Surface staining of mouse splenocytes and peripheral blood cells

APPLICATION NOTE

Authors: John F. Woolley[1,2], PhD
Leonardo Salmena[1,2], PhD

Affiliation:
1. Department of Pharmacology and Toxicology, University of Toronto, Toronto, Canada
2. Princess Margaret Cancer Centre, University Health Network, Toronto, Canada

IN THIS PAPER YOU WILL

Learn about multiparametric and mixed population flow cytometry	Find a brief protocol for preparing mouse splenocytes and peripheral blood for flow cytometry	Learn gating strategies for mixed population flow cytometry

Background

A major advantage of flow cytometry is the ability to analyze complex mixtures with different cell types. Antigen presentation on the surface allows for the identification of discreet cell types within a mixed sample (e.g. blood) simply *via* staining with antibodies conjugated to various fluorophores. Typically, little or no sample preparation is required for surface marker staining other than singlet cell isolation. Thus, flow cytometry has become the gold standard method for analysis of complex cell mixtures, from blood, spleen, lymph or bone marrow. Here, we describe a method to rapidly identify CD3ε -positive and CD19-positive cells from murine spleen and peripheral-blood samples.

Introduction

Measurement of protein expression *via* flow cytometry offers many advantages in both clinical and research settings. Because it allows for multiparametric measurement, reduced sampling can be achieved. This is especially important in clinical monitoring or diagnosis, whereby sampling is often not trivial (e.g. blood or bone marrow draws). It also allows for separating complex/mixed samples to be separated using specific markers, and subsequent measurement of characteristics of interest. This is of particular interest in research using animal models. Such immunophenotyping assays are essential in order to characterize mouse models rapidly and accurately. Monitoring cell populations in blood is also extremely important when using animal models, as it allows rapid assessment of animal health and phenotypic changes throughout the lifespan of the animal. Here we look at CD3ε and CD19, in both the spleen and the peripheral blood. CD3ε is a member of the T cell receptor complex, essential for antigen recognition and signal propagation. It is a commonly used marker for T-cells. CD19 is a surface marker that couples with the antigen receptor of B lymphocytes and decreases the threshold for antigen receptor dependent stimulation. It is commonly used as a marker for B cells. With these two markers we provide a simple and rapid method to identify two discrete populations in murine splenocytes and blood.

Standard Procedure

All mouse experiments were performed using male C57/BL6 young-adult mice (10 weeks). Animals were obtained from the Jackson Laboratory (Bar Harbor, MA, USA). The animals were housed in standard on a 12-hour light-dark cycle and at a temperature of 23°C with free access to food and water in groups of 5 mice. All experimental protocols were approved by the research ethics board of this university and were carried out in compliance with the Canadian Council on Animal Care recommendations.

1. C57/BL6 mice were sacrificed at approximately 10 weeks of age.
2. Peripheral blood was taken from heart immediately *post mortem*. Briefly, a 26-gauge needle was inserted into the heart from the sternum. Approximately 500 µL of blood was drawn slowly and transferred to an EDTA coated tube to prevent clotting.
3. Spleens were isolated *post mortem* and placed in PBS on ice.
4. Splenocytes were collected by mashing the spleen through a 70 µM cell strainer utilizing the thumb-piece of a plunger removed from a 1 mL syringe; single cell splenocyte suspensions were collected in 5 mL of ice-cold PBS.
5. Splenocyte preparation was again passed through a 70 µM cell strainer to remove any remaining debris.
6. Cells were then transferred to a 15 mL centrifuge tube on ice.
7. This was then centrifuged at 400x g for 5 minutes.
8. Splenocytes were resuspended in 10 mL RBC lysis buffer and vortexed briefly. The cells were allowed to incubate for 10 minutes (see Note 1).
9. Cells were then centrifuged at 400x g for 5 minutes and supernatant was removed.
10. Cells were again resuspended in PBS, with 5 % FBS and kept on ice for 20 minutes.
11. Cells were then centrifuged at 400x g for 5 minutes.
12. Cells were resuspended in 100 µL staining buffer and incubated at room temperature in the dark for 20 minutes.
13. Cells were centrifuged 400x g for 5 minutes.
14. Cells were washed once with PBS.
15. Cells were resuspended in 200 µL of PBS.
16. Cells were analyzed on the Cytoflex flow cytometer.
17. Mouse spleen cells or peripheral blood cells stained with CD3ε-alexa fluor 488 and CD19-PerCP-Cy5.5 were gated for mononuclear cells (MNCs) according to FSC vs. SSC intensity.
18. Gated cells were further gated on FSC-area vs. FSC-height to discriminate singlet cells from doublet cells.
19. Singlet cells were then plotted on a dot-plot of CD3ε vs. CD19.

