Staining Index and Spill Over

on

LSRFortessa

Determined using anti-CD8 in an

Experiment for Analysing Lymphocytes.

FACS Core Facility, Aarhus University
2016

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Aim for this study

To get to know our specific flow cytometer – LSRFortessa – better, we decided to calculate Compensation Values and Stain Index for 25 different fluorochromes in an experiment optimized for running human peripheral blood lymphocytes.
It is important to realize, that the compensation values and stain index values are closely related to these settings.

Materials

Antibodies:
25 different fluorochromes conjugated to anti-human CD8 antibody, clone RPA-T8 except for BV605, PerCP-eFlour710, APC (clone SK1), and PE-Cy7 (clone HIT8a). (See page3 for details concerning antibodies, fluorochromes, lasers and emission wavelengths)

MNC separated from human peripheral blood.

Compensation Beads: eBioscience 01-1111-42

Other materials: See the procedure "Antibody staining of surface antigens on cells - using a 96 well plate".

Methods

Lymphocytes and staining.
MNC were separated from human peripheral blood by centrifugation on Histopaque gradient (Sigma). Washed twice in PBS with 0.5% BSA. MNC were frozen in RPMI-1640 containing 10% DMSO and 20% FCS.
MNC were thawed, centrifuged and resuspended in PBS containing 0.5% BSA and 0.09% NaN_3 and stained as described in "Antibody staining of surface antigens on cells using a 96 well plate" (page4) One sample for each antibody-fluorochrome-conjugate and one unstained sample.
OneComp eBeads were stained, as the cells were, but no unstained sample was included, as the bead suspension contains negative as well as positive beads.

An experiment for running lymphocytes
was created by running unstained mononuclear cells on the LSRFortessa, gating the lymphocytes and setting the median fluorescence from each PMT above the lower limit for linearity. Trying to avoid negative values after compensation, voltages were set a bit higher to achieve medians around 100-200.
Compensation beads and MNCs were analyzed in this experiment

Compensation Values
were determined using the beads FCS files and FlowJo Software.
As we wanted to calculate compensation for up to 3 fluorochromes per detector, we had to set up 3 different compensation matrices in FlowJo. After calculating the 3 matrices the values were pooled in one excel spreadsheet.

Stain index
were calculated as $(Median_{pos} - median_{neg}) / (2 \times rSD_{neg})$ after analyzing the lymphocyte gate from the MNC FCS files in FlowJo software.

