

# FLOW CYTOMETRY – BONE MARROW

**Created by:** Jessica Breznik & Dessi Loukov

**Revised Date:** February 7, 2021

Bowdish Lab, McMaster University

Hamilton, ON, Canada

[www.bowdish.ca](http://www.bowdish.ca)

## BACKGROUND

All circulating and circulation-derived tissue immune cells in the body are derived from progenitor cells in the bone marrow, which arise from hematopoietic stem cells. This protocol outlines steps for the processing of bone marrow from mouse femurs, a basic surface stain for detection of monocytes and neutrophils, two approaches for cell surface staining for detection of progenitor populations, and the procedure for intracellular staining for detection of **Ki67**, a marker of cellular proliferation.

The '**monocyte**' surface stain includes antibodies for detection of surface markers **CD45** (leukocyte common antigen), **CD11b** (monocytes/neutrophils), and **Ly6C** (monocytes/neutrophils), as well as antibodies conjugated to the same fluorophore for **CD19** (B cells), **CD3** (T cells), **NK1.1** (natural killer cells), and **Ly6G** (neutrophils), to facilitate their removal from leukocyte populations for the assessment of monocyte populations. Neutrophils may be distinguished from monocytes by their expression of Ly6G. The panel also includes markers of monocyte maturity and migration: **CCR2** (chemokine receptor), **CX<sub>3</sub>CR<sub>1</sub>** (fractalkine receptor), and **F4/80** (macrophages/monocyte maturity marker). **Ly6C<sup>low/-</sup>** monocyte retention in the bone marrow is dependent on expression of CX<sub>3</sub>CR<sub>1</sub>. **Ly6C<sup>high</sup>** monocytes exit the bone marrow in a CCR2-dependent manner.

In the '**basic progenitor**' surface stain, cells expressing lineage markers **CD3**, **B220/CD45R**, **Ter119**, **Ly6G**, and **Gr-1 (Ly6C/Ly6C)** are excluded. **HSPCs** (hematopoietic stem and progenitor cells) are identified by their expression of **Sca-1** (Stem Cells Antigen-1; Ly6A) and **cKit** (CD117; promotes HSC proliferation with cytokines GM-CSF, IL-3, and IL-7, and cell survival). **CLPs** (common lymphocyte progenitors) may be distinguished from other progenitor populations by their expression of **CD127** (IL-7R $\alpha$ ). Lin<sup>-</sup>Sca-1<sup>+</sup>cKit<sup>+</sup> progenitors can be further delineated by their expression of **CD16/32** (Fc $\gamma$  receptors) and **CD34** (marker of hematopoietic stem and progenitor cells): **MEP** (megakaryocytic-erythroid progenitor; CD16/32<sup>-</sup>CD34<sup>-</sup>), **CMP** (common myeloid progenitor; CD16/32<sup>-</sup>CD34<sup>+</sup>), and **GMP** (granulocyte-monocyte progenitor; CD16/32<sup>+</sup>CD34<sup>+</sup>) cells.

The '**monocyte-myeloid progenitor**' surface stain follows the pathway of myeloid development, which is regulated by epigenetic and transcriptional modifications. Cells expressing lineage markers **CD3**, **B220/CD45R**, **Ter119**, and **Ly6G**, as well as **CD127** (IL-7R $\alpha$ ), are excluded. The surface markers **CD115**, **cKit**, and **Flt-3** (all members of the same family of receptor tyrosine kinases) assist in identifying distinct progenitor populations, and **Ly6C** and **CD11b** assist in identifying monocytes. **cKit** is expressed at higher levels on myeloid progenitors than lymphoid progenitors and its expression is lost upon myeloid progenitor differentiation into monocytes. **Flt-3** (also known as CD135/Flk-2) is expressed on immature hematopoietic progenitors and CD34<sup>+</sup>HSCs. Flt-3, via activation with Flt-3 ligand (expressed on the surface of mononuclear cells), stimulates the proliferation and differentiation of HSCs, and this synergizes with cytokines to promote proliferation and survival. **CD115** (also known as CSF-1R, M-CSFR, and c-fms), is expressed by myeloid and monocyte progenitors, monocytes, and macrophages. It binds CSF-1 (M-CSF) and IL-34 and promotes survival, proliferation, and differentiation of macrophages. This staining panel identifies the common myeloid progenitor (**CMP**; CD127<sup>-</sup>Lin<sup>-</sup>cKit<sup>+</sup>Sca-1<sup>+</sup>CD34<sup>+</sup>CD16/32<sup>low</sup>), the macrophage and dendritic cell progenitor (**MDP**; CD127<sup>-</sup>Lin<sup>-</sup>cKit<sup>+</sup>Flt-3<sup>+</sup>CD115<sup>+</sup>Ly6C<sup>-</sup>CD11b<sup>-</sup>), the common monocyte progenitor (**cMoP**; CD127<sup>-</sup>Lin<sup>-</sup>cKit<sup>+</sup>Flt-3<sup>+</sup>CD115<sup>+</sup>Ly6C<sup>+</sup>CD11b<sup>-</sup>), and **monocytes** (CD127<sup>-</sup>Lin<sup>-</sup>cKit<sup>+</sup>Flt-3<sup>-</sup>CD115<sup>+</sup>Ly6C<sup>+</sup>CD11b<sup>+</sup>).

## NOTES

- The number of leukocytes within bone marrow will differ according to the sex, age, housing conditions (i.e. germ-free or SPF), and weight of the mouse (especially with diet manipulation), so it is important to decide whether you will work with samples by taking a specific volume of resuspended bone marrow irrespective of sample source (i.e. per femur), or by counting and using a specific number of cells.
- Use FMO (flow-minus-one) and unstained controls prepared from pooled bone marrow from multiple samples, as well as an extra stained sample, if possible, to guide compensation and gating analysis.

## EQUIPMENT

### ***Bone Marrow Processing:***

- Dissection tools – tweezers, scissors
- Kimwipes or gauze
- 50 mL Falcon tube filled with 70% EtOH (for cleaning supplies)
- 50 mL Falcon tube filled with sterile water (for cleaning supplies)
- 50 mL Falcon tube filled with PBS (for short-term storage of samples)
- 50 mL Falcon tube (empty)
- 1x sterile PBS (prechilled at 4°C and maintained on ice)
- 70% EtOH
- Petri dishes (size is arbitrary)
- 10 mL syringes
- 18G and 26G needles

### ***Bone Marrow Freezing (optional):***

- Fetal calf serum (FCS)
- DMSO
- 2 mL cryovials
- Mr. Frosty or similar freezing container (prechilled to 4°C; isopropanol must be at indicated line and replaced after 5 ‘freeze/thaws’)
- P200 and P1000 sterile tips and pipettors

### ***Flow Cytometry – Staining***

- FACs Wash Buffer
  - 500 mL PBS
  - 2.5 g BSA (kept in the fridge)
  - 5 mL 0.5 M EDTA
  - dissolve and leave overnight in the fridge
  - filter through a 0.2 µM filter using vacuum pump
  - store at 4 °C
- 1-step Fix/Lyse Solution (10X) (eBioscience, sold by ThermoFisher Scientific #00-5333)
  - prepare 1x solution with sterile H<sub>2</sub>O
  - store at 4 °C
- Permeabilization Buffer (10x) (eBioscience, sold by ThermoFisher Scientific #88-8824-00)
  - Prepare fresh 1x solution with sterile H<sub>2</sub>O
- 1x PBS (1.8 mM KH<sub>2</sub>PO<sub>4</sub>, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 137 mM NaCl)
- Fluorophore-conjugated antibodies (see protocol)
- v-bottom 2 mL tubes (or v-bottom plates)

### **Flow Cytometry – Pre-analysis preparation**

- Falcon Round-Bottom Polystyrene Test Tubes with Cell Strainer Snap Cap, 5mL (Falcon, sold by Fisher Scientific #08-771-23)
- Falcon Round-Bottom Polystyrene Test Tubes, 5mL (Falcon, sold by Fisher Scientific # 14-959-6)
- CountBright Absolute counting beads (Invitrogen, sold by ThermoFisher Scientific #C36950)
- OneComp eBeads Compensation Beads (Invitrogen, sold by ThermoFisher Scientific #01-1111-41)
- Fluorophore-conjugated antibodies (see Tables 1-3); eBioscience antibodies are ordered through ThermoFisher Scientific

## PROTOCOL

### **FEMUR BONE MARROW COLLECTION**

NB. Consider whether you should use femur or spine bone marrow. The protocols below are for femur bone marrow extraction. A protocol for spine processing is available on the Bowdish website. Also consider whether the bone marrow should remain sterile (i.e. if planning to freeze or culture). If processing bone marrow solely for flow cytometry, it may not be necessary to work in a Biosafety Cabinet according to the mouse housing conditions and experiment requirements.

### **Removal of leg bones**

- Clean tools by immersing in 70% ethanol in 50 mL conical vial, and then wash tools by dipping in PBS in 50 mL conical vial before/after each sample.
- Euthanize mouse according to standard protocols and experimental procedures.
- Spray down fur on lower back/legs with 70% ethanol.
  - Depending on available time, or if collecting muscles, you may choose to pin the body to a dissection board.
- Using tweezers, lift skin from the middle of the back and cut with scissors to open skin parallel to the spine. Peel skin from back/hip area by gently pulling to either side of cut. Remove skin from legs by grasping top of femur at hip along spine and pulling skin away from the leg and over the foot. Use scissors to cut additional skin if necessary. Be careful to not break/cut the leg bones (and spine if collecting).
  - If working on a dissection board, remove fur and skin from legs by lifting skin at the base of each leg with tweezers and cutting away skin across the thigh and down to the foot.
- Expose the hip joint of each leg and cut above the joint, making sure to not remove the top of the femur. Be very careful not to cut bone as this will compromise the sterility of the bone marrow. Leave the foot attached. *If sterility is important, do not remove muscle until working in a Biosafety Cabinet.* To remove muscle, working parallel to the femur, carefully cut muscle layers away from the bone. Clean bones of remaining muscle tissue using Kimwipes or gauze by gently rubbing the bone area.
  - Alternatively, cut off legs first and then remove skin (and muscle if not concerned with sterility).
- Place leg(s) in a 50 mL tube containing PBS on ice (i.e. one tube per mouse).
- Discard remaining tissues (i.e. place in body bag and freeze to later discard according to facility procedures). Clean and sterilize tools with 70% ethanol; if pins were used, discard into sharps container.

### **Bone marrow processing** \*work in the Biosafety Cabinet and use appropriate procedures if sterility is required\*

- Prepare one 10 mL syringe of cold PBS with a 26G needle and 50 mL falcon tube for each leg.
- Pour out the PBS containing the leg(s) into a petri dish. If necessary, remove muscle as described above.

- If desired to assure sterility, transfer the intact bones with sterile tweezers to a second petri dish containing 70% EtOH for one minute and then to the third petri dish containing sterile PBS (this step should be unnecessary if sterility has been maintained).
- Remove a leg from the PBS with tweezers and cut the ends as follows:
  - Place the scissors just below the joint and adjust the tweezers so they are just above the scissors to prevent the bone from shattering. Cut off the hip joint. Readjusting the scissors and tweezers, cut off the knee joint. DO NOT let the ends of the bones touch anything as this will compromise the sterility of the cells within.
  - Ensure that tools are not coated in ethanol when cutting into bone. This can be avoided by dipping tools into sterile water after cleaning in 70% ethanol.
- Flush out the bone marrow into an empty 50 mL Falcon tube by inserting the 26G needle attached to the 10 mL syringe filled with cold PBS at the knee side. Pass the PBS through the bone into the Falcon tube until the colour of the bone turns from red to white, indicating that all marrow has been expelled. Discard the bone into an empty petri dish. You should be able to see red bone marrow pieces within the PBS in the 50 mL tube. Place the tube on ice.
  - Perform this step for each bone IMMEDIATELY following the cutting of the bone.
  - DO NOT put the bone down between cutting and flushing.
- Repeat steps above for each femur. Samples can be pooled into one Falcon tube if appropriate.
  - Clean tools between mice and use separate 'skin/muscle' and 'bone' tool sets of scissors and tweezers to reduce risk of contamination of bone marrow.
- For each Falcon tube of bone marrow, using a 10 mL syringe attached to an 18G needle, aspirate all liquid and expel to break up clumps of bone marrow. Repeat once.
  - Do not do this more than twice to avoid shearing cells.
- Centrifuge 50 mL Falcon tubes at 1500 rpm for 5 minutes at 4°C.
- Working one tube at a time, discard the PBS supernatant. Samples can be allocated for one purpose or divided for freezing, flow cytometry, and/or cell culture according to experimental requirements.
  - **For freezing**, resuspend pellet in 2 mL FCS and aliquot 1 mL each into 2 mL cryotubes. Add 0.1 mL DMSO dropwise. Freeze in pre-chilled (in fridge) Mr. Frosty overnight at -80°C and then transfer to liquid nitrogen for long term storage.
  - **For culturing**, see bone marrow-derived macrophage protocol available on the Bowdish website.
  - **For flow cytometry**, resuspend cells in up to 500 µL PBS and determine cell count by trypan blue staining using a hemocytometer. Aliquot cells into a 2 mL v-shaped tube, and add FACs wash to ~500 µL. Keep samples on ice/in fridge.
    - Plan to stain  $\sim 0.5 \times 10^6$  cells for the monocyte surface stain, and at least  $\sim 1 \times 10^6$  cells for the progenitor stains, especially if you will also perform intracellular staining.
  - Once all samples are prepared, centrifuge for at 4°C for 5 minutes at 2000 rpm.

