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** (monocytes), and **F4/80** (macrophages/monocyte maturity marker). This stain also includes surface markers **CD19** (B-cell), **CD3** (T-cell), **NK1.1** (natural killer cell), and **Ly6G** (neutrophils), with antibodies conjugated to the same fluorophore, to facilitate their removal from leukocyte populations for the assessment of 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 1xPBS, 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)
- Compensation beads “OneComp eBeads” (Invitrogen Cat. No. 01-1111-42)

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	1x Volume (µL)
Ly6C	AF 488	BioLegend	128022 (HK1.4)	0.125
CCR2	PE	R&D Systems	FAB5538P (475301)	0.4
CD11b	PE-Cy7	eBioscience	25-0112 (M1/70)	0.125
CD45	eFluor 450	eBioscience	48-0451 (30-F11)	0.34
CX3CR1	BV650	BioLegend	194033 (SA011F11)	0.1
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	BioLegend	127621 (1A8)	0.1
FACS Wash				**48.21**
Total volume				**50**

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	1x Volume (µL)
Ly6C	AF 488	Rat IgG2c, κ	BioLegend	400715 (RTK4174)	0.125
CCR2	PE	Rat IgG2b, κ	R&D Systems	IC013P (141945)	0.4
CD11b	PE-Cy7	Rat IgG2b, κ	eBioscience	48-4031 (eB149/10H5)	0.125
CD45	eFluor 450	Rat IgG2b, κ	eBioscience	48-4031 (eB149/10H5)	0.34
CX3CR1	BV650	Mouse IgG2a, κ	BioLegend	400265 (MOPC-173)	0.1
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
FACS Wash					**48.21**
Total volume					**50**

3. Collect blood (100 µL/stain) from mice. Blood is usually collected retro-orbitally by heparinized 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. Add 50 µL of stain, or 50 µL isotype stain (isotype control), or 50 µL FACs Wash Buffer (unstained control) to 2 mL microcentrifuge tubes. For each sample, aliquot 100 µL of blood into the tubes and mix by pipetting. 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 (or FACS Wash Buffer) 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 due to leftover red blood cell debris, 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.

10. Right before flow cytometry analysis, spin samples very briefly and add entire samples on the mesh of flow tubes (blue caps)
    → Using **count beads** for absolute quantification of cells in samples:
    → vortex count beads for 15 sec
    → add 5 µL of beads per sample (not to Isotype ctrl) onto 40 µm mesh of flow tubes (blue lid)
    → add the entire sample to the count beads on the mesh

11. Spin for 5 min at 1500 rpm (room temperature or 4 °C), remove lid, and protect from light

Preparation of compensation

1. Prepare one flow tube (without lid) for each fluorophore (7 tubes) plus 1 for the unstained control.
2. Add 150 µL FACS Wash (300 µL for flow beginners) to each “fluorophore” tube
3. Add 800 µL FACS Wash to the “unstained” tube (100 µL x 8 tubes).
4. Vortex compensation beads for 10 sec.
5. Add 2 drops of compensation beads (3 drops for flow beginner set up) to “unstained” tube (1 drop for every 4-5 fluorophore compensation tubes).
6. Vortex tube and distribute 100 µL from “unstained” tube to each “fluorophore” tube
7. Add 150 µL of FACS Wash (300 µL for flow beginners) to the “unstained” tube
8. Add 0.5 µL of antibody to the respective tube

→ for AF700 use CD19 antibody

→ for BV650 use 0.2 µL of antibody

→ if no negative peak for BV650 is visible during compensation, add a bit of unstained compensation control

9. Gently shake the rack and protect from light by covering with aluminum foil before and during compensation setup.
10. While setting up compensations Ly6C-AF488 positive peak should be as close as possible to 10^4 on the scale (or slightly below). This marker is very bright on the cells and can fall of the scale easily. (Voltage is commonly set to 480-490 on BD-Fortessa in Core Facility).

Recommended Gating: