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STANDARD OPERATING PROCEDURE PACKET

SANGER INSTITUTE
STANDARD OPERATING PROCEDURE

SUBJECT: Peripheral blood leukocyte flow cytometry assay using whole blood

INTRODUCTION:

This SOP details the characterisation of the main leukocyte populations in peripheral blood from mice by 2 panels of 10-colour flow cytometry. The protocol is combined with cell counts obtained from a haematology analyser to derive the absolute cell numbers of various leukocyte populations.

ABBREVIATIONS:

ARA – Advanced Research Assistant
BD – Becton Dickinson
BSA – bovine serum albumin
DCF – data capture form
EDTA – Ethylenediaminetetraacetic acid
FACS – fluorescence activated cell sorting
FSC – forward scatter
PBL – peripheral blood leukocytes
PBS – phosphate buffered saline
PIL - Procedure Individual Licence
PPE – personal protective equipment
PPL - Procedure Project Licence
RCF – relative centrifugal force
SSC – side scatter

HEALTH & SAFETY:

This procedure is covered by the following risk assessment WTSI_2097

- Appropriate personal protective equipment (PPE) is to be worn at all times when handling the samples – white coat and gloves
- New employees, or those returning from a significant period of absence, must be supervised until deemed competent at performing this procedure.
- Individual risk assessments for young persons and new or expectant mothers would be performed to define any exclusions for performing this assay.
- Any employees with disabilities or health issues will undertake pre-employment health questionnaires and seek advice from occupational health.
- Lone working and out of hours working is permitted for those deemed competent although out of hours should be restricted to data acquisition where possible.
- Ergonomic pipettes should be used to minimise the risk of repetitive strain injury as this process involves multiple pipetting steps.
- Users should correctly organise their workspace to minimise stretching and other ergonomic hazards.
- New users are required to be trained in the correct operation of the BD LSRII by the core flow facility staff.

- The BD LSRII flow cytometer contains class III lasers but this instrument is class I by design.
- Under no circumstances should the covers or interlocks on the instrument be overridden to access the lasers.

RESPONSIBILITIES:

All staff performing this procedure are responsible for ensuring that this Standard Operating Procedure (SOP) and accompanying risk assessment has been read and understood. All staff should be trained and competent to perform the procedure.

RESOURCES:

Equipment and reagents:

1. 100 µl EDTA coated blood collection tubes without capillary (Scientific Laboratory Supplies catalogue number 78042)
2. Centrifuge (for plates)
3. BD Falcon 96 well U bottom plates with lid (Sanger stores PLPL0012, BD 353077)
4. Compensation beads (eBioscience Ultracomp eBeads catalogue number 01-2222)
5. FACS buffer (PBS with 0.5% BSA)
6. 1x BD Cell fix (10x Cell Fix BD Biosciences catalogue number 340181) – **hazardous substance contains formaldehyde and sodium azide, prepared by diluting 10x stock with ultrapure water in chemical fume hood wearing safety glasses**
7. 1x RBC lysis buffer (10x BD PharmLyse, BD Biosciences catalogue number 555899) - 1:10 dilution with ultrapure water [remove from fridge to warm up prior to use]
8. Pipettes (single, multi and repeat) and tips
9. Formaldehyde hazardous waste container, plus funnel
10. Timer
11. 4 ml amber glass vials (Sigma catalogue number 27001-U)
12. 0.6 ml clear microcentrifuge tube
13. Antibodies (see appendix B)
14. Vortex
15. Scil Vetabc+ analyser
16. BD LSRII flow cytometer

Associated SOPs/Documentation:

1. SOP0185 – Haematology Vetabc Plus QC and Sample Analysis
2. SOP0153 – Analysis of 16 week PBL data

Staff: This procedure can be performed by one member of staff.

METHOD – Preparing working antibody solution

1. Prepare sufficient amount of antibody-mix with FACS buffer, having calculated the amount of antibodies to be used, based on titration. The tables in appendix 2 describe the staining panels.

2. In a 4 ml amber glass vial, pipette FACS buffer, then add antibodies as indicated for panel 1, then repeat for panel 2 in a separate vial.
3. Label tubes with panel, initials of person who prepared the mix and date.
4. Mix both tubes thoroughly by vortexing and store in fridge until required.
5. The antibody mix is suitable to be used for a maximum of 5 days from preparation and should be disposed of at the end of the week.

METHOD – Setting Up Compensation Controls

Note: Can be done during the 20 minute incubation

6. Collect compbead bottle from the fridge and mix well by vortexing
7. Add 6 drops of beads to a 0.6 ml microcentrifuge tube (these are a mix of binding and non-binding beads)
8. Mix well by vortexing and pipette 15 µl of beads into every compensation control well as indicated in the plate layout (appendix A)
9. Add 0.2 µl of antibody to the appropriate well (see appendix A) and mix
10. Incubate for 5 minutes at room temperature
11. Add 220 µl of FACS buffer to each well. The plate is now ready for acquisition, place in fridge until ready to analyse.

    Note: Compensation controls are performed on a separate plate to the whole blood staining procedure

METHOD – Cell counting and staining whole blood

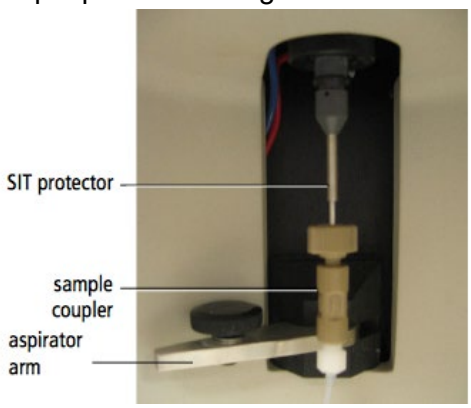
12. Blood is collected according to SOP0046 into 100 µl EDTA tubes.
13. Record the order of samples on a plate template printout.
14. Reverse pipette, with a filter tip, 25 µl of whole blood into two wells of a 96 well U bottom plate according to the plate layout in appendix A.

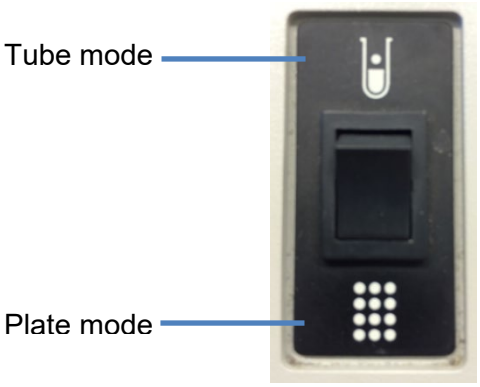
    Note: If there is insufficient blood then pipette 10 µl of whole blood per well and top up with 15 µl FACS buffer.
15. Start up, QC, run samples, clean and shutdown Vetabc Plus according to SOP0185.
    Note: this step should ideally be performed by a second person while the first person proceeds with the PBL assay.
16. Add 25 µl of 2x antibody (as prepared above) to each well using repeat pipetter and mix using multichannel pipette. To prevent contamination of the multichannel pipette, filter tips should be used at all steps.
17. Incubate for 20 minutes at room temperature.
18. **In a certified chemical fume hood** add 150 µl of 1x BD cell fix solution to each well and mix.
19. Incubate at room temperature for 5 minutes.
20. Centrifuge (400 rcf, 3 minutes), **in a certified chemical fume hood dispose of supernatant into cell fix hazardous waste container using a funnel and a flicking action, rinse the funnel with a small amount of water.**

21. Re-suspend pellet in 200 µl of 1x RBC lysis buffer per well. The pellet will be quite hard to re-suspend so mix the solution several times and be careful it does not splash.
22. Incubate for 5 minutes at room temperature, centrifuge (400 rcf, 3 minutes) and **discard supernatant by flicking the waste into the white waste bucket.**
23. Re-suspend in 200 µl of 1x RBC lysis buffer per well.
24. Incubate for 5 minutes at room temperature, centrifuge (400 rcf, 3 minutes) and **discard supernatant by flicking the waste into the white waste bucket.**
25. Wash with 250 µl FACS buffer per well, centrifuge (400 rcf, 3 minutes) and **discard supernatant by flicking the waste into the white waste bucket.**
26. Re-suspend in 250 µl FACS buffer per well. Looking from the top and from the bottom check the 96-well plate for debris and remove them with a pipette.
27. **Add two virkon tablets to the waste liquid in the white waste bucket. Fill it to the top with tap water let it stand for a minimum of 1 hour and then flush down the sink with running water**

METHOD – Data acquisition

28. At the LSRII verify the instrument is in HTS mode and the HTS is connected to the sample probe – see figures below





29. Log into BD FACSDiva.
30. Go to Experiment>New Experiment. Select the most recent experiment template.
31. At the end of the experiment name, add today's date in the format YYYYMMDD using the inspector window.
32. Check that plots are present on both 'global' and 'normal' worksheets. If they are not, open a new experiment and start again, as these cannot be retrieved at a later stage.
33. Open the blood plate (double click on icon in browser window) and delete sample wells as required.
34. Label wells with the correct mouse barcode number (in the Inspector window, click on the 'Well' tab and type the mouse ID in 'Name').
35. Apply application settings.
36. Check that the application settings have been applied, expect FSC voltage around 370, SSC voltage around 340 and FSC threshold 25,000. The application settings are linked to the blood samples but have been tested for beads.

