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STANDARD OPERATING PROCEDURE PACKET

SANGER INSTITUTE
STANDARD OPERATING PROCEDURE

SUBJECT: Peripheral Blood Flow Cytometry Analysis of Major Leukocyte Populations using the buffy coat

INTRODUCTION

This SOP details the characterisation of the main leukocyte populations in peripheral blood from mice by flow cytometry using the buffy coat layer after centrifugation of whole blood. It uses 8-colour flow cytometry with a 2 panel staining system.

ABBREVIATIONS

BSA – bovine serum albumin
FACS – fluorescence activated cell sorting
FMO – fluorescence minus one
PBL – peripheral blood leukocytes
PBS – phosphate buffered saline
PI – propidium iodide
PPL – project licence
RCF – relative centrifugal force
SSC – single stain control

HEALTH & SAFETY

- Appropriate personal protective equipment is to be worn at all times when handling the samples
- Eye wash stations, safety showers and fire extinguishers are to be located within the laboratory
- Appropriate training and familiarization with risk assessment must be undertaken before use of the BD LSRII flow cytometer
- Users should be familiar with the relevant Risk Assessments before commencing the procedure.

RESPONSIBILITIES

All staff performing this procedure are responsible for ensuring that this SOP has been read, understood and where applicable is followed in accordance with the relevant project licence (PPL). All staff should be trained and competent to perform the procedure, where applicable they should also be licensed to perform the procedure.

RESOURCES

Equipment:

1. Eppendorf tubes (1.5ml & 2ml)
2. 96 well Microtiter plate, round bottom
3. Lithium Heparin-containing blood collection tubes (1ml)
4. Timer
5. P1000 and P200 single channel & 30-300 multi channel pipettes + tips
6. Multidispense pipette with 5ml and 25ml Combitips
7. Ice box filled with ice

8. Reservoirs
9. Tube rotator
10. Refrigerated microcentrifuge
11. Plate centrifuge
12. 50ml Falcon tubes + Virkon
13. Vortex

14. Lysis buffer: BD Pharm Lyse (BD 555899) - 1:10 dilution with dH_2O

15. FACS buffer
    - 1000ml 1xPBS (no Ca2+ or Mg2+) + 5g BSA, pH 7.45, filtered (store at 4°C)

16. Propidium Iodide (PI) solution (used to distinguish viable and non-viable cells):
    - 1mg/ml PI in FACS buffer (Sigma, store at 4°C). Prepare a 2x solution by diluting stock 1:200 in FACS buffer. When added to cells this will be further diluted 1:2 to give final concentration of 2.5ug/ml

17. Fc-block-solution:
    - Mouse BD FcBlock (cat no. 553142). Dilute to 10ug/ml in FACS buffer. Stock is 500ug/ml therefore dilute 1:50

18. Single Colour Controls – prepare fresh each week:
    - Remaining cells from all mice are pooled and used for single colour controls. Single colour controls (SCC) contain a single antibody at the dilutions described in appendix 2 (plus PI) and are prepared simultaneously. Fluorescence minus one (FMO) controls are also used as detailed in the tables below. Furthermore, one unstained sample and cells stained only with PI are prepared.

19. Antibodies – prepare fresh each week:
    - Prepare sufficient amount of antibody-mix with FACS buffer, having calculated the amount of antibodies to be used, based on titration. Tables below describe the staining panels.

Table 1: Staining panel 1

Name	Stock	Ref
PerCP-Cy5.5-conjugated Rat anti-mouse CD4 (RM4-5)	1:1000 of 0.2mg/ml	BD 550954
PB-conjugated Rat anti mouse CD3 (17A2)	1:200 of 0.2mg/ml	eBioscience 57-0032-82
APC-conjugated Rat anti-mouse CD25 (PC61)	1:100 of 0.2mg/ml	BD 557192
APC-H7-conjugated Rat anti-mouse CD8a (53-6.7)	1:200 of 0.2mg/ml	BD 560247
PE-conjugated Rat anti-mouse NK1.1 (PK136; for C57BL/6) (panel 1 & 2) OR PE-conjugated Rat anti-mouse NKp46 (29A1.4; for non-C57BL/6 strains)	1:400 of 0.2 mg/ml 1:200 of 0.2 mg/ml	BD 553165 eBioscience 12-3351
FITC-conjugated Rat anti-mouse CD44 (IM7)	1:2000 of 0.5 mg/ml	BD 553133

PE-Cy7-conjugated Rat anti-mouse CD62L (MEL-14)	1:2000 of 0.1 mg/ml	ab25569

Note: highlighted antibodies only added for certain protocols.

Table 2: Staining panel 2

Name	Stock	Ref
FITC-conjugated Rat anti-mouse IgD (11-26c.2a)	1:1000 of 0.5 mg/ml	BD 553439
PerCP-Cy5.5-conjugated Rat anti-mouse Gr1 (RB6-8C5)	1:800 of 0.2 mg/ml	BD 552093
PE-Cy7-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
Alexa Fluor 700-conjugated Rat anti-mouse CD45 (30F11) (panel 1 and 2)	1:1000 of 0.5 mg/ml	BioLegend 103128

FMO 1		**dilution**
CD25	APC	100
CD4	PerCPCy5.5	1000
CD3	Pacific Blue	200
CD8	APC-H7	200
CD44	FITC	2000
CD62L	PE-Cy7	2000
CD45	Alexa-F700	1000
FMO 2		**dilution**
NK1.1	PE	400
CD4	PerCPCy5.5	1000
CD3	Pacific Blue	200
CD8	APC-H7	200
CD44	FITC	2000
CD62L	PE-Cy7	2000
CD45	Alexa-F700	1000

Note: highlighted antibodies only added for certain protocols.

Associated SOPs/ documentation:

- SOP0078 – LSRII operation
- SOP0099 - FlowJo analysis of PBL data (Mac)

Staff: This procedure requires one member of staff

CYTOMETER CONFIGURATION INFORMATION

Table 3. BD LSR II configuration

Laser	P	Longpass Dichroic Mirror	Bandpass Filter	Fluorochrome	*blood staining panel 1*	*blood staining panel 2*
Blue: 488nm	**G**	-	488/10		Side scatter	Side scatter
	F	505LP	530/30	FITC	CD44	IgD
	E	550LP	575/26	PE	NK1.1 (or NKp46)	NK1.1 (or NKp46)
	D	600LP	610/20	PI (Texas Red)	Propidium iodide	Propidium iodide
	B	685LP	695/40	PerCP-Cy5.5	CD4	Gr1
	A	755LP	780/60	PE-Cy7	CD62L	CD19
Violet: 405nm	**B**	-	450/50	Pacific Blue	CD3	CD11b
Red: 633nm	**C**	-	660/20	APC	CD25	
	B	710LP	730/45	Alexa Fluor 700	CD45	CD45
	A	755LP	780/60	APC-H7	CD8a	

PROCEDURE

1. Collect blood via retro-orbital bleeds within heparin coated tubes. Centrifuge samples at 5000RCF, 10 min, 8°C, remove the plasma layer and immediately proceed to next step to avoid clot formation.

2. For each sample, pipette 300 µl of blood into a 2ml Eppendorf tube, taking care to transfer leukocytes from the buffy coat (plasma/RBC interface).

3. Add 1.5ml lysis buffer to each tube and place on tube rotator (20rpm) for 8 minutes at room temperature (start timer when tubes go on to rotator).

4. Centrifuge the tubes (400RCF, 3 min, 10°C). Discard the supernatants into a tube containing a small amount of Virkon.

5. Re-suspend the pellet in 1.5ml of lysis buffer and place on tube rotator (20rpm) for 8 minutes at room temperature (RT).

6. Centrifuge the tubes (400RCF, 3 min, 10°C) and discard the supernatants. Re-suspend each cell pellet in 500µl FACS buffer.

7. Vortex each tube briefly at medium speed. Use quick pulses to break up the pellet.

8. Transfer 100µl of the cell suspension, avoiding any clumps, into a U-bottomed 96 well into both Tube 1 and Tube 2 positions (see Appendix 1 for plate layout).

9. Pool the remaining cell suspensions (100 µl from ~20 samples) in a 2ml Eppendorf tube.

10. Pipette 100µl of this pooled sample into the 16 wells reserved for unstained

cells, single color controls (SCC) and Fluorescence Minus One (FMO) controls.

11. After plating out add 100µl FACS buffer per well.

12. Centrifuge the plate (400RCF, 3 min, RT). Discard the supernatants.

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

14. Centrifuge the plate (400RCF, 3 min, RT). Discard the supernatant.

15. Resuspend the cells in **50µl** FcBlock per well and incubate for 15 minutes **on ice**.

16. Add 150µl FACS buffer to each well and centrifuge the plate (400RCF, 3 min, RT). Discard the supernatant.

17. Resuspend the cells in **50µl** Antibody-Mix as seen in tables 1 and 2.

18. To SSC 1 (unstained) & SSC 2 (PI) – this is wells A1 and B1, working vertically – add 50ul FACS buffer each.

19. To SSC wells 3-14 (see Appendix 2) and FMO wells 1 & 2 (G2 & H2), add 50µl of the appropriate SSC or FMO.

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

21. Add **50µl** PI solution per well (**excluding unstained cells - SSC1, well A1**) and then incubate for a further 5 minutes **on ice** in the dark.

22. Centrifuge the plate (400RCF, 3 min, RT). Discard the supernatant.

23. Wash the cells by adding 200µl FACS buffer per well and centrifuge the plate (400RCF, 3 min, RT). Discard the supernatant.

24. Repeat this wash once more. [If necessary cells can be fixed following this stage, see below]

25. Re-suspend the cells in 200µl FACS buffer and store on ice in the dark until acquisition.

26. A “stopping gate” of living (PI negative) CD45 positive cells is defined in the FACSDiva Software and is set to collect 30000 events. Data will be acquired until this number is reached.
    a. See **SOP0078 - LSRII operation** for how to setup the experiment and QC steps
    b. Once acquisition is finished, run a cleaning plate and set the cytometer to **Standby mode**. The LSRII will continue to produce waste whilst on run mode and can overfill the waste container.

27. Complete interpretation of the results, including compensation, using the FlowJo software (See SOP0099 - FlowJo analysis of PBL data (Mac)).

Fixation of Cells

In the event that cells cannot be analysed on the day of collection (e.g. flow cytometer inoperable) then cells can be fixed and stored for up to 96h (preferably within 48h) before analysis without significant change in data

Fixation Procedure

- Prepare Formaldehyde fixation buffer by diluting 37% formaldehyde (Sigma, cat. Number F1635) 1:10 in PBS (no Ca2+ or Mg2+)
- Following the final 2 wash steps after staining (see above), resuspend cells in 100ul of fixation buffer per well
- Cover plate and incubate at 4°C for 15 minutes
- Add 100ul of FACS buffer per well and centrifuge plate (400RCF, 3 min, RT). Discard the supernatant
- Wash the cells by adding 200µl FACS buffer per well and centrifuge the plate (400RCF, 3 min, RT). Discard the supernatant
- Re-suspend the cells in 200µl FACS buffer and store at 4°C in the dark until acquisition

Appendix 1 - Plate layout

	1	2	3	4	5	6	7	8	9	10	11	12
A	SSC1	SSC9	TUBE1	TUBE1	TUBE1	TUBE1	TUBE1	TUBE1	TUBE1	TUBE1	TUBE1	TUBE1
B	SSC2	SSC10	TUBE2	TUBE2	TUBE2	TUBE2	TUBE2	TUBE2	TUBE2	TUBE2	TUBE2	TUBE2
C	SSC3	SSC11	TUBE1	TUBE1	TUBE1	TUBE1	TUBE1	TUBE1	TUBE1	TUBE1	TUBE1	TUBE1
D	SSC4	SSC12	TUBE2	TUBE2	TUBE2	TUBE2	TUBE2	TUBE2	TUBE2	TUBE2	TUBE2	TUBE2
E	SSC5	SSC13	TUBE1	TUBE1	TUBE1	TUBE1	TUBE1	TUBE1	TUBE1	TUBE1	TUBE1	TUBE1
F	SSC6	SSC14	TUBE2	TUBE2	TUBE2	TUBE2	TUBE2	TUBE2	TUBE2	TUBE2	TUBE2	TUBE2
G	SSC7	FMO1	TUBE1	TUBE1	TUBE1	TUBE1	TUBE1	TUBE1	TUBE1	TUBE1	TUBE1	TUBE1
H	SSC8	FMO2	TUBE2	TUBE2	TUBE2	TUBE2	TUBE2	TUBE2	TUBE2	TUBE2	TUBE2	TUBE2

Appendix 2

Single Stain Controls

	Well	STAIN	DILUTION
SSC1	A1	unstained	N/A
SSC2	B1	PI	1:250
SSC3	C1	NK1.1 (or NKp46)-PE	1:400 (or 1:200)
SSC4	D1	CD4-PerCP-Cy5.5	1:1000
SSC5	E1	CD3-Pacific Blue	1:200
SSC6	F1	CD25-APC	1:100
SSC7	G1	CD8-APC-H7	1:200
SSC8	H1	CD45-Alexa Fluor 700	1:1000
SSC9	A2	IgD-FITC	1:1000
SSC10	B2	Gr1-PerCP-Cy5.5	1:800
SSC11	C2	CD19-PE-Cy7	1:1000
SSC12	D2	CD11b-Pacific Blue	1:800
SSC13	E2	CD44-FITC	1:2000
SSC14	F2	CD62L-PE-Cy7	1:2000

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STANDARD OPERATING PROCEDURE

SUBJECT: Use of BD LSRII Flow Cytometer

INTRODUCTION
This document outlines the general procedures involved in analysis of stained blood samples by the BD LSRII flow cytometer.

ABBREVIATIONS
PBL – peripheral blood leukocytes
FACS – fluorescence activated cell sorting
PPL – project licence
CS&T – cytometer setup and tracking
HTS – high throughput sampler

HEALTH & SAFETY
- Appropriate personal protective equipment is to be worn at all times when handling the samples
- Eye wash stations, safety showers and fire extinguishers are to be located
- Appropriate training must be undertaken for use of the BD LSRII flow cytometer
- Users should be familiar with the relevant Risk Assessments before commencing the procedure:
    - **RA003** Hazardous substances (subsection RA0003.8)
    - **RA005** Biological Hazards (subsection RA005.5)
    - **RA007**Musculoskeletal (subsection RA007.9)

RESPONSIBILITIES
All staff performing this procedure are responsible for ensuring that this SOP has been read, understood and where applicable is followed in accordance with the relevant PPL. All staff should be trained and competent to perform the procedure, where applicable they should also be licensed to perform the procedure.

RESOURCES
Equipment:
- BD LSRII flow cytometer
- 96 well Microtiter plate, round bottom containing stained samples
- 96 well plate for cleaning panel

Staff: This procedure requires one member of staff

Associated documents and SOPs:
- SOP0079 - PBL Flow cytometry assay
- SOP0099 - FlowJo analysis of PBL data (Mac)

Procedure

- Before switching on LSRII, make sure waste is empty and sufficient FACS flow is present in the tank (under the cytometer)

- Switch on computer and log in.

- Switch on LSRII. This also powers up high throughput sampler (HTS) and FACS flow pump) - **NOTE: LSRII requires minimum of 30 minutes warm up time.** Wait for LSRII connection to stabilize.

- Analyser is cleaned and cytometer setup and tracking (CS&T) beads are run.

- Log into BD FACSDiva software.

- Go to Experiment>New Experiment and load experiment template.

- Rename appropriately in Inspector window (e.g. 2 panel 01_03_12)

- Apply application settings.