#### **BONE MARROW SURFACE STAINING FOR FLOW CYTOMETRY**

- As outlined in the introduction, the 'monocyte' stain (**Table 1**) is for identification of monocytes, the 'basic progenitor' stain (**Table 2**) is for identification of HSPC, CLP, GMP, MEP, and CMP populations, while the 'monocyte progenitor' stain (**Table 3**) is for identification of those populations as well as a further assessment of monocyte-myeloid progenitors.
- Antibodies should be titrated according to specific experimental requirements and cytometer use.
- For the 'monocyte' stain (**Table 1**), you should consider whether blocking with CD16/32 Fc block (cat#101302, BioLegend) for 10 minutes, with a subsequent wash, and then staining samples, will reduce any non-specific binding of antibodies. DO NOT block cells in the progenitor stains as CD16/32 is included within the staining panels.

**Protocol:**

- Resuspend cells in 100  $\mu$ L FACS wash.
- Add 50  $\mu$ L of bone marrow surface stain made in FACS wash as outlined in the tables on the next page.
- Mix by gently pipetting. Incubate for 30 minutes in the dark at 4°C.
- Add 1x Fix-Lyse up to 2 mL and incubator for 10 minutes at room temperature in the dark.
- Centrifuge at 4°C for 5 minutes at 2000 rpm. Remove supernatant.
- Resuspend cells in ~2 mL 1x PBS.
- Centrifuge at 4°C for 5 minutes at 2000 rpm. Remove supernatant.
  - Intracellular staining protocol can start here.
- Resuspend cells in 200  $\mu$ L FACS wash and store at 4°C in the dark until ready to run samples.

**Table 1.** Fluorophore-conjugated antibody mix for ‘monocyte’ surface stain

Marker	Conjugated Fluorophore	Company	Catalogue No. and Clone	1x Volume ( $\mu$ L)
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	AF 700	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

**Table 2.** Fluorophore-conjugated antibody mix for ‘basic progenitor’ surface stain

Marker	Conjugated Fluorophore	Company	Catalogue No. and Clone	1x Volume ( $\mu$ L)
Sca-1 (Ly6A)	PE-Dazzle 594	BioLegend	108137 (D7)	0.5
CD117 (cKit)	BV421	BioLegend	105827 (2B8)	0.5
CD127 (IL7R $\alpha$ )	PerCP-Cy5.5	BioLegend	121114 (A7R34)	0.5
CD34	APC	BioLegend	128606 (HM34)	0.5
CD16/32	BV711	BioLegend	101337 (93)	0.125
Gr-1	PE-Cy7	eBioscience	25-5931-83 (RB6-8C5)	0.25
CD3	PE-Cy7	eBioscience	25-0031-82 (145-2C11)	0.125
Ter119	PE-Cy7	eBioscience	25-5921-81 (TER-119)	0.125
B220/CD45R	PE-Cy7	eBioscience	25-0452-81 (RA3-6B2)	0.125
CD11b	PE-Cy7	eBioscience	25-0112-81 (M1/70)	0.25
			FACS Wash	47.25
			Total volume	50

**Table 3.** Fluorophore-conjugated antibody mix for ‘monocyte-myeloid progenitor’ surface stain

Marker	Conjugated Fluorophore	Company*	Catalogue No. and Clone	1x Volume (µL)
CD115 (M-CSF R)	AF488	eBioscience	53-1152-80 (AFS98)	0.5
CD135 (Flt-3/Flk-2)	PE	BioLegend	135305 (A2F10)	0.5
Sca-1 (Ly6A)	PE-Dazzle 594	BioLegend	108137 (D7)	0.5
CD127 (IL7Rα)	PerCP-Cy5.5	BioLegend	121114 (A7R34)	0.5
Ter119	PE-Cy7	eBioscience	25-5921-81 (TER-119)	0.125
CD3	PE-Cy7	eBioscience	25-0031-82 (145-2C11)	0.125
B220/CD45R	PE-Cy7	eBioscience	25-0452-81 (RA3-6B2)	0.125
CD117 (cKit)	BV421	BioLegend	105827 (2B8)	0.5
Ly6C	BV510	BioLegend	128033 (HK1.4)	0.125
CD16/32	BV711	BioLegend	101337 (93)	0.125
CD34	AF 647	BioLegend	128606 (HM34)	0.25
Ly6G	AF 700	BioLegend	127622 (1A8)	0.125
CD11b	APC-Cy7	BioLegend	101225 (M1/70)	0.125
FACS Wash				46.375
Total volume				50

**NB.** A live-dead stain is not used in these protocols as the viability of cells collected from fresh bone marrow is generally high, but if there are concerns about viability, this can be determined (i.e. by Trypan blue staining and hemocytometer) and the staining panels should be adjusted to incorporate a viability dye or live-dead marker stain. In particular, if planning to use frozen bone marrow, a marker of viability should be incorporated, and it should be carefully considered whether it is most appropriate to stain a specific number of ‘live’ cells (determined by Trypan blue staining and hemocytometer) rather than a specific sample volume.

#### **BONE MARROW INTRACELLULAR STAINING FOR KI67 STAINING** (optional)

Intracellular staining can be performed without stimulation with permeabilization after surface staining.

If intracellular staining is performed, samples should be run on the cytometer on the same day.

- Starting point:
  - If starting intracellular immediately after surface staining, remove the ~2 mL 1x PBS wash after centrifugation.
  - If samples were resuspended in 200 µL FACS wash and stored in fridge, add ~300 µL FACS wash and centrifuge at 4°C for 5 minutes at 2000 rpm. Remove supernatant.
- Resuspend cells in 500 µL room temperature 1x Permeabilization Buffer.
- Incubate in the dark for 30 minutes at room temperature.
- Centrifuge at RT for 5 minutes at 2000 rpm. Remove supernatant.
- Prepare antibody mix in 1x Permeabilization Buffer (see table). Resuspend cell pellets in 50 µL of antibody mix, and incubate in the dark for 30 minutes at room temperature.
- Add ~2 mL FACS wash and centrifuge for 5 minutes at 2000 rpm.
- Resuspend cells in 200 µL FACS wash and store at 4°C in the dark until ready to run samples.

**Table 4.** Fluorophore-conjugated antibody mix for intracellular Ki67 staining

Marker	Conjugated Fluorophore	Company	Catalogue No. and Clone	1x Volume (µL)
Ki67	Brilliant Violet 605	BioLegend	652413 (16A8)	0.25
FACS Wash				47.75
Total volume				50

### **PREPARATION OF SAMPLES FOR FLOW CYTOMETRY ANALYSIS**

- After pipetting several times to resuspend cells, transfer total volume onto the mesh (40 µm) of 5 mL polystyrene Falcon tubes (blue lids) (or filter through mesh into a U-bottom plate).
- Centrifuge tubes at 1500 rpm for 5 min at 4°C to remove cell clumps. Liquid should be at the bottom of the tube, not on the mesh, after centrifugation.
- Protect from light by covering with aluminum foil.
- If using **count beads** for absolute quantification of cells in samples:
  - Vortex count beads vial for 15 seconds and add 5 µL of beads per sample.
  - Record concentration of count beads – this varies batch-to-batch.

### **PREPARATION OF COMPENSATIONS**

- Prepare one 5 mL Falcon tube (without lid) for each fluorophore and the unstained control.
- Add 150-300 µL FACS Wash to each “fluorophore” tube.
- Add 1 mL FACS Wash to the “unstained” tube (100 µL x # of fluorophore tubes).
- Vortex OneCompBeads vial for 10 seconds.
- Add compensation beads into the “unstained” tube (1 drop for every 4-5 fluorophore tube).
- Vortex tube and distribute 100 µL from “unstained” tube to each “fluorophore” tube.
- Add 150-300 µL of FACS Wash (300 µL for flow beginners) to the “unstained” tube.
- Add 0.5 µL of appropriate antibody to each tube.
- Gently shake the rack and protect from light by covering with aluminum foil.

**\*See general flow cytometer protocol for further instructions\***

## ANALYSIS/SOFTWARE

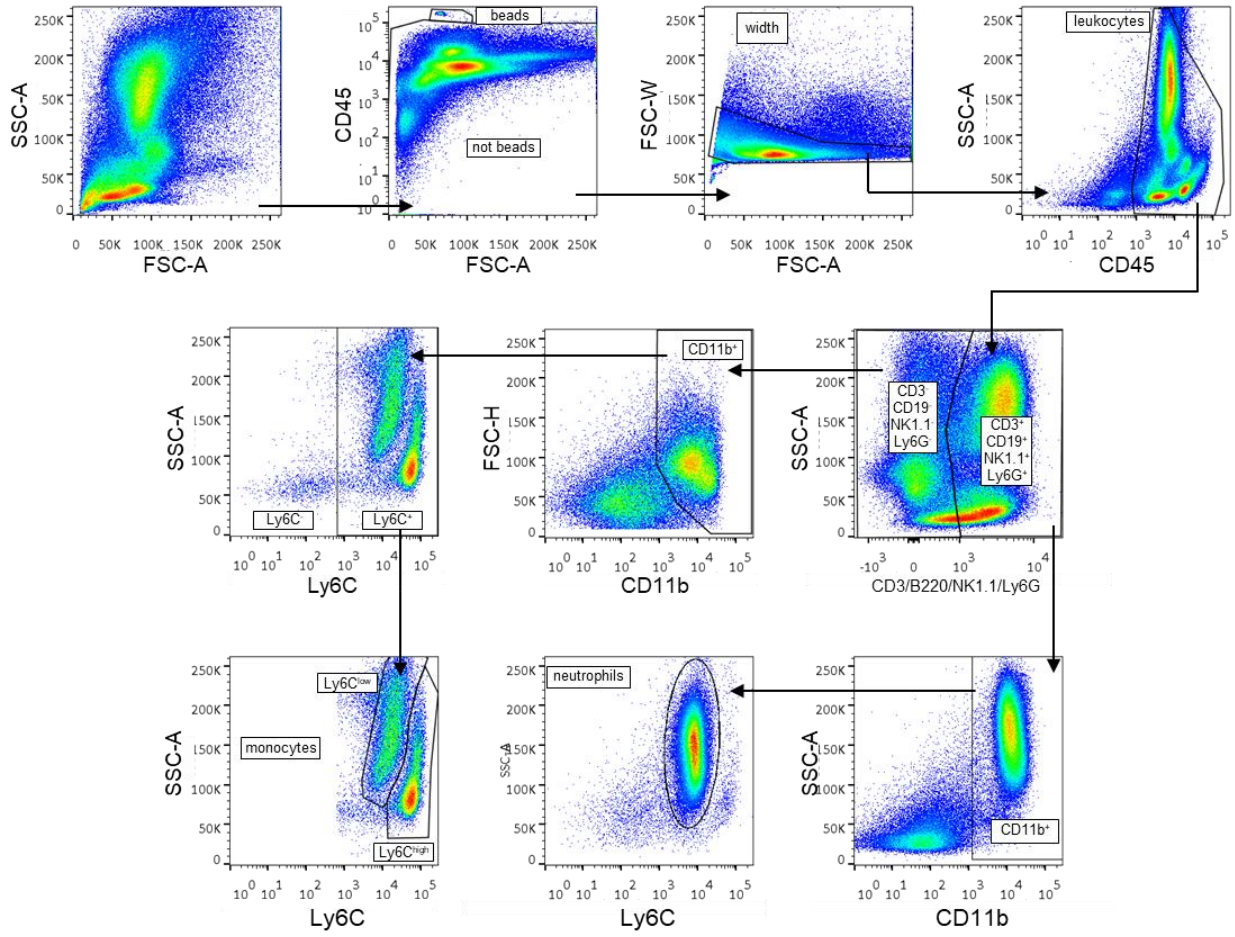
- Machine: LSR Fortessa (or other suitable cytometer with appropriate lasers and configuration)
- Software: FlowJo (TreeStar)
- Gating: See attached diagram as a reference for surface staining. This varies depending on which markers are included within the stain, as well as whether intracellular staining was included within the analysis.