37. Place the compensation plate onto HTS platform (make sure A1 is in correct position) and press the 'Run' button on the analyser.
38. Open the 'comp controls plate' and select the first well on the plate layout.
39. Acquire the first well of compensation controls and check that the beads are visible on the plots on the global worksheet (comp samples tab).
40. Ensure that you then select "record" to save the data.
41. Highlight the rest of the compensation control wells and select "run selected wells", it will record 5000 events per well as determined by the experiment layout.
42. In the 'normal' worksheet view, move and resize the P1 gate on the Unstained tab so it is set correctly around the beads, then right click the gate and select "apply to all compensation controls".
43. In each of the single stain control tabs, check that the P2 gate is set correctly around the positive bead population, most will need to be resized and/or moved to the correct population.
44. Calculate compensation by selecting Experiment>Compensation setup>Calculate compensation
45. Select "link and save" (the default file name is fine).
46. Place the blood plate containing samples onto HTS platform (make sure A1 is in correct position).
47. Open the blood samples plate and select the first panel 1 well.
48. Check the global worksheet is showing the panel 1 analysis, change to this if necessary.
49. Acquire this panel 1 sample to check the staining and that the cells are on scale.
50. In theory the same settings will work for both the compensation beads and the blood samples due to the way the application settings have been created, if necessary adjust FSC/SSC voltages or the FSC threshold as required.
51. Verify that the FSC-H vs FSC-A singlet doublet exclusion (top left plot) and CD45 vs SSC gates (top right plot) are set correctly; adjust the position of the gates as required or the voltages. You should expect >80% CD45+ events.
52. If this looks good record this sample, a stopping gate of 80,000 CD45+ singlet leukocyte events is set in the template.
53. Repeat the process for a panel 2 well, to ensure all gates are set correctly on this analysis sheet (panel 2 analysis).
54. Highlight the rest of the samples on the plate and click run wells

Note: The plate can now be left to run but should be checked on regularly to make sure no clogs or other problems have occurred.

55. Export the data as an FCS file (FCS 3.0 option, default) onto a USB stick.
56. Run a cleaning plate on the LSRII.
57. **When finished, <u>put the LSRII in standby mode</u>. 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 PPMS and leave the LSRII on standby.
58. Analyse data in FlowJo X according to SOP0153

Troubleshooting

- 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 bottom 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:
    - Prime the sample probe, can do this a few times (Click HTS/Prime)
    - Run a cleaning plate
    - If in any doubt, seek advice from a member of Core facility staff
- Sample mixing and correct running can be verified by checking the dot plot showing 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, top up affected wells with 200 µl FACS buffer, mix with a pipette and run them again (select overwrite data). Normally there are still enough cells to give usable data.

Appendix A: plate layout

	1	2
A	US	PE-Cy7 CD11b
B	FITC	BV421 NK1.1
C	PE	V450 Ly6G
D	PE-CF594 CD62	BV510 CD4
E	PE-CF594 CD19	APC
F	PerCP-Cy5.5 abTC	Alexa 700 CD45
G	PerCP-Cy5.5 Ly6C	APC-Cy7 CD8a
H	PE-Cy7 KLRG	

	1	2	3	4	5	6	7	8	9	10	11	12
A	1	5	9	13	17	21	25	29	33	37	41	45
B	1	5	9	13	17	21	25	29	33	37	41	45
C	2	6	10	14	18	22	26	30	34	38	42	46
D	2	6	10	14	18	22	26	30	34	38	42	46
E	3	7	11	15	19	23	27	31	35	39	43	47
F	3	7	11	15	19	23	27	31	35	39	43	47
G	4	8	12	16	20	24	28	32	36	40	44	48
H	4	8	12	16	20	24	28	32	36	40	44	48

	Compbeads
	Samples

Appendix B: antibody panels

Panel 1

Antibody and fluorochrome		Channel	Dilution	Supplier	Catalogue #	Clone
CD44	FITC	FITC	2000	BD	561859	IM7
CD25	PE	PE	500	Biolegend	102008	PC61
CD62L	PE-CF594	PE-Texas Red	2000	BD	562404	MEL-14
TCRαβ	PerCP-Cy5.5	PerCP-Cy5.5	600	Biolegend	109228	H57-597
KLRG1	PE-Cy7	PE-Cy7	500	Biolegend	138416	2F1
CD161/NK1.1	BV421	Pacific Blue	600	Biolegend	108732	PK136
CD4	BV510	AmCyan	3000	Biolegend	100553	RM4-5
TCRγδ	APC	APC	600	Biolegend	118116	GL3
CD45	Alexa 700	Alexa 700	600	Biolegend	103128	30-F11
CD8a	APC-H7	APC-Cy7	200	BD	560182	53-6.7

Panel 2

Antibody and fluorochrome		Channel	Dilution	Supplier	Catalogue #	Clone
Ly6B	FITC	FITC	1000	Serotec	MCA771FB	7/4
I-A/I-E	PE	PE	4000	Biolegend	107608	M5/114.15.2
CD19	PE-CF594	PE-Texas Red	2000	BD	562291	ID3
Ly6C	PerCP-Cy5.5	PerCP-Cy5.5	5000	Biolegend	128012	HK1.4
CD11b	PE-Cy7	PE-Cy7	2000	Biolegend	101216	M1/70
Ly6G	V450	Pacific Blue	600	BD	560603	1A8
IgD	BV510	AmCyan	2000	BD	563110	11-26c.2a
CD115	APC	APC	500	Biolegend	135510	AF598
CD45	Alexa 700	Alexa 700	600	Biolegend	103128	30-F11

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STANDARD OPERATING PROCEDURE

SUBJECT: Analysis of 16 week PBL flow cytometry data

INTRODUCTION

This document outlines the method for analysing data generated from the 16 week PBL flow cytometry screen using FlowJo software.

ABBREVIATIONS

CD – cluster of differentiation
DCF – data capture form
FSC – forward scatter
HTS – high throughput sampler
PBL – peripheral blood leukocytes
PIL – procedure individual licence
QC – quality control
SSC – side scatter
TCR – T cell receptor
WBC – white blood cell

HEALTH & SAFETY

This procedure has been classified as not containing any hazards. It does involve the use of computers and as such a DSeasy display screen equipment assessment might be required.

RESPONSIBILITIES

All staff performing this procedure are responsible for ensuring that this SOP has been read, understood and all staff should be trained and competent to perform the procedure.

RESOURCES

Equipment:

- Mac with FlowJo software installed

Staff: This procedure requires one member of staff

Associated documents:

- 16 week PBL 2panel calculation template.xltx

METHOD

Arranging the Data for Analysis

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

2. Open the FlowJo analysis template.

3. Drag and drop the raw data folder into the FlowJo workspace. All files in that folder that FlowJo recognises as samples will be imported and should be sorted automatically into groups for samples and compensation.

Note: If it looks like this has not happened properly, double click on the name of the groups and select the staining protocol to define the sample groups

4. Save the FlowJo file as a workspace (.wsp file) in your analysis folder with the date of the run (e.g. 16wk PBL analysis 20180918.wsp).

Checking compensation

5. Select the first group containing your samples, “panel 1”. Right click the matrix icon  next to the first sample name and select ‘Edit compensation matrix’

6. Enlarge the window, change preview population to ‘singlets’, untick ‘Overlay uncompensated’ box.

7. Click ‘edit’ then ‘yes’ in the pop up to edit the matrix

8. Edit the matrix numbers until you are happy that the compensation for each pair of fluorochromes looks correct. Close the window when you are satisfied (you don’t need to click ‘save’).
    - The matrix icon  will change colour to match the new matrix so you know which has been applied
    - Do this for both panels (edit the matrix from a sample in the second group, “panel 2”). The matrix icon for the second group should then have a different colour to the first 

Note: If an individual sample requires different compensation to the rest, right click on the coloured matrix icon  next to that sample and select ‘reapply acquisition matrix’. The matrix icon will go grey  , then right click it again and select ‘edit compensation matrix’, follow steps 8 to 10 to change the compensation. The matrix icon then changes to a different colour for that sample  to indicate different compensation has been applied.