- Open Plate 1 in Browser window. Plate layout is shown in Appendix 1.

- Click in well A3 (this corresponds to panel 1, mouse 1) to highlight well. In Inspector, click on 'Well' tab and type the mouse ID in 'Name'. Repeat this for well B3 (corresponding to panel 2, mouse 1)
  Note: the mouse ID can be copy and pasted.

- Repeat this for all wells, delete wells that are not required.

- Place 96-well U-bottom plate containing samples onto HTS platform (make sure A1 is in correct position) and press the 'Run' button on the analyser.

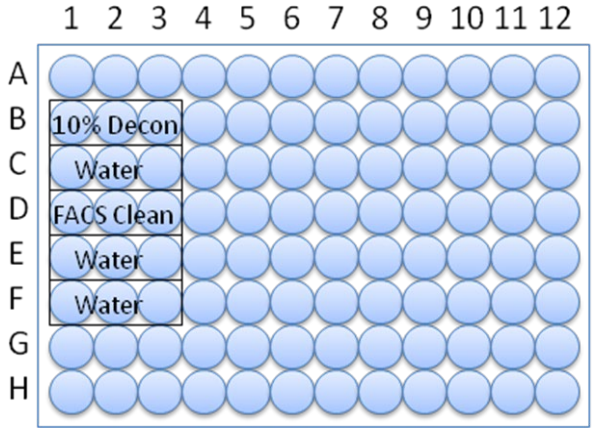
- In plate, highlight well A8 (this contains CD45 stained cells,) and click 'acquire'

- Check the cells appear in the correct position on the FSC/SSC plot, the CD45+ peak is within the gate (alter if necessary) and that the percentage of live cells is 75% or higher.

- If all these are ok, press 'record'.

- Highlight the rest of the controls and samples and press 'run wells'

- When the run is finished, a cleaning plate needs to be run (approx. 15 mins). Open the cleaning experiment, double click on the plate icon. Fill the wells detailed below with the relevant cleaning solutions and place on the HTS platform. Highlight all wells (B1-F3) and select 'run wells' on Acquisition Dashboard (click OK to overwrite if prompted).



- **When finished, put the LSRII in standby mode. The LSRII will continue to produce waste whilst on run mode and can overfill the waste container.**
    - If there are no further users booked that day, the analyser and computer can be switched off.
    - Otherwise, log out of FACSDiva and leave the LSRII on standby.
    - If the run is finished early, let a member of Core facility staff know and they can inform the next user.

- **Export data.** Under File go to 'Export FCS'. Click OK in next pop-up window, make sure destination drive is correct (in D drive) and click OK. Transfer data to USB stick
- Complete interpretation of the results, including compensation, using the FlowJo software (See SOP0099 - FlowJo analysis of PBL data (Mac))

QC during data acquisition

- While the plate is running, check on it periodically to make sure it is progressing as expected. If the plots start looking odd, for example, all cells start to become squashed against the FSC/SSC plot axis, or very few events appear, there may be a blockage. Stop the plate as soon as you notice a problem. You can try a few things to fix this:
    - Run a cleaning panel
    - Prime the sample probe
    - If in any doubt, seek advice from a member of Core facility staff.
- Sample mixing and correct running can be verified by adding a new dot plot with Time on the x axis and Alexa Fluor 700 on the y axis and will normally be a flat tight line, large alterations to the flow rate will be indicated by broadening of the line or gaps appearing.
- Make a note on the plate layout sheet about any issues that occur during the run. This will help when it comes to the data analysis and gives a valid reason if any results need to be QC failed.
- If a blockage or other problem has caused data from some wells to be unusable, and if it can be resolved on the day then you can top up affected wells with 180ul FACS buffer and run them again (overwrite data). Normally there are still enough cells to give usable data.

Appendix 1: Plate layout

For ease of preparation the following plate layout should be used:-

	1	2	3	4	5	6	7	8	9	10	11	12
A	SSC 1	SSC 9	Tube 1	Tube 1	Tube 1	Tube 1	Tube 1	Tube 1	Tube 1	Tube 1	Tube 1	Tube 1
B	SSC 2	SSC 10	Tube 2	Tube 2	Tube 2	Tube 2	Tube 2	Tube 2	Tube 2	Tube 2	Tube 2	Tube 2
C	SSC 3	SSC 11	Tube 1	Tube 1	Tube 1	Tube 1	Tube 1	Tube 1	Tube 1	Tube 1	Tube 1	Tube 1
D	SSC 4	SSC 12	Tube 2	Tube 2	Tube 2	Tube 2	Tube 2	Tube 2	Tube 2	Tube 2	Tube 2	Tube 2
E	SSC 5	SSC 13	Tube 1	Tube 1	Tube 1	Tube 1	Tube 1	Tube 1	Tube 1	Tube 1	Tube 1	Tube 1
F	SSC 6	SSC 14	Tube 2	Tube 2	Tube 2	Tube 2	Tube 2	Tube 2	Tube 2	Tube 2	Tube 2	Tube 2
G	SSC 7	FMO 1	Tube 1	Tube 1	Tube 1	Tube 1	Tube 1	Tube 1	Tube 1	Tube 1	Tube 1	Tube 1
H	SSC 8	FMO 2	Tube 2	Tube 2	Tube 2	Tube 2	Tube 2	Tube 2	Tube 2	Tube 2	Tube 2	Tube 2

SSC	**Antibody**	**fluorochrome**		**Mouse ID**								
SSC 1	unstained	none	1	MDD4	11	MDD4	21	MDD4	31	MDD4	41	MDD4
SSC 2	PI	PI	2	MDD4	12	MDD4	22	MDD4	32	MDD4	42	MDD4
SSC 3	NK1.1 (or NKp46)	PE	3	MDD4	13	MDD4	23	MDD4	33	MDD4	43	MDD4
SSC 4	CD4	PerCPCy5.5	4	MDD4	14	MDD4	24	MDD4	34	MDD4	44	MDD4
SSC 5	CD3	Pacific Blue	5	MDD4	15	MDD4	25	MDD4	35	MDD4	45	MDD4
SSC 6	CD25	APC	6	MDD4	16	MDD4	26	MDD4	36	MDD4	46	MDD4
SSC 7	CD8	APC H7	7	MDD4	17	MDD4	27	MDD4	37	MDD4	47	MDD4
SSC 8	CD45	Alexa F700	8	MDD4	18	MDD4	28	MDD4	38	MDD4	48	MDD4
SSC 9	IgD	FITC	9	MDD4	19	MDD4	29	MDD4	39	MDD4		
SSC 10	Gr1	PerCPCy5.5	10	MDD4	20	MDD4	30	MDD4	40	MDD4		
SSC 11	CD19	PECy7										
SSC 12	CD11b	Pacific Blue		Note: If plate 2 required, set up mice #41, 42, etc in wells A3/B3, A4/B4, etc								
SSC13	CD44	FITC										
SSC14	CD62l	PE-Cy7										

Appendix 2: Loader Settings

	samples	**cleaning wells**
Flow rate	1ul/sec	3ul/sec
Sample volume	150ul	200ul
Mixing volume	100ul	100ul
Mixing speed	180ul/sec	180ul/sec
Number of mixes	2-5	2
Wash volume	400ul	400ul

SANGER INSTITUTE
STANDARD OPERATING PROCEDURE

SUBJECT: FlowJo analysis of PBL data (using a Mac)

INTRODUCTION
This document outlines the method for analyzing data generated from the PBL screen via flow cytometry using the FlowJo software and uploading the data to the WTSI Mouse database.

ABBREVIATIONS
PBL – peripheral blood leukocytes
SSC – single stain control
FMO – fluorescence minus one

HEALTH & SAFETY
- RA007 – Musculoskeletal; *Section RA007.1; RA007.11*

RESPONSIBILITIES
All staff performing this procedure are responsible for ensuring that this SOP has been read, understood and where applicable is followed in accordance with the relevant PPL. All staff should be trained and competent to perform the procedure, where applicable they should also be licensed to perform the procedure.