## LINKS AND REFERENCES

- Challen, G. A., Boles, N., Lin, K. K.-Y., & Goodell, M. A. (2009). Mouse hematopoietic stem cell identification and analysis. *Cytometry. Part A : The Journal of the International Society for Analytical Cytology*, 75(1), 14–24.  
<http://doi.org/10.1002/cyto.a.20674>
- Hettinger, J., Richards, D. M., Hansson, J., Barra, M. M., Joschko, A. C., Krijgsveld, J., & Feuerer, M. (2013). Origin of monocytes and macrophages in a committed progenitor. *Nature Immunology*, 14(8), 821–830.  
<http://doi.org/10.1038/ni.2638>
- Toxavidis, V., Tigges, J., & Alberich Jorda, M. Hematopoietic Bone Marrow Cell Sorting Without Compromise: 7 Lasers, Eight Colors, Six Way Sorting A Novel Approach. Beckman Coulter White Paper. Available from  
<https://www.beckman.com/gated-media?mediald={38BF4CEA-379E-4B3A-8D98-F0932C9ABFA5}>
- Loukov, D., Naidoo, A., Puchta, A., Marin, J.L.A., Bowdish, D.M.E. (2016). Tumor necrosis factor drives increased splenic monopoiesis in old mice. *Journal of Leukocyte Biology*, 100(1):121-9.  
<http://doi.org/10.1189/jlb.3MA0915-433RR>