Name	Statistic	#Cells	QC
Panel 2_F9_F09_055.fcs		88856	
singlets	97.9	86983	
Panel 2_M02338927_F06_052.fcs		88177	
singlets	98.2	86621	
Panel 2_M02339246_H05_059.fcs		87128	
singlets	97.5	84972	
Panel 2_M02339247_F05_051.fcs		88884	
singlets	97.5	86697	

Gate hierarchy:

Panel 1

- FSC-A vs FSC-H (singlets)
    - CD45 vs time (HTS issues)
        - CD45 vs SSC-A (leukocytes)
            - Total T cells
            - gd T cells
            - NK cells
                - KLRG1+ mature NK cells
            - NKT cells
            - ab T cells
                - CD8+ ab T cells
                    - CD4+KLRG1+ ab T cells
                    - CD8+CD44+ Teff memory cells
                - CD4+ ab T cells
                    - CD4+KLRG1+ ab cells
                    - CD4+CD44+ Teff memory cells
                    - CD4+CD25+ Tregs

Panel 2

- FSC-A vs FSC-H (singlets)
    - CD45 vs time (HTS issues)
        - CD45 vs SSC-A (leukocytes)
            - B cells
                - IgD+ mature B cells
            - NOT B cells
                - Neutrophils
                    - Eosinophils (NOT neutrophils)
                    - Monocytes (NOT neutrophils)
                        - Ly6Chi monocytes
                        - Ly6Clo monocytes

Note: On any axis, click the T icon to allow adjustment of axis scaling using the slider, making sure all events can be seen and giving better visualisation of the populations. Normally, setting the linear scale on the time plot to 120 will allow good visualisation of all the data. For all fluorescent parameters the width basis can be changed, ensuring the scale is set to BiExp

Open plots

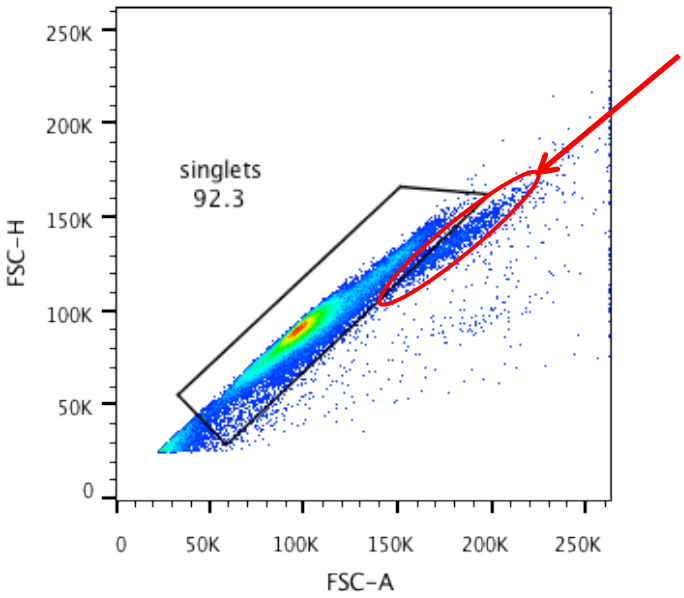
Panel 1

9. Right click on the Panel 1 group and click the check box for synchronised, click apply changes and close. Open all plots as described in the following steps, but do not move any gates until they are all open and you have selected a representative sample to set gates on while synchronised.

10. Now the plots are synchronised the gates can be changed as described in the steps below and any changes will be applied to the whole group, minimising the number of gates that need to be changed on individual samples.

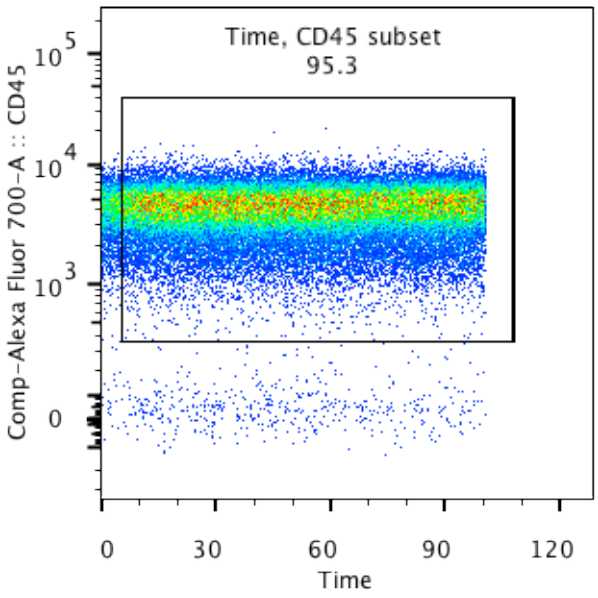
Note: once synchronised ensure the file is saved at regular intervals, changing the gates when synchronised takes a lot of memory and FlowJo can often crash at this stage

11. Open the first sample.
    - X axis = FSC-A; Y axis = FSC-H
    - gate = singlets
    - If this gate is not set correctly you will see double positive events in the plots which do not occur biologically e.g. cells positive for both TCRαβ and TCRγδ.

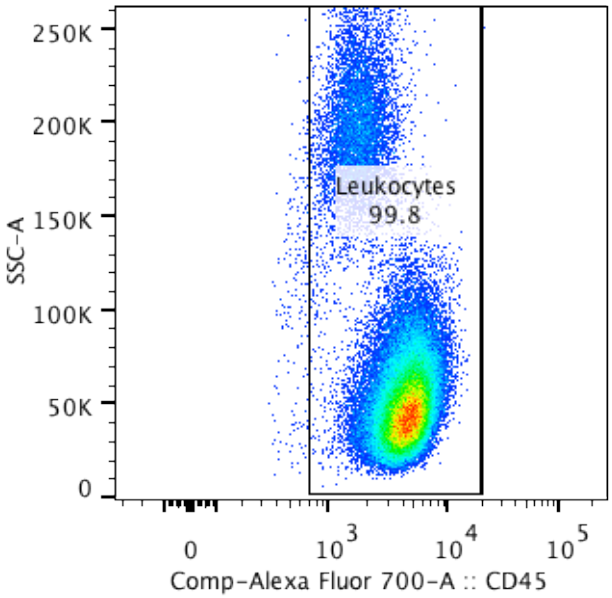


Doublets like these are what you want to avoid

12. Open ‘singlets’.
    - X axis = Time; Y axis = Alexa700-CD45;
    - gate = Time, CD45 subset
    - Here you want to avoid any fluctuations in the event rate. This will often mean taking the middle section of the events. If the gate is hard to set or the CD161(NK1.1) vs TCRab gates are hard to set, duplicate the view and change the Y axis to Pacific Blue-CD161. As this is the lowest wavelength fluorochrome in the panel for excitation and emission this is the worst affected by an unstable flow via the HTS. From here you can determine the correct time region to use and set the gate according to this.
    - Click “T” > customize > set the max to 120. Click apply all samples.

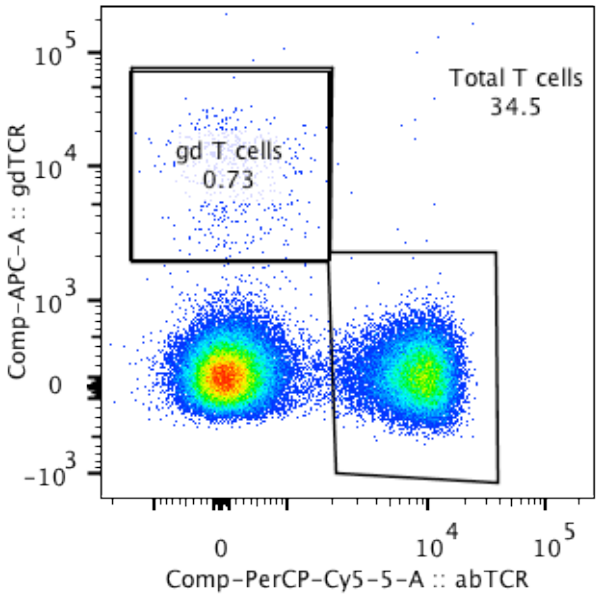


13. Open ‘Time, CD45 subset’.
    - X axis = Alexa700-CD45; Y axis = SSC-A;
    - gate = leukocytes
    - Ensure this gate is pushed off the top of the SSC-A axis to ensure all events are accounted for

