Standard Operating Procedure	Title: FACS analysis of peripheral blood cells	
	Doc. Number: ESLIM_018_001 Rev No.	Date Issued: 01/06/04

1.0 Purpose:

1.1 The characterisation of of main leukocyte subsets in peripheral blood cells from mice by flow cytometry.

Reliable values of frequencies of leukocyte clusters are very much dependent on the appropriate preparation, acquisition and gating of leukocytes. This method involves ammonium chloride erythrocyte lyses, thus preventing interference by larger amounts of erythrocytes. For all stainings, a monoclonal antibody to the cell surface glycoprotein CD45 is used to be able to create a CD45+ gate, allowing discrimination of leukocytes from debris, erythrocytes and thrombocytes. Furthermore, staining with propidium iodide (PI) gates out dead cells. Samples are acquired until a number of 30000 living CD45+ cells is reached for each sample (ref Adler and Busch 2007, submitted). Data are compensated analysed and using FlowJo software.

This protocol is part of the EMPReSSslim pipeline and is carried out on a cohort of 10 males and 10 females (age matched) at a time.

2.0 Scope:

2.1 Individuals who have been trained, and are competent in performing the procedures described herein must follow this procedure.

2.2 Any queries, comments or suggestions, either relating to this SOP in general or to a specific problem encountered during a procedure, should be addressed to the Project Leader.

2.3 Any deviances from this protocol must be reported to the Project Leader.

3.0 Safety Requirements:

3.1 General laboratory procedures should be followed, which include: no eating, no chewing gum, no drinking, and no applying of cosmetics in the work area. Laboratory coats must be worn at all times in the work area, unless the protocol specifically describes the appropriate attire for the procedure.

4.0 Associated Documents:

4.1 ESLIM_028_001: Titration of antibody solutions for FACS analysis

4.2 ESLIM_018_001_Appendix_1: FACS analysis – Appendix 1: Definition of main lineages

4.3 ESLIM_024_001: Blood collection by retro-orbital puncture

5.0 Notes:

5.1. The selection of antibody-conjuagte and the design of the staining panel has to be adapted to each laboratory, depending on the flow cytometer in the facility. It is possible to use a broader antibody-panel, as long as the gating strategy (appendix) is respected.

6.0 Quality Control:

6.1 Before the acquisition of the samples, run a sample of calibration beads, sphero Ultra Rainbow, 3 µm ϕ (spherotech Cat. URFP-30-2), in order to test the regular running of the flow cytometer (LSR II).

7.0 Equipment:

7.1 Microhematocrit tube

7.2 Centrifuge (enabling centrifugation of tubes/Microtiter plate, and temperature control)

7.3 7 colour Flow cytometer (LSR II)
Example of configuration in table 1:

Table 1:

Laser	P	Longpass Dichroic Mirror	Bandpass Filter	Fluochrome	blood staining 1	blood staining 2
Blue: 488nm	G	-	488/10		Side scatter	Side scatter
	F	505LP	530/30	FITC		IgD
	E	550LP	575/26	PE		NK1.1/U5A 2
	D	600LP	610/20		Propidium Iodide	Propidium Iodide
	B	685LP	695/40	PerCPCy55	CD4	Gr1
	A	755LP	780/60	PECy7		CD19
Violet: 405nm	B	-	450/50	Pacific Blue	CD3	CD11b
Red: 633nm	C	-	660/20	APC	CD25	CD5
	B	710LP	730/45	Alexa Fluor 700	CD45	CD45
	A	755LP	780/60	APC-Alexa750	CD8a	

7.4 FlowJo software (for data analysis)

8.0 Supplies:

8.1 Eppendorf tubes

8.2 Microtiter plate, round bottom

8.3 Heparin: Liquemin® N 25000 (Roche)

8.4 NH4Cl-Tris (room temperature)

8.5 FACS buffer
1000ml PBS, 5g BSA, 660µl NaN_3 30%, pH 7.45, filtered

8.6 Lysis buffer:
45ml sol.A + 5ml sol.B. –
sol.A: 1000ml aqua dest., 9.1 g (=0.17M) NH4Cl, filtered
sol.B: 1000ml aqua dest., 28.8g Tris-HCl, pH 7.5, filtered.

8.7 Propidium Iodide (PI) solution:
1:500 of stock solution
2mg/ml Propidium Iodide in PBS (stored at -20°C)

8.8 Fc-block-solution:
1:500 of stock solution (2mg/ml) in FACS buffer

8.10 Antibodies:
Prepare sufficient amount of antibody-mix within FACS buffer, having calculated the amount of antibodies to be used, based on titration (see EMPReSS SOP 6_001 Revision 0: Titration of antibody solutions for FACS analysis). Tables 2 and 3 describe the staining panels for EMPReSSslim pipeline.