RESOURCES
Equipment:
- Mac with FlowJo software installed

Staff: This procedure requires one member of staff

METHOD

Arranging the Data for Analysis

- Copy the raw data from the memory stick onto the Mac in an appropriate folder

- Open the FlowJo application and a new workspace.
  Current version is FlowJo 9.6

- Drag and drop the raw data folder into the FlowJo workspace. All files in that folder that FlowJo recognizes as samples will be imported. Delete the separate group that appears in the top pane (below 'All Samples').

- Click on the 'Create/Define Groups' button  . In the dialog box that appears, enter 'SSC' in the 'Name' field.

- Under 'FCS Header Keywords' select $SRC contains 'SSC'. This should move all SSCs to the correct group.

- Tick the 'Keep this window open to create more groups' box and press 

- Repeat this to create groups named 'panel 1' and 'panel 2', with $SRC containing 'panel 1' or 'panel 2' as appropriate.

- Save the flow jo analysis (manually or if prompted) with the date of the run (e.g. 10.09.13.jo)

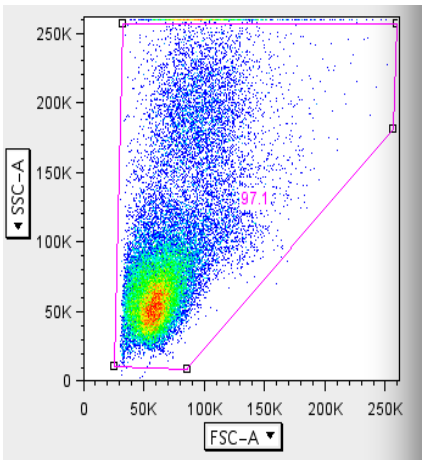
Setting up the Compensation Matrix

Gates

- Select the SSC group. Double click on the first sample to open its FSC vs SSC plot.

- Use the polygon tool  to draw a gate containing the majority of the cells (ignore those right on the edges). Name the gate 'leukocytes'.

Example plot

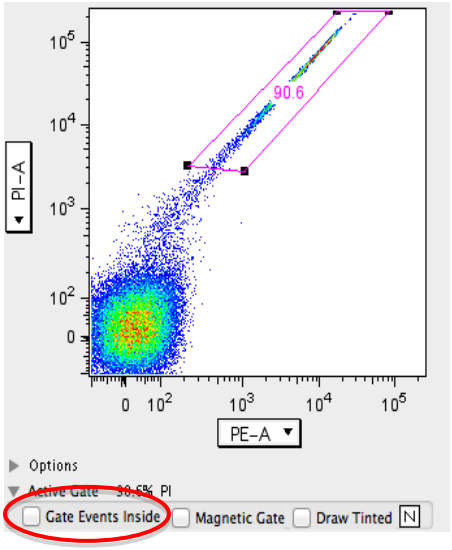


- Double click inside the gate you just created to open a new plot on those cells.
  - Change X-axis to PE-A
  - Change Y-axis to PI-A

- Draw a polygon gate  around the double positive population and name the gate 'PI'.

- In 'Active gate', uncheck 'gate events inside' (so the gated events are excluded when you click on the gate in the next step)

Example plot



- Highlight the two gates (leucocytes and PI) under the sample in the sample panel of the workspace (hold shift and click) and drag them to the 'SSC' group in the group panel.

- Double click in the PI gate to open a plot of the events outside the gate.
  - Change Y-axis to SSC-A
  - Change X-axis to the relevant fluorochrome for that SSC (check the plot title for which SSC you are on and cross-check with the table below for the fluorochrome)

SSC	Antibody	Fluorochrome
SSC 1	unstained	none
SSC 2	PI	PI – PE-PI
SSC 3	NK1.1 (or NKp46)	PE
SSC 4	CD4	PerCPCy5.5
SSC 5	CD3	Pacific Blue
SSC 6	CD25	APC
SSC 7	CD8	APC-Cy7 (APC-H7)
SSC 8	CD45	Alexa-F700
SSC 9	IgD	FITC
SSC 10	Gr1	PerCPCy5.5
SSC 11	CD19	PECy7
SSC 12	CD11b	Pacific Blue
SSC13	CD44	FITC
SSC14	CD62L	PE-Cy7

- Draw a rectangle gate  around the positive population and name to include the fluorochrome and respective antibody (e.g. 'Pacific Blue – CD3')

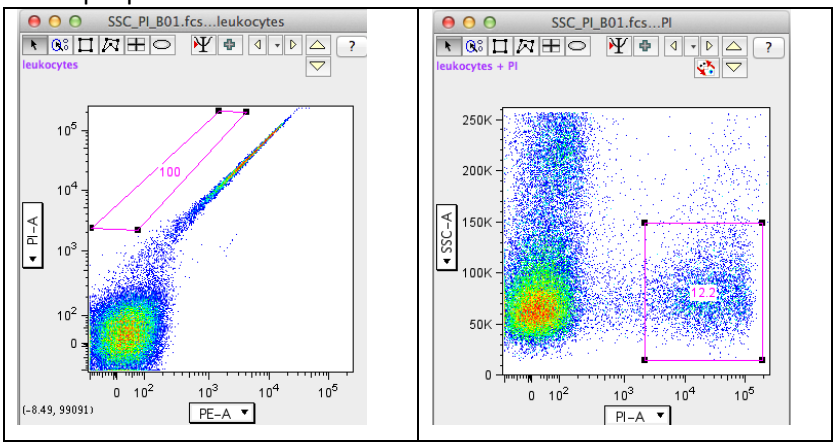
- Hold the 'Alt' key and click the forward button  to cycle all the plots to those for the next sample.
  - Change X-axis to the fluorochrome for this SSC (see table below)

- Draw a rectangle gate  around the positive population and name to include the fluorochrome and respective antibody as before

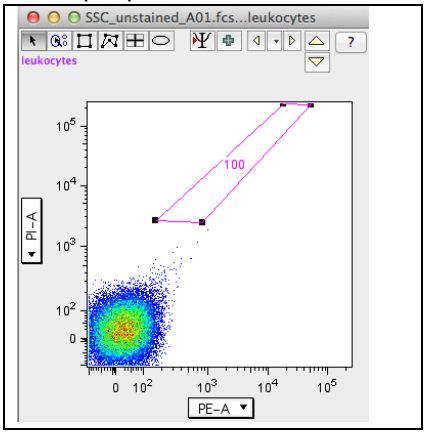
- Cycle through and repeat the above steps for all the SSCs
  - Make gates quite tight around the densest part of the positive population, ignoring most of the 'debris' surrounding it
  - Always take the brightest population if there is more than one
  - If there are two at the same intensity, gate around both of them
  - For the PI sample, move the PI positive gate off the events (in plot 2) to view them and be able to gate on them (in plot 3)
  - Unstained sample – no gates needed

- See example plots of all compensation gates in tables below.

