 minutes. Remove the supernatant by carefully pipetting or by slow vacuum aspiration. **If samples are red in colour, you can repeat the wash again.*

8. Resuspend the pellet in 200 µL FACS Wash Buffer.

9. Store the samples in the dark at 4˚C until flow cytometry analysis.

SUGGESTED GATING STRATEGY


SSC-A
FSC-A
FSC-W
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CD45+
95,8
Comp-e-Fluor 450-A
Comp-PE-CyZ-A
M
3,94
N
7,34
Comp-Alexa Fluor 700-A
Ly6C-
22,3
Ly6C+
60,3
Comp-Alexa Fluor 488-A
Actual Neutrophils
97,9
LOW
26,9
HI
73,1
Count