Materials & Methods

Material required but not supplied

Microcentrifuge, micro-pipetteman (P10, P20, P200, P1000), dissection kit, 70 µM cell strainer, 26-gauge needles and syringes, 15 mL centrifuge tubes, 1.5 mL microcentrifuge tubes, 2 mL EDTA coated microcentrifuge tubes

Reagents

1. Red Blood Cell (RBC) lysis buffer: 155 mM Ammonium Chloride (NH_4Cl), 12 mM Sodium Bicarbonate ($NaHCO_3$) and 0.1 mM EDTA were prepared in double distilled H_2O.
2. Phosphate buffered saline (PBS): 8g NaCl, 0.2g KCl, 1.44 g Na_2HPO_4, 0.24g KH_2PO_4 in 1 L of double distilled H_2O. pH 7.4.
3. Staining Buffer: 5 % FBS in PBS. 100 µL staining buffer contains 400 ng CD19-PerCP-Cy5.5 and 500 ng CD3e-AlexaFluor 488.
4. Staining Buffer: 5 % FCS in PBS, made fresh. If not made fresh, an antimicrobial agent such as sodium azide (at a concentration of 0.1 % *v/v*) should be added.

Laser	405nm					488nm					638nm		
Fluor	Krome Orange	Pacific Blue	V610	V660	V780	Alexa Fluor 488	PE	ECD	PerCP-Cy5.5	PC7	APC	APC AF700	APC AF750
Marker						CD3ε			CD19				
Clone						145-2C11			1D3				

Sample prep

Sample Type	Species	Age of specimen	Prep Method
Mouse splenocytes	C57/BL6	12 weeks	
Mouse Peripheral blood	C57/BL6	12 weeks	
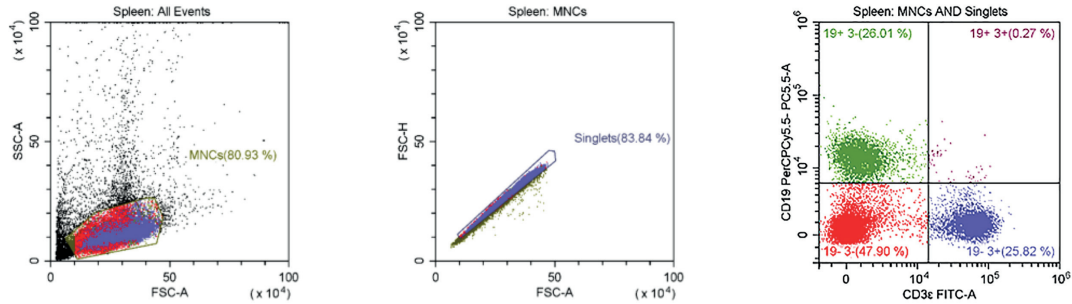
Tube Name: Spleen

Sample ID:

Population	Events	% Total	% Parent
⌄ ● All Events	30170	100.00%	100.00%
⌄ ● MNCs	24418	80.93%	80.93%
● Singlets	20473	67.86%	83.84%
⌄ ● MNCs AND Singlets	20473	67.86%	67.86%
● 19+ 3+	56	0.19%	0.27%
● 19+ 3-	5325	17.65%	26.01%
● 19- 3-	9806	32.50%	47.90%
● 19- 3+	5286	17.52%	25.82%
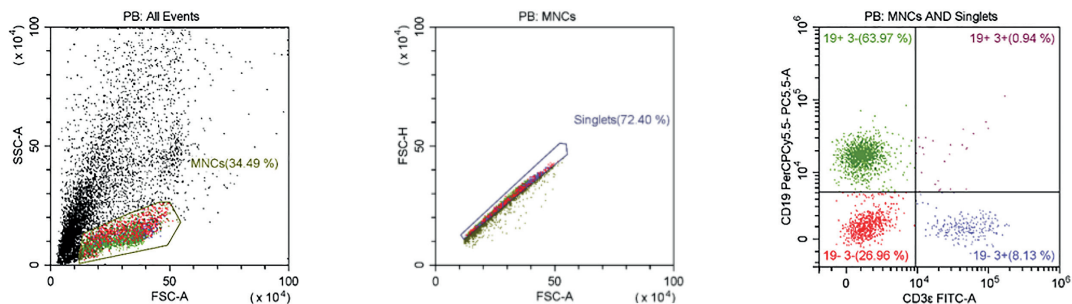
Tube Name: PB

Sample ID:

Population	Events	% Total	% Parent
⌄ ● All Events	11082	100.00%	100.00%
⌄ ● MNCs	3822	34.49%	34.49%
● Singlets	2767	24.97%	72.40%
⌄ ● MNCs AND Singlets	2767	24.97%	24.97%
● 19+ 3+	26	0.23%	0.94%
● 19+ 3-	1770	15.97%	63.97%
● 19- 3-	746	6.73%	26.96%
● 19- 3+	225	2.03%	8.13%

Figure 1 Legend: Surface staining of mouse splenocytes and peripheral blood cells with CD3ε and CD19.

(a) Gating strategy for C57/BL6 murine splenocytes on a FSC *vs.* SSC dot-plot. Viable cells were further gated to remove possible doublet cells on a dot-plot for FSC-area *vs.* –height. Singlet cells were then analyzed for CD3ε and CD19 expression, in the FL1 and FL3 channels respectively.

(b) a) Gating strategy for C57/BL6 murine peripheral blood cells on a FSC *vs.* SSC dot-plot. Viable cells were further gated to remove possible doublet cells on a dot-plot for FSC-area *vs.* –height. Singlet cells were then analyzed for CD3ε and CD19 expression, in the FL1 and FL3 channels respectively.

Results

In both the peripheral blood and spleen samples, cells were gated to exclude debris and the majority of erythrocytes that were not lysed in the RBC lysis step. Typically, not all RBCs will be removed by this lysis but enough will have been removed to allow for efficient gating. Cells were also gated to remove any doublets present. We then examined both the CD3ε and CD19 staining on a dot-plot. Here we see excellent separation of both CD3ε-positive and CD19-positive cells. We see effectively no double-positive cells and thus can easily identify the T- and B-cell populations, with particularly good separation in the spleen sample.

This rapid detection of these two important populations could also be joined with other surface markers to identify more populations such as monocytes, macrophages, dendritic cells etc.

The ability to identify two populations in this way, simply and rapidly, allows for monitoring of these populations. This can be done, of course, with tail bleeds also throughout the life of the mice.

Notes

It is desirable to removed erythrocytes from spleen mononuclear cell preparations prior to flow cytometry experiments as large numbers of RBCs in the sample can occlude populations of interest. A small number of RBCs remaining in the sample will not prove difficult to gate out however, so partial lysis of RBCs is sufficient and should be optimized depending on the individual experiment being performed.

Reagent Details

Reagent	Supplier	Order Details
CD3ε-Alexa Fluor 488	Biolegend	Cat. # 100321
CD19-PerCP-Cy5.5	eBiosciences	Cat. # 45-0193-80

For Research Use Only. Not for use in diagnostic procedures.