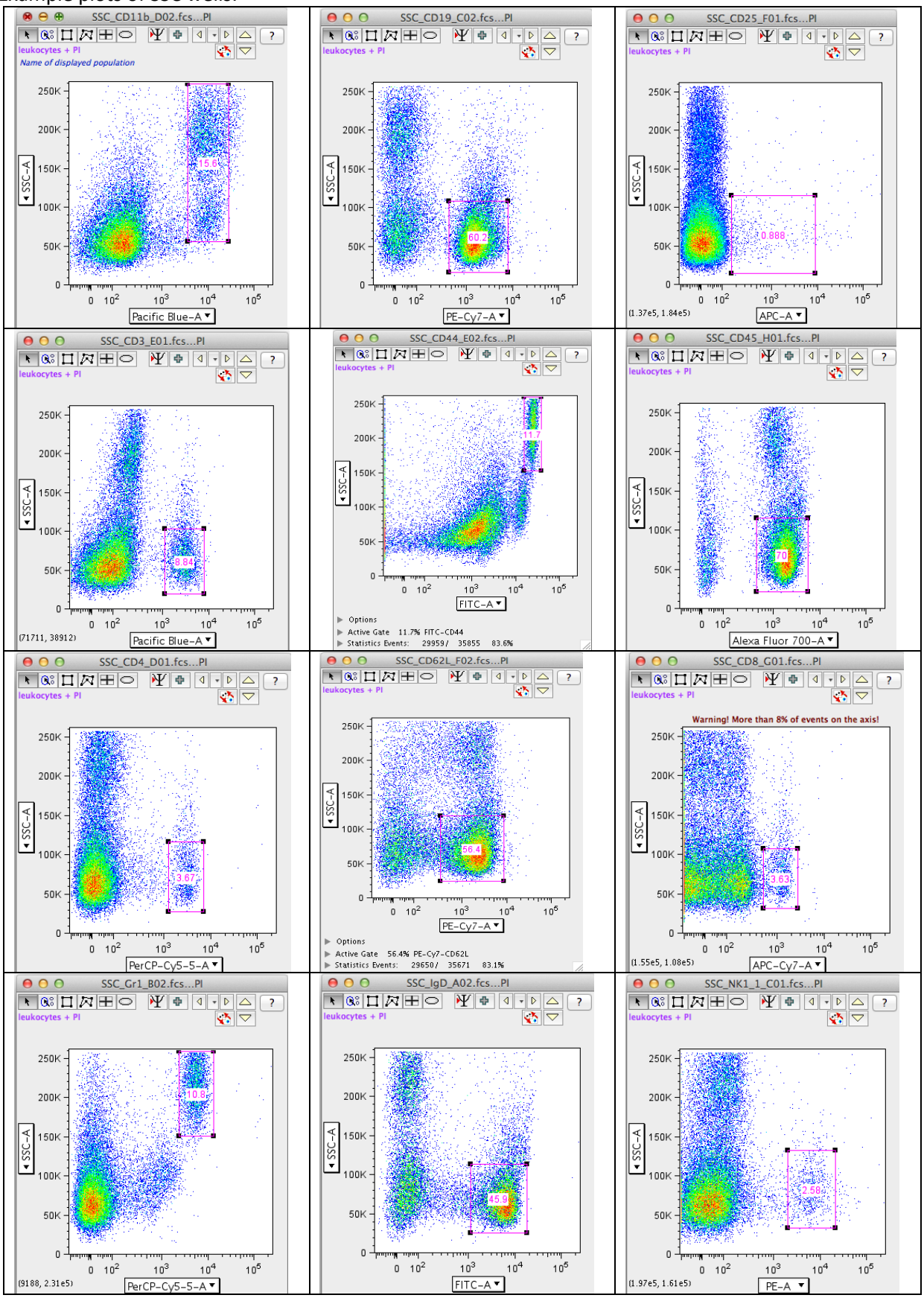
Example plots for PI control well:



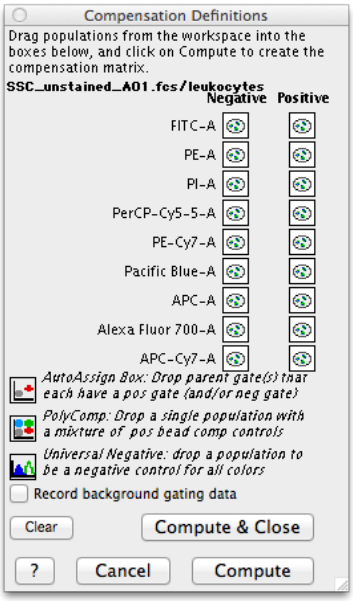
Example plot for unstained control well:



Example plots of SSC wells:



Defining the matrix



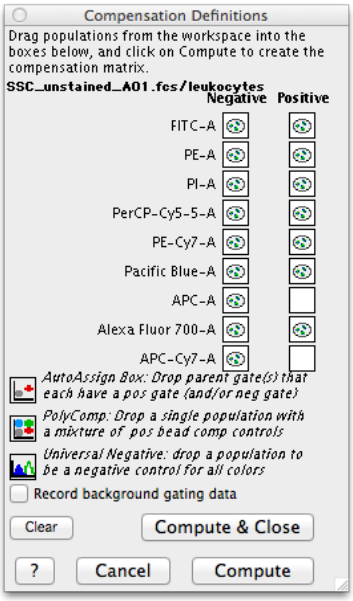
- Go to Platform>Compensate Sample>Define new matrix

- Drag the positive populations you have just created for panel 1 to the corresponding boxes in the window that opens (all boxes should get filled)
  - CD3 – Pacific Blue
  - CD4 – PerCP-Cy5.5
  - CD8 – APC-H7
  - CD25 – APC
  - CD44 – FITC
  - CD45 – Alexa-F700
  - CD62L – PE-Cy7
  - NK1.1 – PE
  - PI – PI

- Drag the unstained 'leukocytes' gate to the 'Universal Negative' box

- Click Compute and name the matrix with the date e.g. 'panel 1 7.03.12'

- Click Clear and now drag the positive populations for panel 2 to the corresponding boxes (2 boxes should be left empty at the end)
  - NK1.1 – PE
  - IgD – FITC
  - Gr1 – PerCP-Cy5.5
  - CD45 – Alexa-F700
  - CD19 – PE-Cy7
  - CD11b – Pacific Blue
  - PI – PI



- Drag the unstained 'leukocytes' gate to the 'Universal Negative' box

- Click Compute & Close and name the matrix with the date e.g. 'panel 2 7.03.12'

If there is a problem with the SSCs/compensation for the run you are analyzing, it is possible to use a recent compensation matrix. Go to Platform>Compensate sample>Import Matrix from Other Workspace.
Note: the 'other workspace' needs to be open for this to work.

Set up for Panel 1

- Drag FMO1 and FMO2 into the panel 1 group.

- Click on the 'panel 1' group in the group panel then go to Platform>Compensate Group>panel 1 7.03.11 (or whatever you named your panel 1 matrix)
  - A line should appear on the left of the samples to indicate compensation has been applied
  - FMO1 and FMO2 will jump out of the Panel 1 group again but this is ok

- Click on the FMO2 sample in the 'All samples' group and go to Platform>BiExponential Transformation>Custom Transformation then click 

in the window that appears.
  - This changes the scale at the lower end of the X-axis to expand the view of data in the lower regions to make it easier to view all the cells.

Apply gates to samples

- Open a recent workspace (File>Open recent) and click on the panel 1 group in it

- Highlight all the gates under one sample (but not the sample itself) and drag them to FMO2 in the current workspace

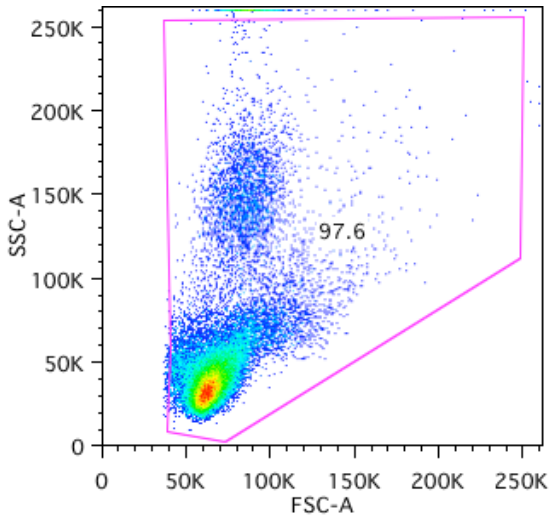
Opening plots for Panel 1

Notes: For each plot, check the gates encompass the correct populations, tweak if necessary
Try not to have gates sitting right on the axes