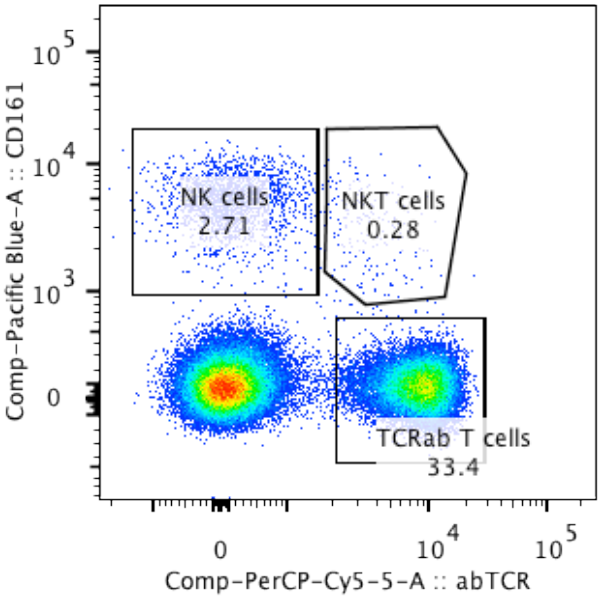


14. Open ‘leukocytes’ and duplicate this new plot once more. 

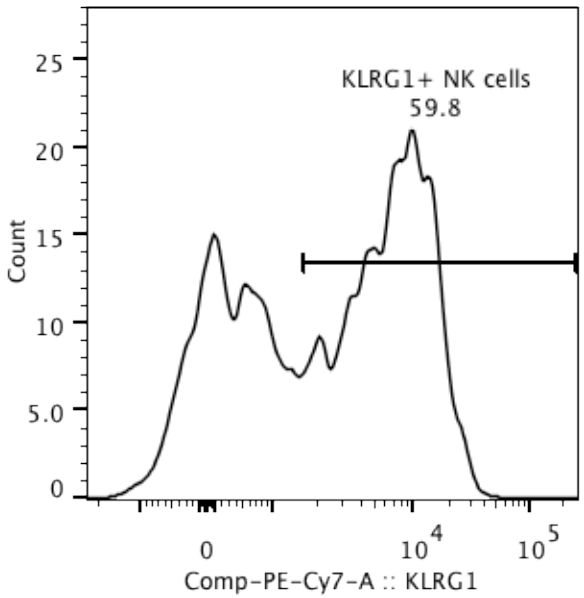
15. For the first plot, X axis = PerCPCy5.5-abTCR; Y axis = APC-gdTCR
    - gate = Total T cells
    - gate = gd T cells



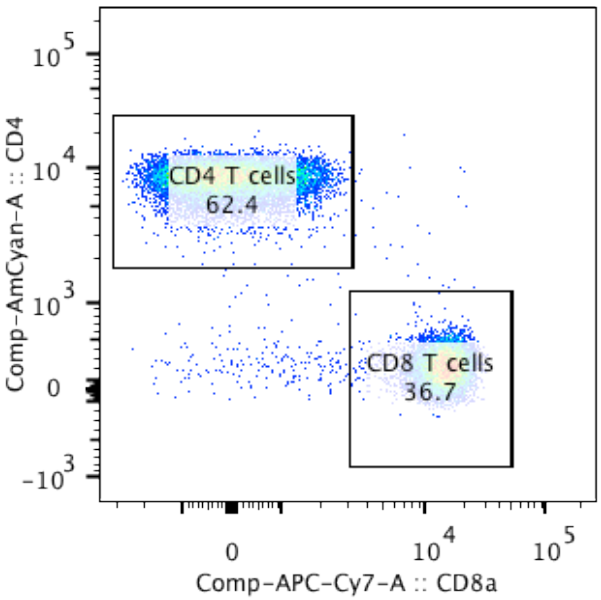
16. For the second plot, X axis = PerCPCy5.5-abTCR; Y axis = PacificBlue-CD161
    - gate = NK cells
    - gate = NKT cells
    - gate = TCRab T cells
    - Between these two plots check that the sum of NKT, gd T cells and TCRab T cells is ±0.1% of Total T cells.



17. Open ‘NK cells’
    - X axis = PE-Cy7-KLRG1; Y axis = histogram
    - gate = KLRG1+ NK cells
    - This gate has been set using a FMO control and shouldn’t be changed. Make a note in the QC column of the FlowJo workspace window if the whole population has shifted and is KLRG1^{hi}.

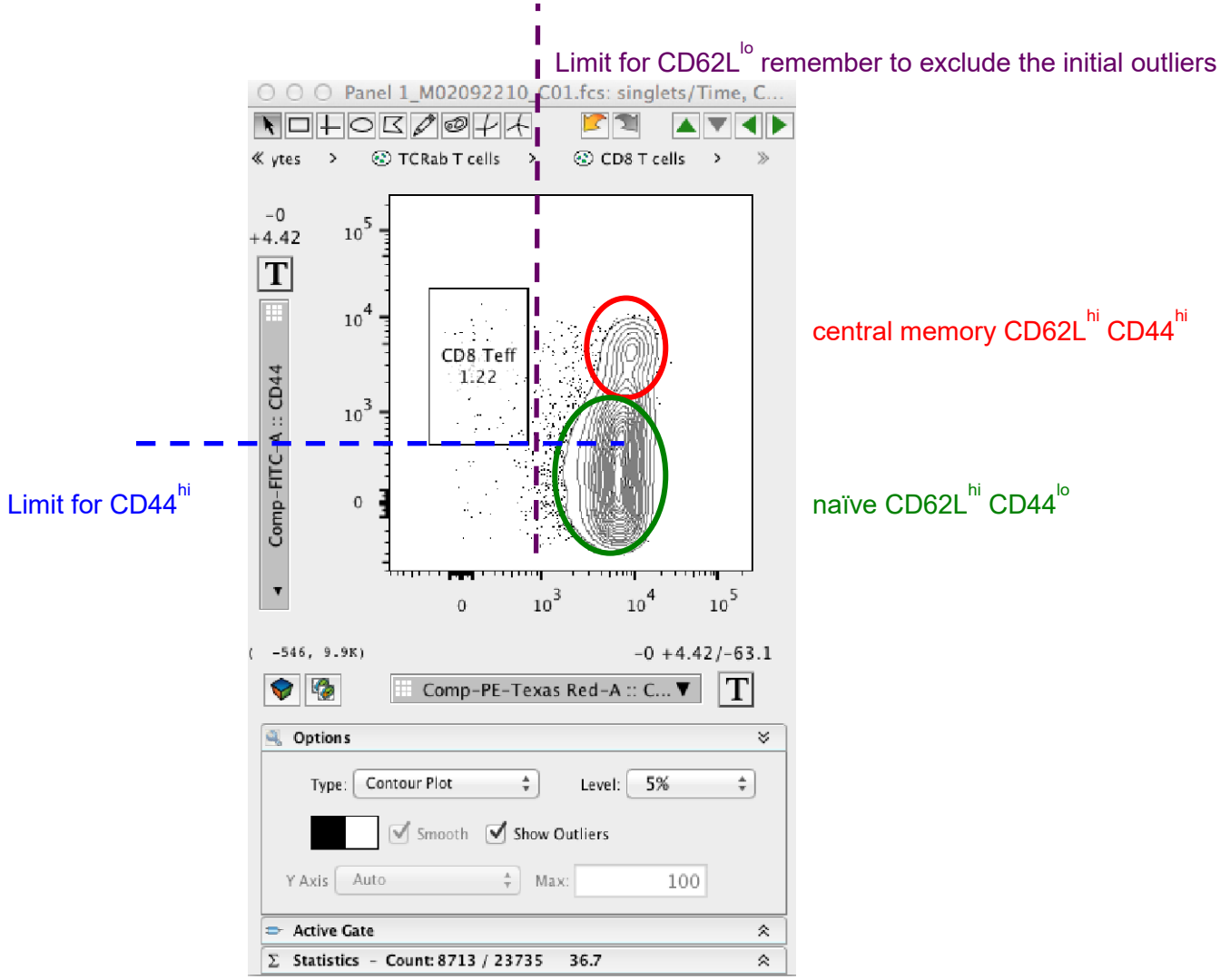


18. Open ‘TCRab T cells’.
    - X axis = APC-Cy7-CD8a; Y axis = AmCyan-CD4
    - gate = CD8 T cells
    - gate = CD4 T cells
    - Here you should have very few CD4 CD8 double negative cells (they do not circulate) if the population is quite noticeable this might indicate the TCRab T cell gate is set too far to the left and should be adjusted