Table 2: Staining panel 1-mix:

Name	**Stock**	**Ref**
PerCpCy55-conjugated Rat anti-mouse CD4 (RM4-5)	1:1000 of 0.2 mg/ml	BD 5500954
PB-conjugated Rat anti mouse CD3 (17A2)	1:100 of 0.2 mg/ml	eBioscience 57-0032-82
APC-conjugated Rat anti-mouse CD25 (PC61)	1:100 of 0.2 mg/ml	BD 557192
APC-Alexa750-conjugated Rat anti-mouse CD8a (53-6.7)	1:400 of 0.1 mg/ml	Caltag MCD0827
Alexa Fluor 700-conjugated Rat anti-mouse CD45 (30F11)	1:2000 of 0.5 mg/ml	BioLegend 103128

Table 3: Staining panel 2-mix

Name	**Stock**	**Ref**
FITC-conjugated Rat anti-mouse IgD (11-26c.2a)	1:1000 of 0.5 mg/ml	BD 553439
PE-conjugated Rat anti-mouse NK-T/NK (U5A2-13)	1:200 of 0.2 mg/ml	BD 550082
PE-conjugated Rat anti-mouse NK1.1 (PK136) (Note: to be used only for C57BL/6 mice)	1:200 of 0.2 mg/ml	BD 553165
PerCpCy55-conjugated Rat anti-mouse Gr1 (RB6-8C5)	1:800 of 0.2 mg/ml	BD 552093
PECy7-conjugated Rat anti mouse CD19 (ID3)	1:1000 of 0.2 mg/ml	BD 552854
PB-conjugated Rat anti mouse CD11b (M1/70)	1:800 of 0.1 mg/ml	Caltag RM2828
APC-conjugated Rat anti-CD5 (53-7.3)	1:2000 of 1mg/ml	BD 550035
Alexa Fluor 700-conjugated Rat anti-mouse CD45 (30F11)	1:2000 of 0.5 mg/ml	BioLegend 103128

8.13 Single Colour Control:

Rest of blood from all mice is pooled and used for single colour controls. Single colour controls (SCC) contain a single antibody and propidium iodide (PI), and are prepared simultaneously. Furthermore, one unstained sample (also containing PI) is prepared.
In table 4 is the antibody used only for single colour controls in order to compensate PI-channel):

Table 4: PI channel compensation antibody

Name	**Stock**	**Ref**
PE-Alexa Fluor 610-conjugated Rat anti-mouse CD4 (RM4-5)	1:100 of 0.1 mg/ml	Caltag MCD0422

9.0 Procedure:

This protocol is carried out on a cohort of 20 mice (10 males and 10 females). It uses 7 colour flow cytometry with a 2 panel staining system.

9.1 Put 10µl of Heparin into an Eppendorf tube.

9.2 Collect 300µl of blood from the tail vein of the mouse (see EMPReSS SOP 3_011 Revision 0: Blood collection by tail venipuncture) and mix gently with the heparin. Repeat for all the mice in the cohort.

9.3 Centrifuge the samples (530RCF, 3 min, 10°C) and take 50-100µl of the plasma from each sample for further analysis.

9.4 Resuspend each cell pellet in 500µl NH4Cl-Tris (room temperature) and filter the solutions through a nylon net into separate 1.5ml Eppendorf tubes.

9.5 Shake the tubes for 10 minutes at room temperature

9.6 For each mouse sample, pipette 200µl of the cell suspension into 2 wells of a U-bottomed 96-well plate.

9.7 Pool the remaining cell suspensions and pipette 100µl into 18 wells for single colour controls (SCC) and 1 well for an unstained control.

9.8 Centrifuge the plate (530RCF, 3 min, 10°C). Discard the supernatants.

9.9 Resuspend the cells in 200µl NH4Cl-Tris (RT) per well and shake the plate for 10 minutes at room temperature.

9.10 Centrifuge the plate (530RCF, 3 min, 10°C). Discard the supernatant.

9.11 Resuspend the cells in 200µl FACS buffer per well.

9.12 Centrifuge the plate (530RCF, 3 min, 10°C). Discard the supernatant.

9.13 Resuspend the cells in 50µl Fc-block-solution per well, and incubate on ice for 20 minutes.

9.14 Add 150µl FACS buffer to each well and centrifuge the plate (530RCF, 3 min, 10°C). Discard the supernatant.

9.15 Resuspend the cells in 50µl Antibody-Mix as follows:

i. In the 2 wells that originally contained 200µl cells use the Antibody mix for staining panel 1 and staining panel 2 respectively.
ii. In the18 wells which originally contained 100µl pooled cells, use a different single antibody for each well. This is the SCC.
Note: to compensate the PI-channel antibody conjugated to PE-Alexa Fluor 610 is used.
iii. The 1 remaining well is resuspended in 50µl FACS buffer as an unstained control.

9.16 Protect from the light and incubate on ice for 15 minutes.

9.17 Add 50µl PI solution per well and then incubate in the dark, on ice, for a further 5 minutes.

9.18 Add 100µl FACS buffer to each well and centrifuge the plate (530RCF, 3 min, 10°C). Discard the supernatant.

9.19 Wash the cells by adding 200µl FACS buffer per well and centrifuge the plate (530RCF, 3 min, 10°C). Discard the supernatant. Repeat this a wash further four times.

9.20 Resuspend the cells in 100µl FACS buffer and store at 4°C until data acquisition.

9.21 Define a "stopping gate" of living CD45 positive cells as 30 000 living cells. Data will be acquired until this number is reached. Storage gate is defined as 'all events'.

9.22 Complete interpretation of the results, including compensation, using the FlowJo software.

10.0 Supporting information

11.0 History Review:

17 Aug 08: Track changes accepted and SOP numbers updated