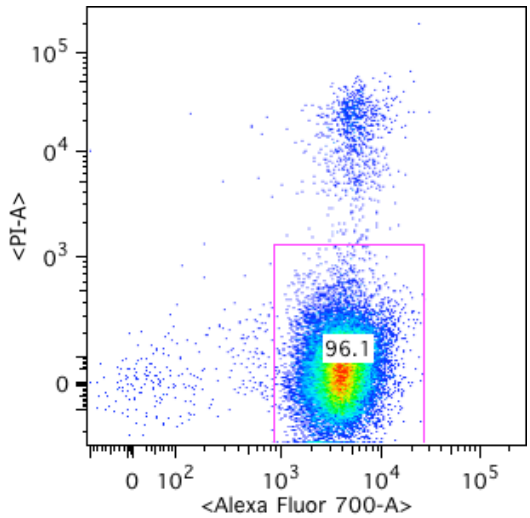
Gate hierarchy for Panel 1

- CD45+ vs PI
  - Total T cell CD3+
  - NKT & NK cells
  - CD8 vs CD4 T cells
    - CD8 vs CD3
      - CD8+CD44+CD62L-
    - CD4 vs CD3
      - CD4+CD44+CD62L-
      - CD25

1. Double click the FMO2 sample (e.g FMO_2_H02.fcs).
   This opens **Plot 1: 'Leukocytes' – Example plot:**



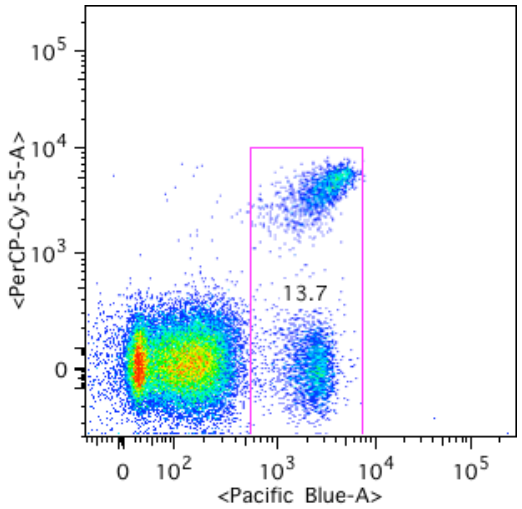
2. Double click inside the 'Leukocytes' gate.
   This opens **Plot 2: CD45+ cells with PI+ cells gated out – Example plot:**



AlexaFluor700 = CD45, PI = PI

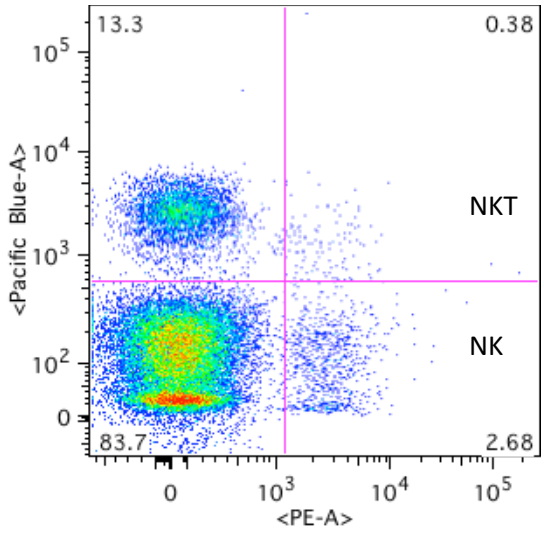
3. Double click inside 'CD45+ cells' gate.
   This may open **Plot 3: Total CD3 T cells – Example plot:**

Note: If a different plot opens, it will be plot 5. Simply change the axes to the ones shown below (X=Pacific Blue, Y = PerCP-Cy5.5) and carry on as normal from here.



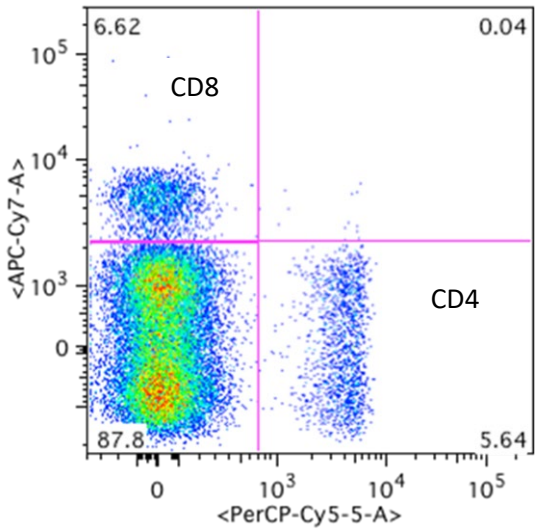
PerCP-Cy5-5 = CD4, Pacific Blue = CD3

4. Duplicate Plot 3 using the 'plus' icon in the gate's tool bar 
   Change the X axis to PE-A (NK1.1) and the Y-axis to Pacific blue (CD3).
   This is **Plot 4: NK and NKT cells – Example plot:**

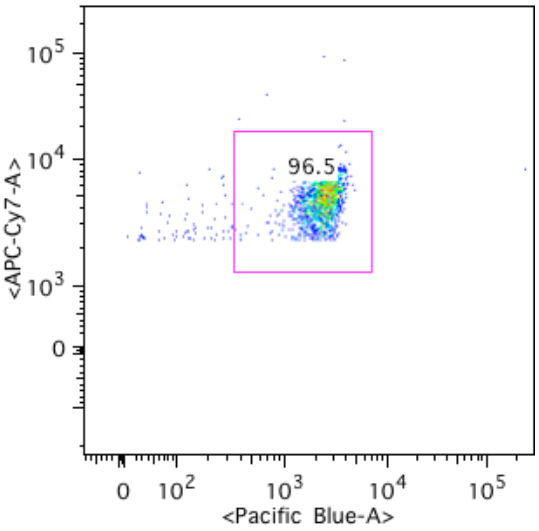


If this plot looks 'overcompensated' go to Platform>Compensate sample>Edit/Save matrix and change the value at the PE/Pacific blue intercept to zero.

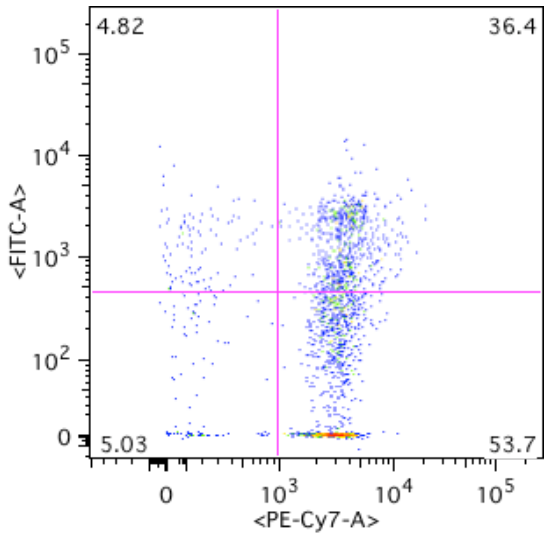
5. Duplicate plot 3 using the  button. Change the X axis to PerCP-Cy5-5 (CD4) and the Y-axis to APC-Cy7 (CD8).
   This is **Plot 5: CD8 verses CD4 T cells – Example plot:**