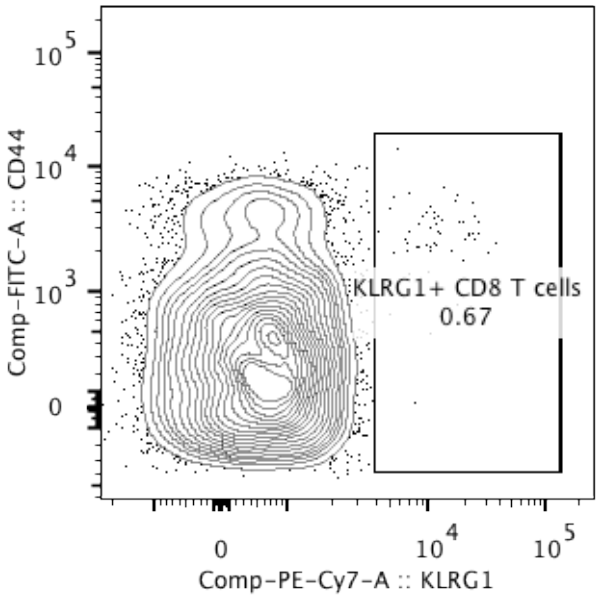


19. Open ‘CD8 T cells’ and duplicate this new plot once more. 

20. For the first plot, X axis = PE-TexasRed-CD62L; Y axis = FITC-CD44
    - gate = CD8 Teff, change the display to ‘contour’ using the options window if not already in this format, and check the ‘show outliers’ box
    - This gate is quite hard to set. Use the naïve (CD62L^{hi} CD44^{lo}) and central memory (CD62L^{hi} CD44^{hi}) populations to help guide where to place the gate.
    - The effector memory T cells you are after are (CD62L^{lo} CD44^{hi}). Place the gate to the left of the main population on the CD62L axis and use top of the contour for the naïve cells to determine the CD44 level (see plot below).
    - Make a note in the QC column of the FlowJo workspace window if there is CD62L shedding which makes this gate hard to set. In the event of shedding the Teff gate should be moved higher on the CD44 axis to avoid the CD44^{lo} CD62L^{lo} population that is a processing artefact (this can be caused by a difficult bleed or incubation too long in RBC lysis buffer).

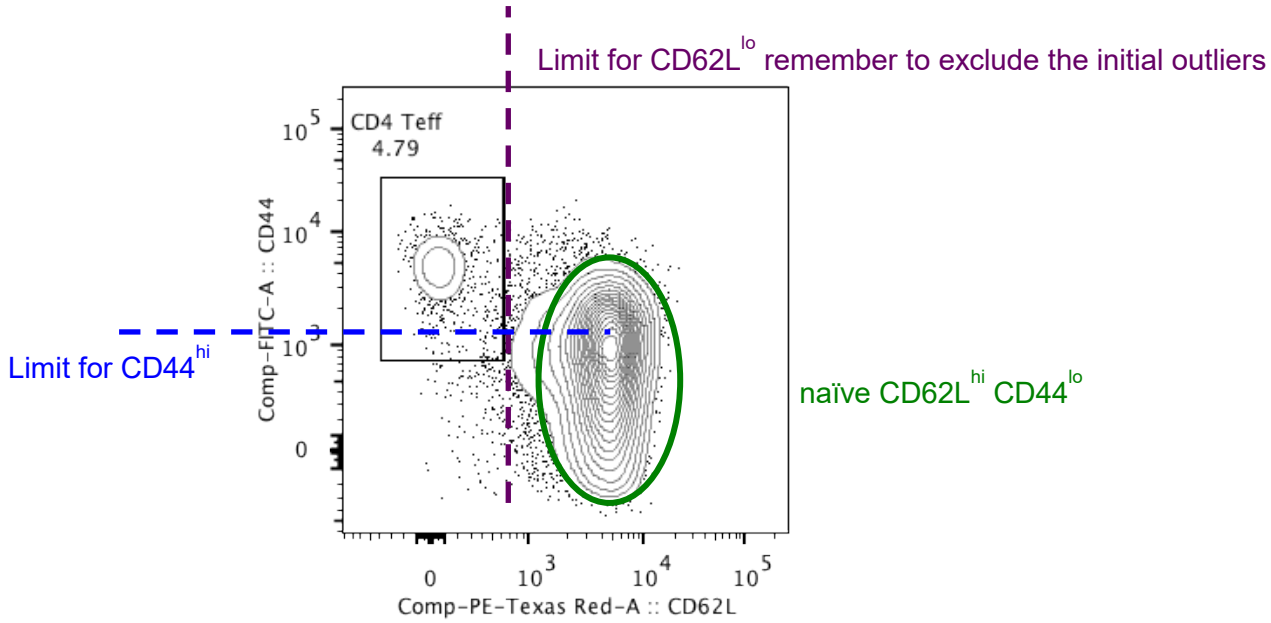


21. For the second plot, X axis = PE-Cy7-KLRG1; Y axis = FITC-CD44
    - gate = KLRG1+ antigen experienced CD8 T cells. Change the display to 'contour' using the options window if not already in this format, and check the 'show outliers' box
    - Here the gate is extended over the entire CD44 range (although the majority should be CD44^{hi}) and is set just to the right of the initial outliers from the main KLRG1 negative population.
    - Make a note in the QC column of the FlowJo workspace window if the whole population has shifted and is KLRG1^{hi} that prevents the gate being placed.

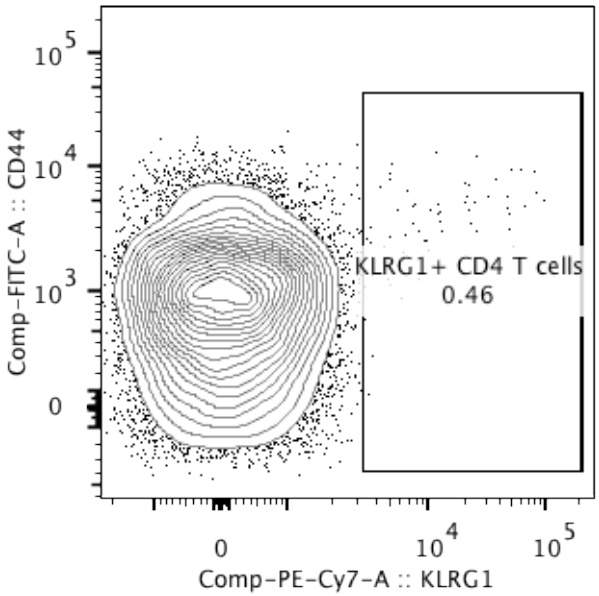


22. Open 'CD4+' and duplicate this new plot twice 

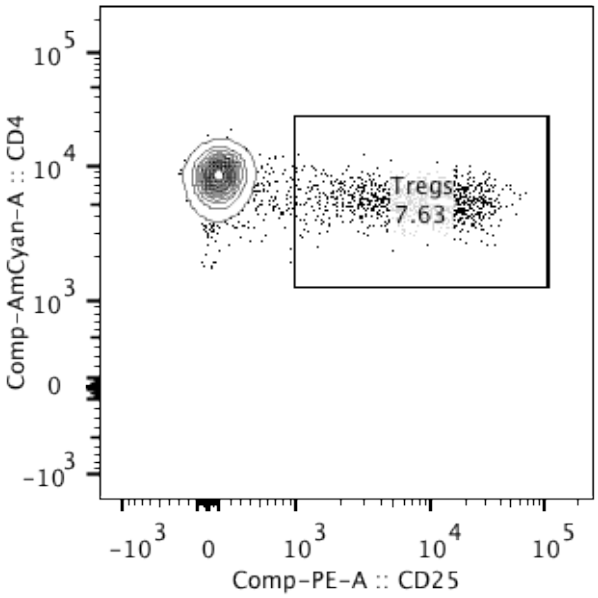
23. For the first plot, X axis = PE-TexasRed-CD62L; Y axis = FITC-CD44
    - gate = CD4+CD44 Teff mem
    - This gate is quite hard to set. Use the naïve CD62L^{hi} CD44^{lo} cells to help guide where to place the gate.
    - The effector memory T cells you are after are (CD62L^{lo} CD44^{hi}). Place the gate to the left of the main population on the CD62L axis and use the central point of the contour for the naïve cells to determine the CD44 level.
    - Make a note in the QC column of the FlowJo workspace window if there is CD62L shedding which makes this gate hard to set. In the event of shedding the Teff gate should be moved higher on the CD44 axis to avoid the CD44^{lo} CD62L^{lo} population that is a processing artefact (this can be caused by a difficult bleed or incubation too long in RBC lysis buffer).

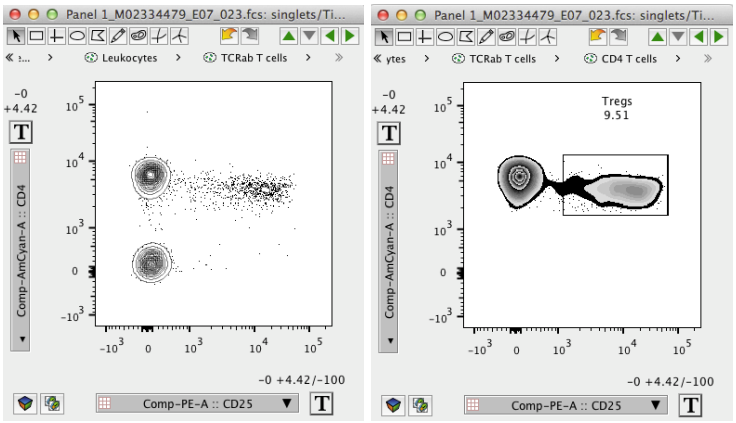


24. For the second plot, X axis = PE-Cy7-KLRG1; Y axis = FITC-CD44
    - gate = KLRG1+ antigen experienced CD4 T cells
    - Here the gate is extended over the entire CD44 range (although the majority should be CD44^{hi}) and is set just to the right of the initial outliers from the main KLRG1 negative population.
    - Make a note in the QC column of the FlowJo workspace window if the whole population has shifted and is KLRG1^{hi} that prevents the gate being placed.



25. For the third plot, X axis = PE-CD25; Y axis = AmCyan-CD4
    - gate = Tregs
    - If this gate is difficult to place, click on ‘TCRab T cells’ in the gating path to see the CD4- population and set gate to the right of this, or change the display to zebra plot using the options window (see below).





26. When all the gates are set correctly for a representative sample, uncheck the synchronised option for the group to allow gates to be adjusted for individual samples.

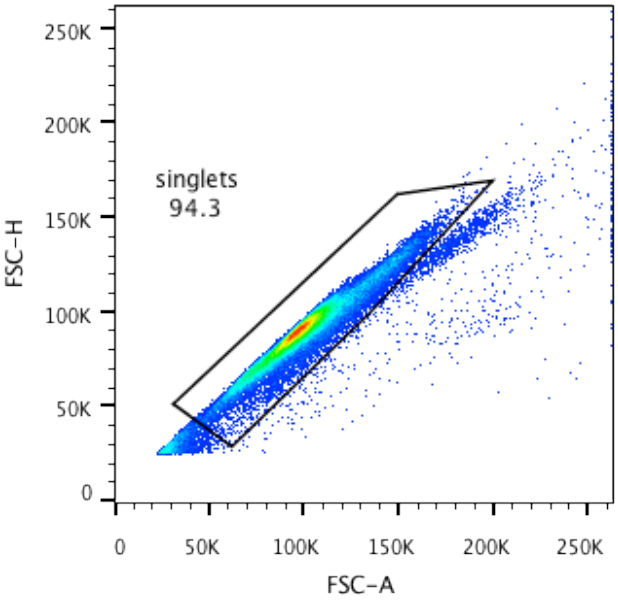
27. Hold Shift and click the forward button  to cycle through all the samples, checking the populations fall into the gates correctly, and tweaking any if necessary.

28. If staining looks abnormal add a comment to the QC column in the main FlowJo window, this will appear on the export for data analysis later

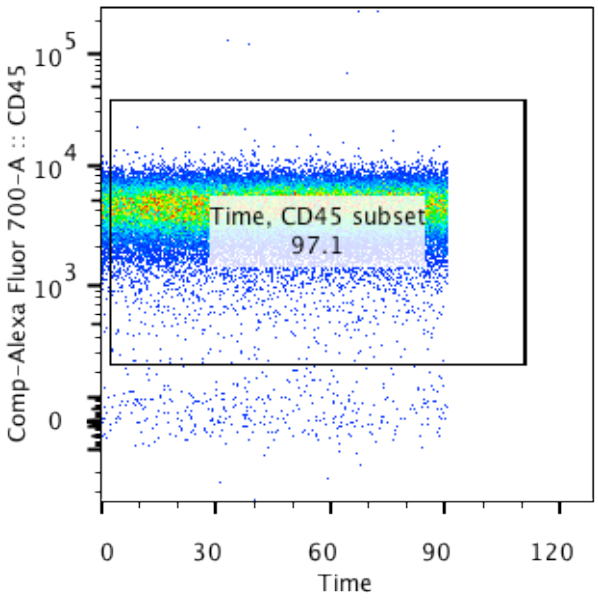
29. Once you have been through all samples, save the workspace file again, and close all the plots.

Panel 2

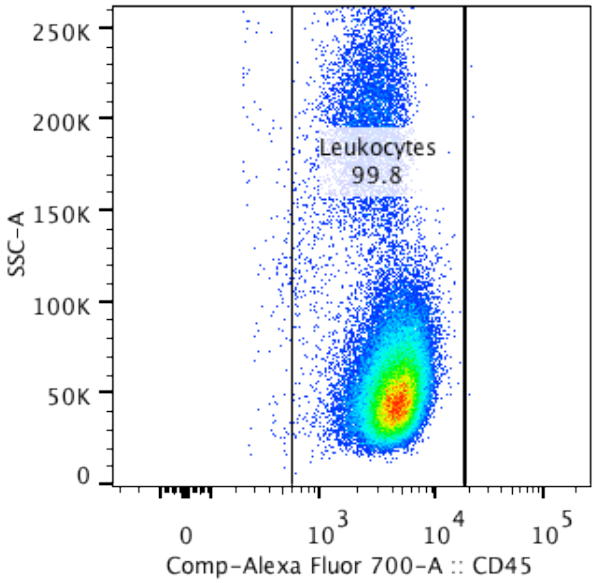
30. Open the first sample
    - X axis = FSC-A; Y axis = FSC-H
    - gate = singlets
    - If this gate is not set correctly you will see double positive events in the plots which do not occur biologically e.g. cells positive for both CD19 and Ly6B



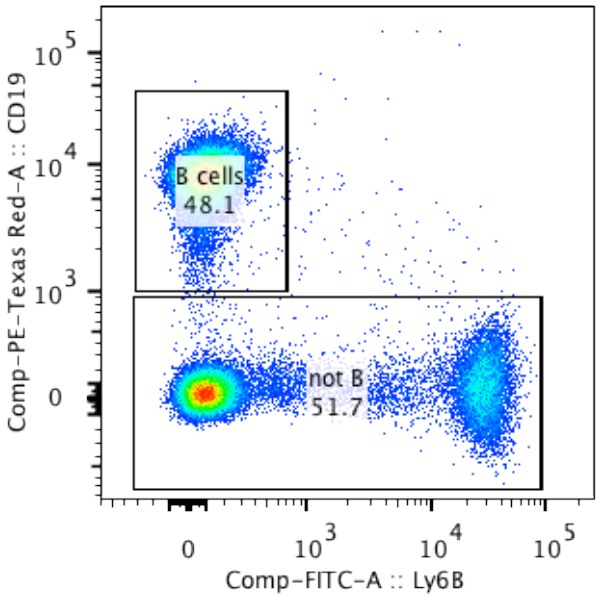
31. Open 'singlets'.
    - X axis = Time; Y axis = Alexa700-CD45;
    - gate = Time, CD45 subset
    - Here you want to avoid any fluctuations in the event rate, this will often mean taking the middle section of the events.



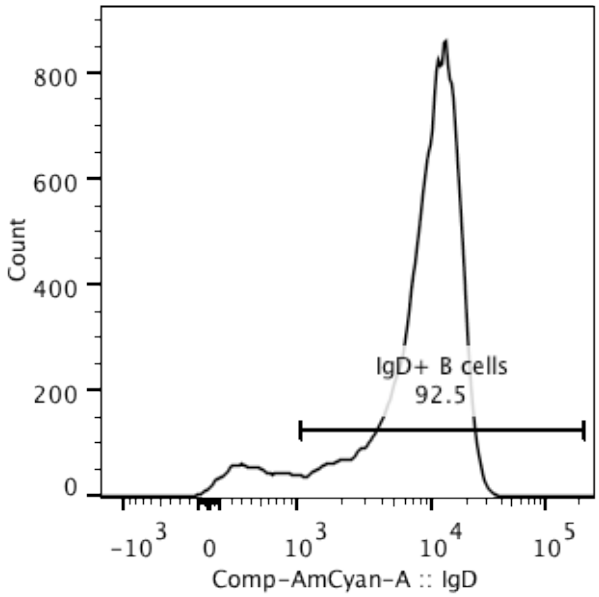
32. Open ‘Time, CD45 subset’
    - X axis = Alexa700-CD45; Y axis = SSC-A;
    - gate = leukocytes
    - Ensure this gate is pushed off the top of the SSC-A axis to ensure all events are accounted for