# Flow Cytometry Analysis - Bone Marrow 'Monocyte' Gating

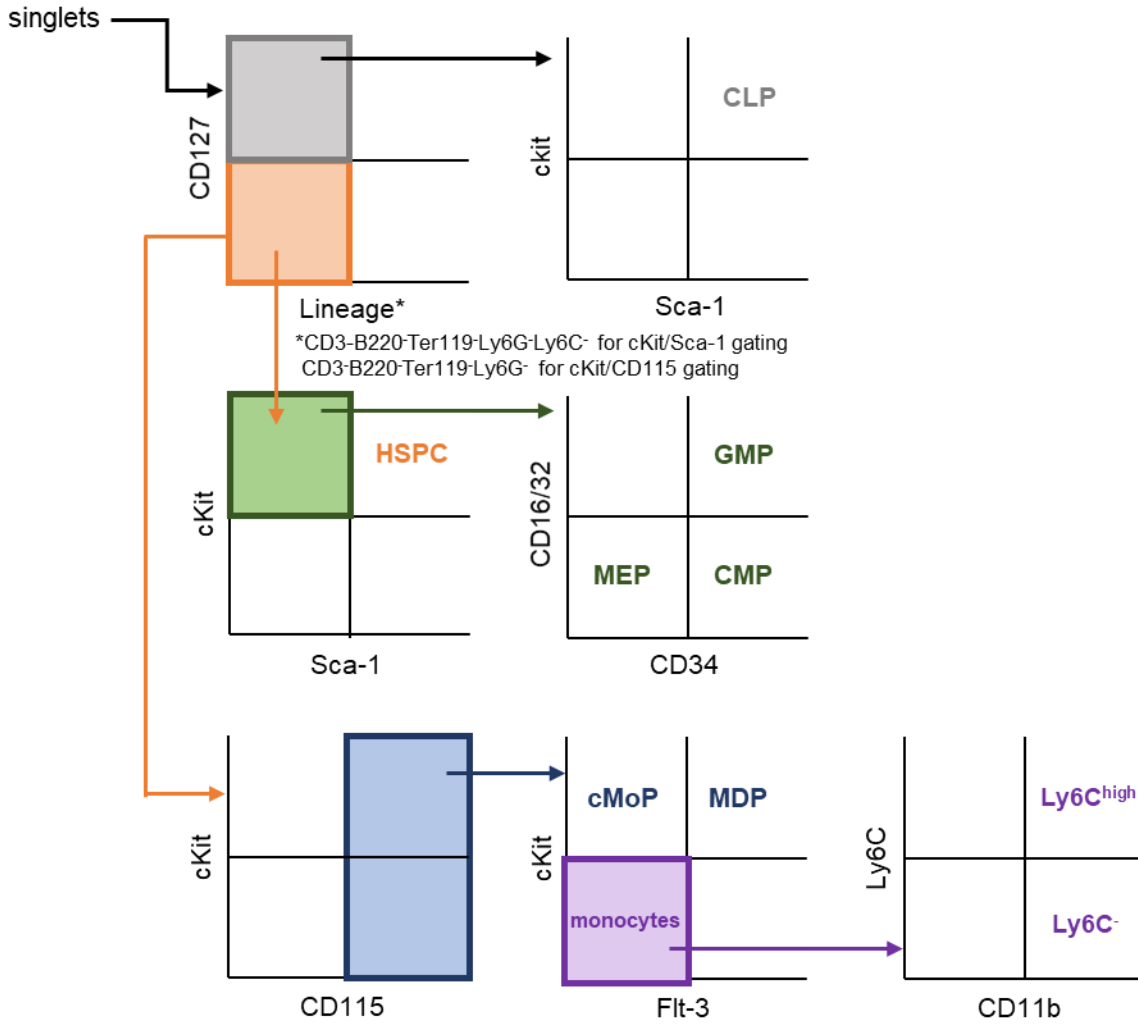
## Bone Marrow Monocyte Surface Stain





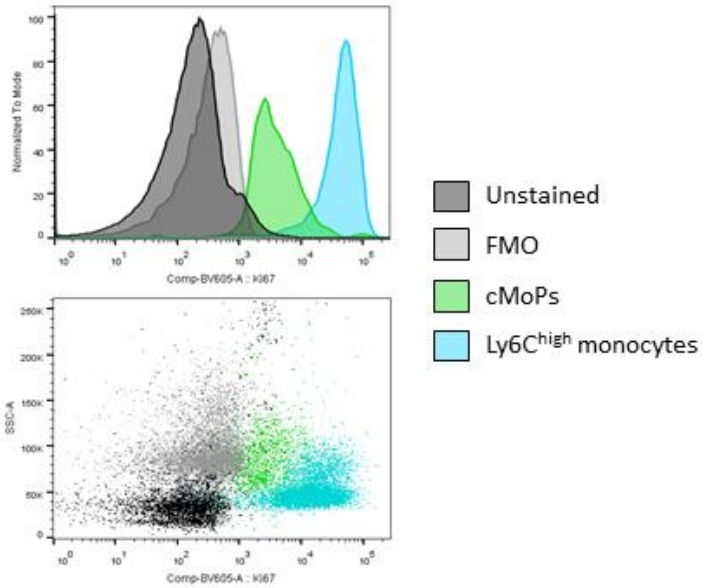
# Flow Cytometry Analysis - Bone Marrow 'Progenitor' Gating

## Bone Marrow Progenitor Stains



## Flow Cytometry Analysis - Bone Marrow Gating – Intracellular Staining

### Intracellular Staining for Ki67 - example



Intracellular staining of Ki67 should be assessed by measuring geometric mean fluorescence intensity.