6. Double click on the top left (CD8) quadrant in plot 5.
   This is **Plot 6: CD8 verses CD3 T cells – Example plot:**

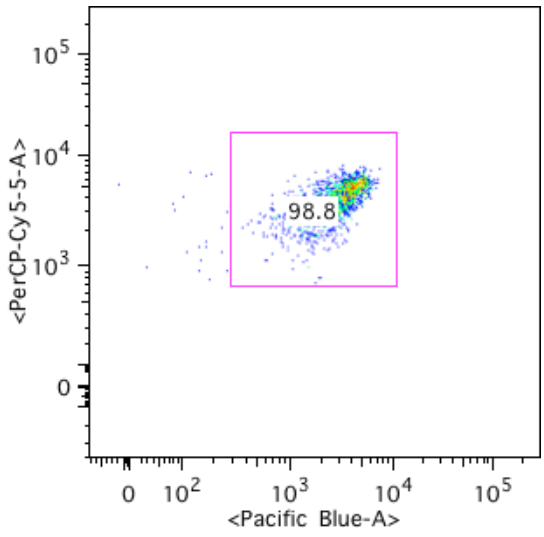


7. Double click in the plot 6 gate.
   This is **Plot 7: CD8+CD44+CD62l- T cells – Example plot:**



FITC = CD44, PE-Cy7 = CD62L

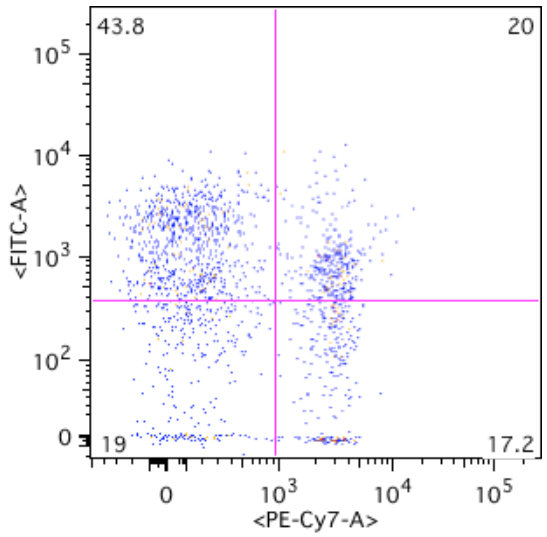
8. Go back to plot 5 and double click on the bottom right (CD4) quadrant.
   This is **Plot 8: CD4 verses CD3 T cells – Example plot:**



9. Double click in the plot 8 gate.

This may open **Plot 9: CD4+CD44+CD62l- T cells – Example plot:**

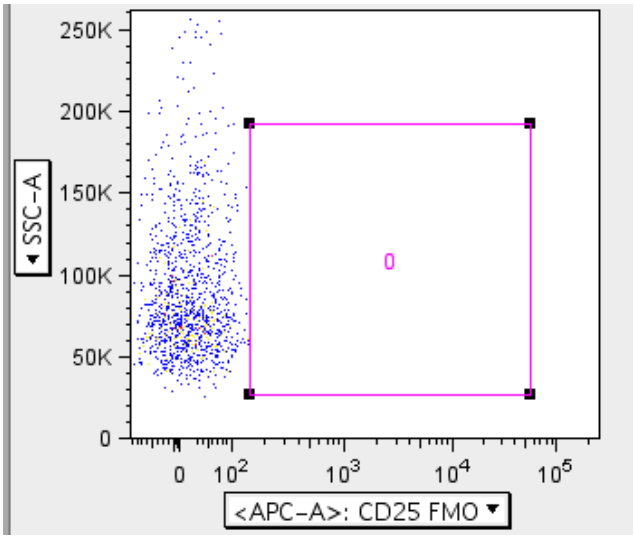
Note: If a different plot opens, this will be Plot 10 below. In this case, follow the instructions below Plot 10 to open Plot 9



FITC = CD44, PE-Cy7 = CD62L

10. Duplicate plot 9. Change the X-axis to APC-A (CD25) and the Y-axis to SSC-A.
    This is **Plot 10: CD4+CD25+ Treg cells - Example plot:**

Note: If this plot opens instead of plot 9, duplicate plot 10. Then change the X-axis to PE-Cy7-A and the Y-axis to FITC-A. This will give you plot 9 as above.



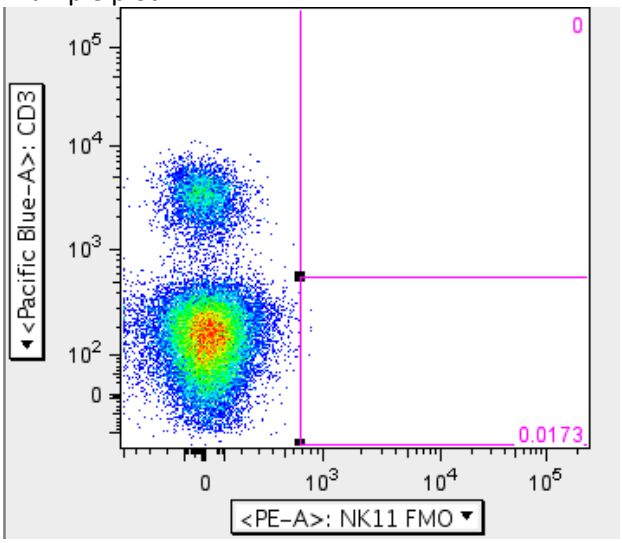
- Move this gate until the statistic inside it is just at zero (one or two outliers are ok).

- Highlight all gates under FMO2 and drag to FMO1.

- Hold Alt and click the back button  to change all plots to those for FMO1.

- Tweak the gates on plot 4 (NK and NKT cells) to meet the edge of the main bulk of cells (below 0.03% in bottom right quadrant).

Example plot:

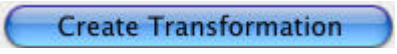


- Highlight all gates under FMO1 and drag to the panel 1 group.

- Double click on the 'Panel 1' group title. Tick 'Synchronize group's gates' then 'Apply changes'.
- Now hold Alt and click the forward button  to cycle through the samples, checking the populations fall into the gates correctly. Once you have been through all samples, close all the plots.

Set up for panel 2

- Click on the 'panel 2' group in the group panel then go to Platform>Compensate Group>panel 2 7.03.11 (or whatever you named your panel 2 matrix)
  - A line should appear on the left of the samples to indicate compensation has been applied

- Click on a sample and go to Platform> BiExponential Transformation>Custom Transformation then click 

in the window that appears.

- Open a recent workspace (File>Open recent) and click on the panel 2 group.

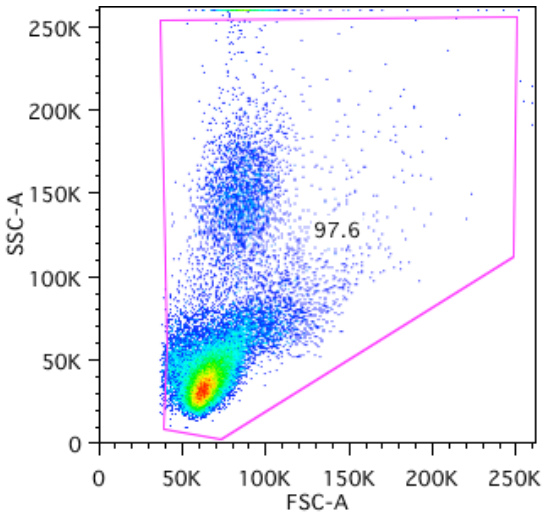
- Highlight (by holding Shift and clicking) all the gates within one sample from the recent workspace (make sure it is a panel 2 sample!) and drag them to the top sample in the current workspace

Opening plots for Panel 2

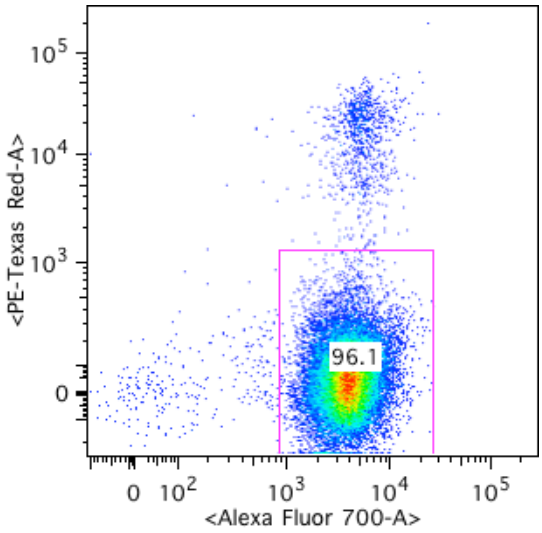
Gating hierarchy for Panel 2

- FSC vs SSC
  - CD45+ vs PI
    - Granulocytes & Monocytes
      - Purified monocytes
    - B cells CD19+
      - Mature B cells CD19+ IgD+