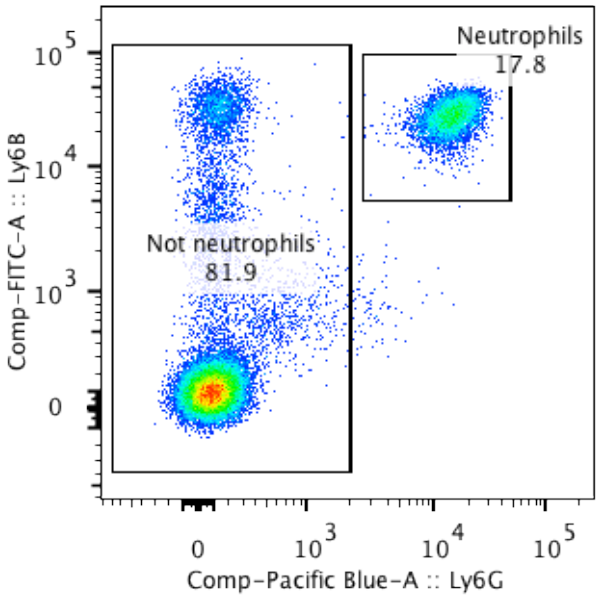
33. Open ‘leukocytes’
    - X axis = FITC-Ly6B; Y axis = PE-TexasRed-CD19
    - gate = B cells
    - gate = not B
    - If a mouse has granulocytosis, the neutrophils are the main Ly6B^{hi} population and the size of the ‘not B’ gate might need to be increased to include them all



34. Open ‘B cells’
    - X axis = AmCyan-IgD; Y axis = histogram
    - gate = IgD+ B cells
    - This gate has been set using a FMO control and shouldn’t be changed.

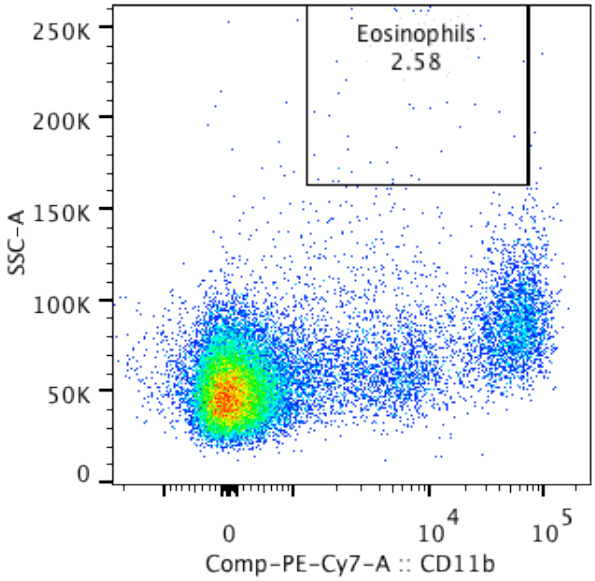


35. Open ‘not B’
    - X axis = PacificBlue-Ly6G; Y axis = FITC-Ly6B
    - gate = Neutrophils
    - gate = Not neutrophils
    - If a mouse has granulocytosis, the neutrophils can be >60% of the events on this plot and in that case the gate will need to be adjusted as best as possible

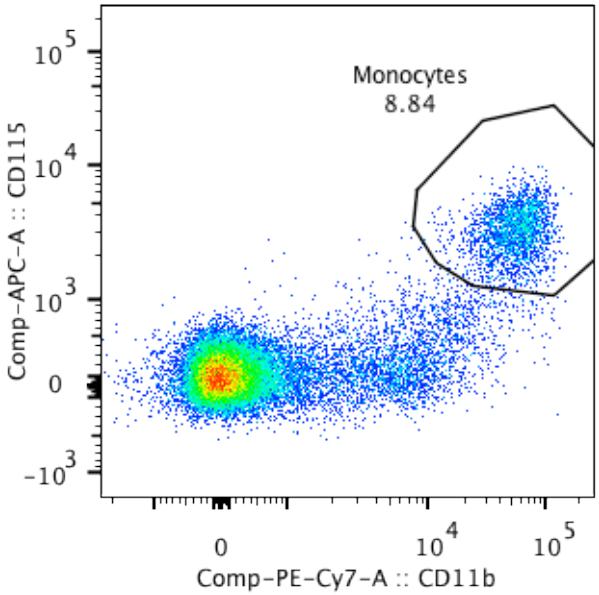


36. Open ‘Not neutrophils’ and duplicate this new plot once 

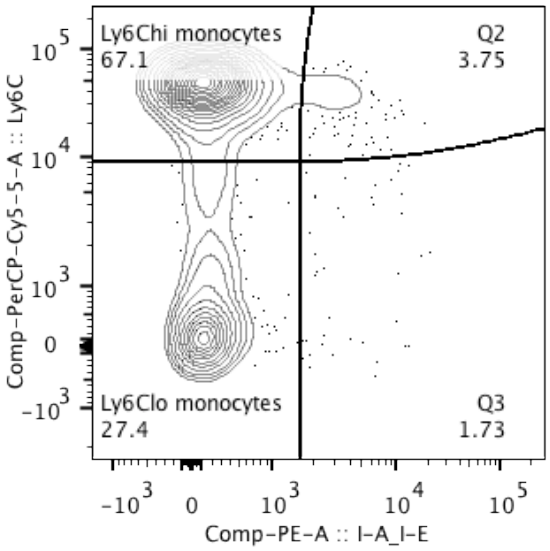
37. For the first plot, X axis = PE-Cy7-CD11b; Y axis = SSC-A
    - gate = Eosinophils
    - Ensure this gate is pushed off the top of the SSC-A axis to ensure all events are accounted for
    - If a sample was KLRG1 high in panel 1, it is likely it will also be shifted and appear CD11b bright for panel 2. In this case, the gate can normally be set but needs to be moved further to the right.



38. For the second plot, X axis = PE-Cy7-CD11b; Y axis = APC-CD115
    - gate = Monocytes
    - If a sample was KLRG1 high in panel 1, it is likely it will also be shifted and appear CD11b bright for panel 2. In this case the gate can normally be set but needs to be moved further to the right.
    - This is the gate that needs to be adjusted the most in panel 2.



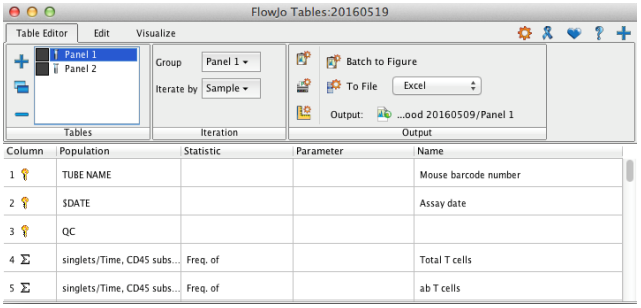
39. Open ‘Monocytes’
    - X axis = PE-I-A_I-E; Y axis = PerCP-Cy5.5-Ly6C – Change the display to ‘contour’ using the options window if not already in this format, and check the ‘show outliers’ box.
    - gate = Ly6C^{hi} monocytes
    - gate = Ly6C^{lo} monocytes
    - If there are >5% of events in the I-A/I-E high gates make a note of this in the QC column of the FlowJo workspace.



40. Ensure this sample looks representative and synchronise the panel 2 group to make changes to the core gates. Once set, unsynchronise.

41. Hold Shift and click the forward button  to cycle through all 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 and save the workspace.

42. Open the table editor  , this is already set up to output the data in the necessary format to excel spreadsheets, one for panel 1 and one for panel 2. Click to the right of “output” to change the destination folder to your analysis folder and add the date of the run to the file name, click “save” in this window to save the location. Then click “to file” in the table editor window to export the excel file. Select panel 2 on the left to select the panel 2 data and repeat the steps to export it.