1. Double click the first 'panel 2_Sample ID' in Panel 2 group
   This opens **Plot 1: 'Leukocytes' – Example plot:**



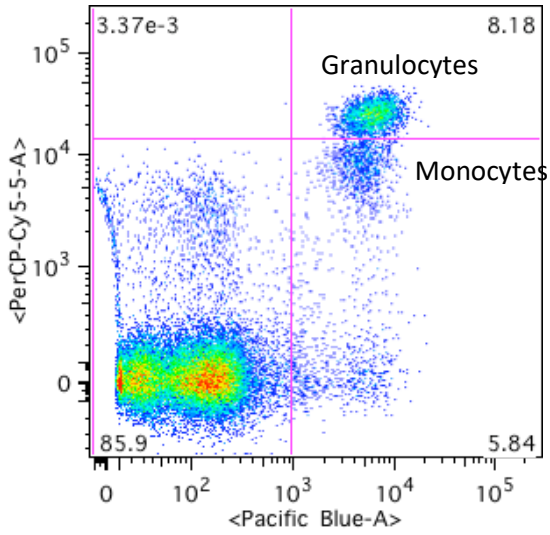
2. Double click inside the 'Leukocytes' gate.
   This opens **Plot 2: CD45+ cells with PI+ cells gated out – Example plot:**



PE-Texas Red = PI, AlexaFluor700 = CD45

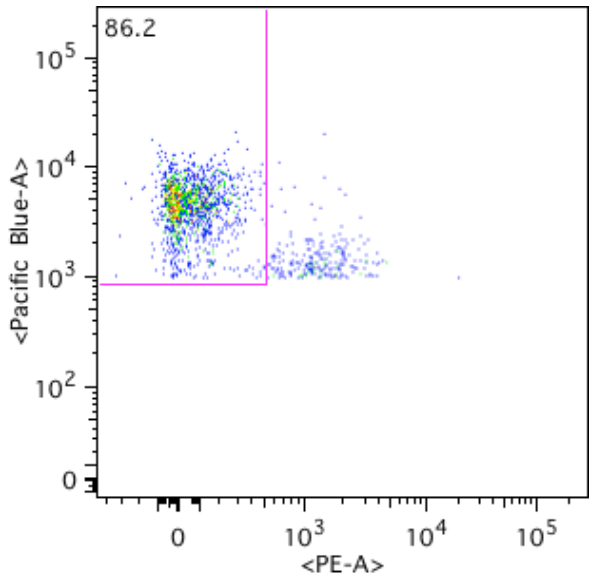
3. Double click inside 'CD45+ cells' gate.
   This may open **Plot 3: Granulocytes & monocytes (contaminated with NK cells) – Example plot:**

Note: If a different plot opens, it will be plot 5. Simply change the axes to those shown below (X=Pacific Blue, Y = PerCP-Cy5.5) and carry on as normal from here.



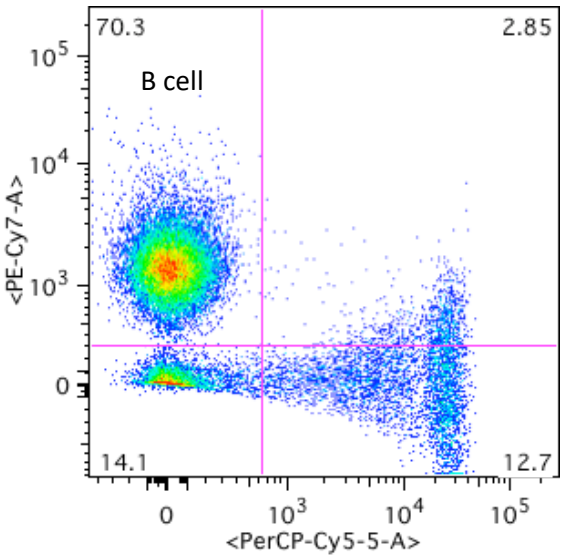
PerCP-Cy5-5 = Gr1, Pacific Blue = CD11b

4. Double click in 'Monocytes (contaminated)' quadrant, plot 3
   This is **Plot 4: 'Purified' Monocytes – Example plot:**

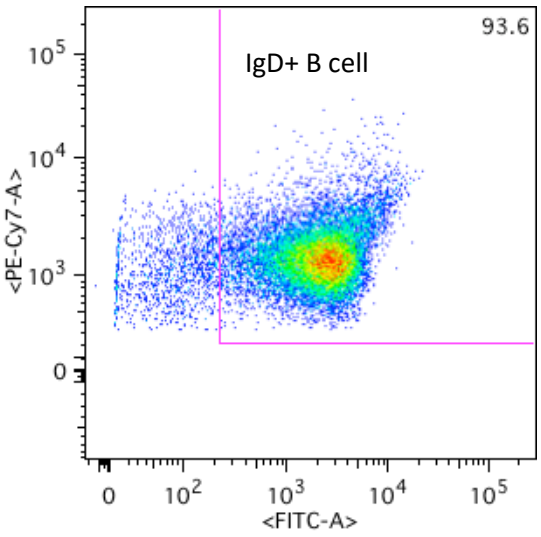


Pacific Blue = CD11b, PE = NK1.1

5. Duplicate plot 3 using the  button. Change the X axis to PerCP-Cy5-5 (Gr1) and the Y-axis to PE-Cy7 (CD19).
   This is **Plot 5: total B cells – Example plot:**



6. Double click on B cell quadrant (plot 5) to give PE-Cy7 (CD19) verses FITC (IgD)
   This is **Plot 6: Mature IgD+ B cells – Example plot:**



- Highlight all gates under the sample you are working on and drag to the panel 2 group
  - Do not synchronize gates for panel 2, as tweaking to individual samples may be required

- Now hold Alt and click the forward button  to cycle through the samples, checking the populations fall into the gates correctly, and tweaking any if necessary. Once you have been through all samples, close all the plots.

Setting up the table

- Click the table editor button  in the workspace window
- Open a recent workspace (File>Open recent) and open the table editor in that too.
- In the recent workspace, click on the 'panel 1' table and go to Table>Copy Table
- Go to the table editor in the current workspace and go to Table>Paste Table.
- Repeat this for the 'panel 2' table the close the recent workspace.
- Highlight the panel 1 group in the workspace and click  in the table editor window to create the table for panel 1.
- Click  in the new window to copy the table to the clipboard and paste it into a new Excel spreadsheet.
- Close the panel 1 table and move to panel 2 in the table editor.
- Highlight the panel 2 group in the workspace and click  in the table editor window to create the table for panel 2.
- Click  in the new window to copy the table to the clipboard and paste it into an Excel spreadsheet starting next to the previously entered data.

Excel

- Rearrange the columns, tidy up the sample IDs so they are in the format 'M00123456' and add in two columns next to the sample ID, titled 'sex' and 'genotype' and fill these in. Add a column on the far left (column A) and fill in with the date of the run.
- The column should be in the following order:

Assay date
Sample
Sex
Genotype
Total CD3
CD4
CD25 Treg
CD8
NKT
NK

CD4+ CD44+ CD62L-
CD8+ CD44+ CD62L-
B cell
IgD+ B cell
Granulocytes
Monocytes
Panel 1 viable CD45 count
Panel 2 viable CD45 count

- Save the file in **.csv** format and upload to the database.

QC during analysis

- An 'abnormal plots' or 'Equipment failure' comment can be added to with further details such as 'blockage' or 'laser issue'.
- An 'abnormal plots' comment can be combined with 'poor staining' to distinguish staining issues from analyser issues.
- If there are <10,000 events for a panel then all results for that panel are QC failed with the comment 'Insufficient events'