43. Save the FlowJo file and exit the programme.

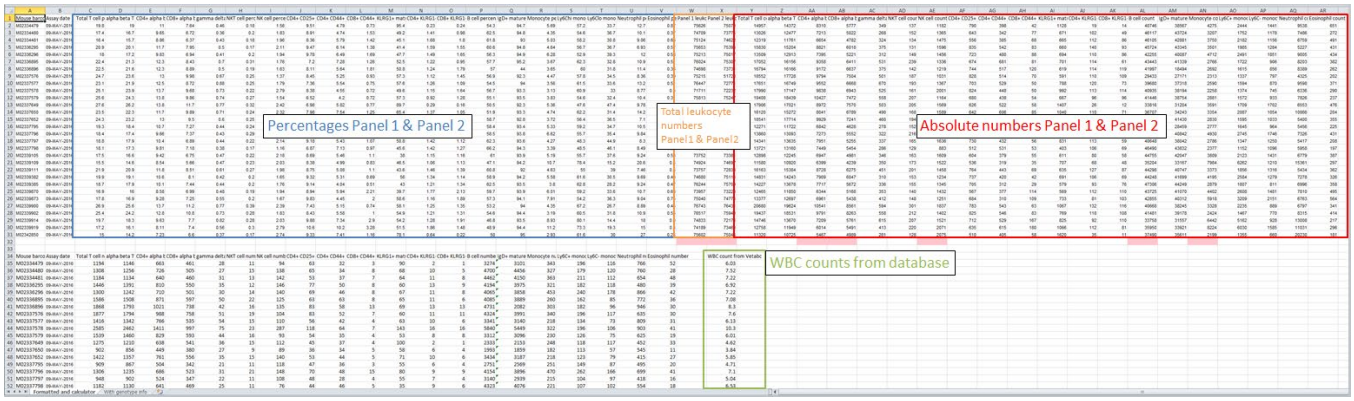
Using the QC checker and calculation template

44. Open the panel 1 and panel 2 output spreadsheets.

45. Check that all barcodes match between the sheets and that they are in the same order.

46. Check that the following percentage columns add up to >95%: Total t cell + NK cell + B cell + monocyte + neutrophil + eosinophil
    If not then there may be a gating problem somewhere.
    - This could be due to low event count making the data unreliable for a panel
    - If a lot of samples appear wrong the sample order between the two sheets might not be the same so go back and double check the barcodes and order

47. Open the “16 week PBL 2panel calculation template” file and paste in the data under the correct headings in the upper part of the “formatted and calculator” tab from the output files.



48. Auto formatting will indicate any samples where event number falls below the required minimum and QC is required. See the ‘QC during analysis’ section at the end of this SOP for details on these thresholds.

49. In the database, run the report “ABC Plus Results” filtering by assay date and sorting by mouse (ascending). Download the report to an excel file and ensure that the barcodes are in the same order as the flow data.

50. Copy and paste the WBC counts to the correct column in the lower part of the “formatted and calculator” tab in the “16 week PBL 2panel calculation template” file and the cell counts for each population will be automatically calculated

51. Paste the columns of data in the required order for upload to the database into a new file and save as a .csv file.

52. Upload this file to the database
    Note: remember to QC fail any data that was flagged in the calculation template or from your comments during FlowJo analysis.

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.
- The calculation template has auto formatting that highlights cells in red that are below certain thresholds for event counts to indicate that they and offspring populations should be QC failed – remember to fail both the % and the cell count parameter with the comment ‘insufficient events’:
    - Leukocyte events < 10,000 = QC fail whole panel
    - CD4+ ab T cell count < 2000 = fail CD4+CD25+Tregs, CD4+CD44+Teffmem, CD4+KLRG1+ ab T cells
    - CD8+ ab T cell count < 1000 = fail CD8+CD44+Teffmem, CD8+KLRG1+ ab T cells
    - NK cell count < 500 = fail KLRG1+ mature NK cells
    - B cell count < 3000 = fail IgD+ mature B cells
    - Monocyte cell count < 1000 = fail Ly6Chi and Ly6Clo monocytes
- For KLRG1 shifts that meant gates could not be set, fail all KLRG1 related parameters with the comment ‘KLRG1 shift, cannot set gates’

	A	B	C	D	E	F	G	H	I	J	K
1	Mouse barco	Assay date	Total T cell percentage	alpha beta T cell percentage	CD4+ alpha beta T cell percentage	CD8+ alpha beta T cell percentage	gamma delta cell percentage	NKT cell percentage	NK cell percentage	CD4+ CD25+ alpha beta regulatory T cell percentage	CD4+ CD44+ CD62L- alpha beta effector T cell percentage
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32	Mouse barco	Assay date	Total T cell number	alpha beta T cell number	CD4+ alpha beta T cell number	CD8+ alpha beta T cell number	gamma delta cell number	NKT cell number	NK cell number	CD4+ CD25+ alpha beta regulatory T cell number	CD4+ CD44+ CD62L- alpha beta effector T cell number
33			=(C2/100)*($X34*1000)	=(D2/100)*($X34*1000)	=(E2/100)*($X34*1000)	=(F2/100)*($X34*1000)	=(G2/100)*($X34*1000)	=(H2/100)*($X34*1000)	=(I2/100)*($X34*1000)	=((J2/100)*($E2/100))*($X34*1000)	=((K2/100)*($E2/100))*($X34*1000)
34			0	0	0	0	0	0	0	0	0
35			0	0	0	0	0	0	0	0	0
36			0	0	0	0	0	0	0	0	0
37			0	0	0	0	0	0	0	0	0
38			0	0	0	0	0	0	0	0	0
39			0	0	0	0	0	0	0	0	0
40			0	0	0	0	0	0	0	0	0
41			0	0	0	0	0	0	0	0	0
42			0	0	0	0	0	0	0	0	0
43			0	0	0	0	0	0	0	0	0
44			0	0	0	0	0	0	0	0	0
45			0	0	0	0	0	0	0	0	0
46			0	0	0	0	0	0	0	0	0
47			0	0	0	0	0	0	0	0	0
48			0	0	0	0	0	0	0	0	0
49			0	0	0	0	0	0	0	0	0
50			0	0	0	0	0	0	0	0	0
51											

	L	M	N	O	P	Q	R	S
1	CD8+ CD44+ CD62L- alpha beta effector T cell percentage	KLRG1+ mature NK cell percentage	CD4+ KLRG1+ alpha beta T cell percentage	CD8+ KLRG1+ alpha beta T cell percentage	B cell percentage	IgD+ mature B cell percentage	Monocyte percentage	Ly6Chi monocyte percentage
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32	CD8+ CD44+ CD62L- alpha beta effector T cell number	KLRG1+ mature NK cell number	CD4+ KLRG1+ alpha beta T cell number	CD8+ KLRG1+ alpha beta T cell number	B cell number	IgD+ mature B cell number	Monocyte number	Ly6C+ monocyte number
33	=((L2/100)*($F2/100))*($X34*1000)	=((M2/100)*($I2/100))*($X34*1000)	=((N2/100)*($E2/100))*($X34*1000)	=((O2/100)*($F2/100))*($X34*1000)	=(P2/100)*($X34*1000)	=((Q2/100)*($P2/100))*($X34*1000)	=(R2/100)*($X34*1000)	=((S2/100)*($R2/100))*($X34*1000)
34	0	0	0	0	0	0	0	0
35	0	0	0	0	0	0	0	0
36	0	0	0	0	0	0	0	0
37	0	0	0	0	0	0	0	0
38	0	0	0	0	0	0	0	0
39	0	0	0	0	0	0	0	0
40	0	0	0	0	0	0	0	0
41	0	0	0	0	0	0	0	0
42	0	0	0	0	0	0	0	0
43	0	0	0	0	0	0	0	0
44	0	0	0	0	0	0	0	0
45	0	0	0	0	0	0	0	0
46	0	0	0	0	0	0	0	0
47	0	0	0	0	0	0	0	0
48	0	0	0	0	0	0	0	0
49	0	0	0	0	0	0	0	0
50	0	0	0	0	0	0	0	0
51								

	T	U	V	W	X	Y	Z	AA	AB	AC	AD	AE
1	Ly6Clo monocyte percentage	Neutrophil percentage	Eosinophil percentage	Panel 1 leukocyte count	Panel 2 leukocyte count	Total T cell count	alpha beta T cell count	CD4+ alpha beta T cell count	CD8+ alpha beta T cell count	gamma delta cell count	NKT cell count	NK cell count
2												
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32	Ly6C- monocyte number	Neutrophil number	Eosinophil number
33	=((T2/100)*($R2/100))*($X34*1000)	=(U2/100)*($X34*1000)	=(V2/100)*($X34*1000)
34	0	0	0
35	0	0	0
36	0	0	0
37	0	0	0
38	0	0	0
39	0	0	0
40	0	0	0
41	0	0	0
42	0	0	0
43	0	0	0
44	0	0	0
45	0	0	0
46	0	0	0
47	0	0	0
48	0	0	0
49	0	0	0
50	0	0	0
51			

WBC count

	AF	AG	AH	AI	AJ	AK	AL	AM	AN	AO
1	CD4+ CD25+ alpha beta regulatory T cell count	CD4+ CD44+ CD62L- alpha beta effector T cell count	CD8+ CD44+ CD62L- alpha beta effector T cell count	KLRG1+ mature NK cell count	CD4+ KLRG1+ alpha beta T cell count	CD8+ KLRG1+ alpha beta T cell count	B cell count	IgD+ mature B cell count	Monocyte count	Ly6C+ monocyte count
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51										

	AP	AQ	AR
1	Ly6C- monocyte count	Neutrophil count	Eosinophil count
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