Results can be seen at pages 5 The Compensation Matrix and 6 Stain Index

Antibody overview

Laser	Well	µl/test	ab	FL	klon	Cat #	Lot #	Exp date	**Comp. matrice nr. ***	**Filter**	**Filter**
	A1	5ul	CD8	V450	RPA-T8	BD560347	3039752	2015-01	**1**	**442/46**	**V1**
	A2	5ul	CD8	BV421	RPA-T8	BD562429(25test)	4290912	2018-10	**2**	**442/46**	**V1**
	A3	5ul	CD8	V500	RPA-T8	BD560775	3235542	2015-03	**1**	**525/50**	**V2**
	A4	5ul	CD8	BV510	RPA-T8	BD563256	4080668	2015-07	**2**	**525/50**	**V2**
	A5	5ul	CD8	eVolve605	RPA-T8	eBio83-0088-41	E24358-102	2015-11	**1**	**610/20**	**V3**
	A6	5ul	CD8	BV 605	SK1	BD564116	-	-	**2**	**610/20**	**V3**
	A7	5ul	CD8	BV650	RPA-T8	BD563822	4115843	2017-09	**1**	**660/20**	**V4**
	A8	5ul	CD8	BV711	RPA-T8	BD563676	4108877	2018-02	**1**	**711/25**	**V5**
	A9	5ul	CD8	BV786	RPA-T8	BD563824	5098928	2016-07	**1**	**785/80**	**V6**
	A10	20ul	CD8	FITC	RPA-T8	BD561947	4028695	2015-12	**1**	**530/30**	**B1**
	A11	5ul	CD8	AF488	RPA-T8	BD557704	4171577	2015-10	**2**	**530/30**	**B1**
	A12	5ul	CD8	BB515	RPA-T8	BD564527	4255561	2015-12	**3**	**530/30**	**B1**
	B1	5ul	CD8	PerCP-Cy5.5	RPA-T8	BD560662	4339888	2016-10	**1**	**710/50**	**B2**
	B2	5ul	CD8	PerCP-eFlour710	SK1	eBio46-0087-41	E10832-1633	2017-02	**2**	**710/50**	**B2**
	B3	20ul	CD8	PE	RPA-T8	BD561949	43044878	2020-08	**1**	**586/15**	**GG1**
	B4	5ul	CD8	PE-eFlour610	RPA-T8	eBio61-0088-41	E19357-101	2016-02	**1**	**615/20**	**GG2**
	B5	5ul	CD8	PE-CF594	RPA-T8	BD562311	3346941	2015-11	**2**	**615/20**	**GG2**
	B6	10ul	CD8	PE-Cy5	HIT8a	BD555636	2097558	2015-03	**1**	**660/20**	**GG3**
	B7	5ul	CD8	PE-Cy7	RPA-T8	BD560917	3287705	2015-04	**1**	**780/60**	**GG5**
	B8	10ul	CD8	APC	RPA-T8	BD555369	12780	2014-03	**1**	**670/30**	**R1**
	B9	5ul	CD8	AF647	RPA-T8	BD557708	3252624	2015-04	**2**	**670/30**	**R1**
	B10	0.2ul	CD8	AF700	RPA-T8	BD557945	3316609	2015-10	**1**	**730/45**	**R2**
	B11	5ul	CD8	APC-eFlour780	RPA-T8	eBio47-0088-41	E08444-1634	2016-09	**1**	**780/60**	**R3**
	B12	5ul	CD8	APC-H7	SK1	BD560273	4238532	2016-02	**2**	**780/60**	**R3**
	C1	5ul	CD8	APC-Cy7	RPA-T8	BD557760	4045963	2015-12	**3**	**780/60**	**R3**

* Which color goes to which Compensaton Matrix calculation.

To calculate compensation matrices, we have to make 3 matrices as we test up to 3 colors in the same channel.

Antibody staining of surface antigens on cells using a 96 well plate

Notes

This is a general protocol, which does not concern specific antibodies, however it takes into consideration: Incubation, washing and fixation.

Consider blocking before staining.

In this protocol the incubation is performed at RT. If you want to incubate on ice the incubation time should be expanded to 30-60 min and the centrifuge should be cold.

In each well there will be approximately 15µl left after pouring off your supernatant.

MATERIAL

1. Stain buffer: PBS pH 7.4 with 0.5% BSA and 0.09% Na-azide
2. Fluorocrome conjugated antibodies and maybe isotype controls
3. Cells at 1-5x10^6/ml in stain buffer
4. A round bottom microwell plate (not for cell culturing)
5. Fixation buffer: PBS pH 7.4 with 0.9% formaldehyde

PROCEDURE

Be cautious at any time not to mix fluid from one well to another when pouring fluid off. Even a tiny bit of antibody can stain your cells.

1. Make a scheme showing which antibodies in which wells. If viability test is not a part of your panel you should make a sample just with your viability marker
2. Adjust your cell suspension to 1-5x 10^6/ml in stain buffer. Blocking could be performed now
3. Add antibodies to the wells
4. Transfer 100µl cell suspension to each well and pipette up and down 5 times times to mix cells and antibodies.
5. Incubate in the dark 15-30 min at RT. Some cell types will require incubation 30-60 min at 4 C^0
6. Add 100 µl washing buffer to each well.
7. Centrifuge the plate at 350g for 2 min at RT (or cold)
8. Place a thick tissue flat on the table
9. Pour off the supernatant in one sliding movement and press the plate briefly against the tissue before you turn the plate bottom downwards again
10. Loosen the cell pellet by a gentle knocking on the side of the plate
11. Add 200µl stain buffer to each well using a multichannel pipette, resuspend cells using the pipette.
12. Repeat from 7-10 twice
13. Fix and resuspend the cells by adding 200µl of fixation buffer to each well and pipette up and down 5 times
14. Place a lid on the plate and keep it cold and dark until analysis
15. The use of tandem dyes will limit the storage time to 24 hours. We recommend to analyse as quickly as possible.

LSRFortessa **Compensation Matrix** for"Lymphocyte Settings" See these settings below **More than one stain per PMT is shown!**
Staining performed May 2015 and Feb 2016

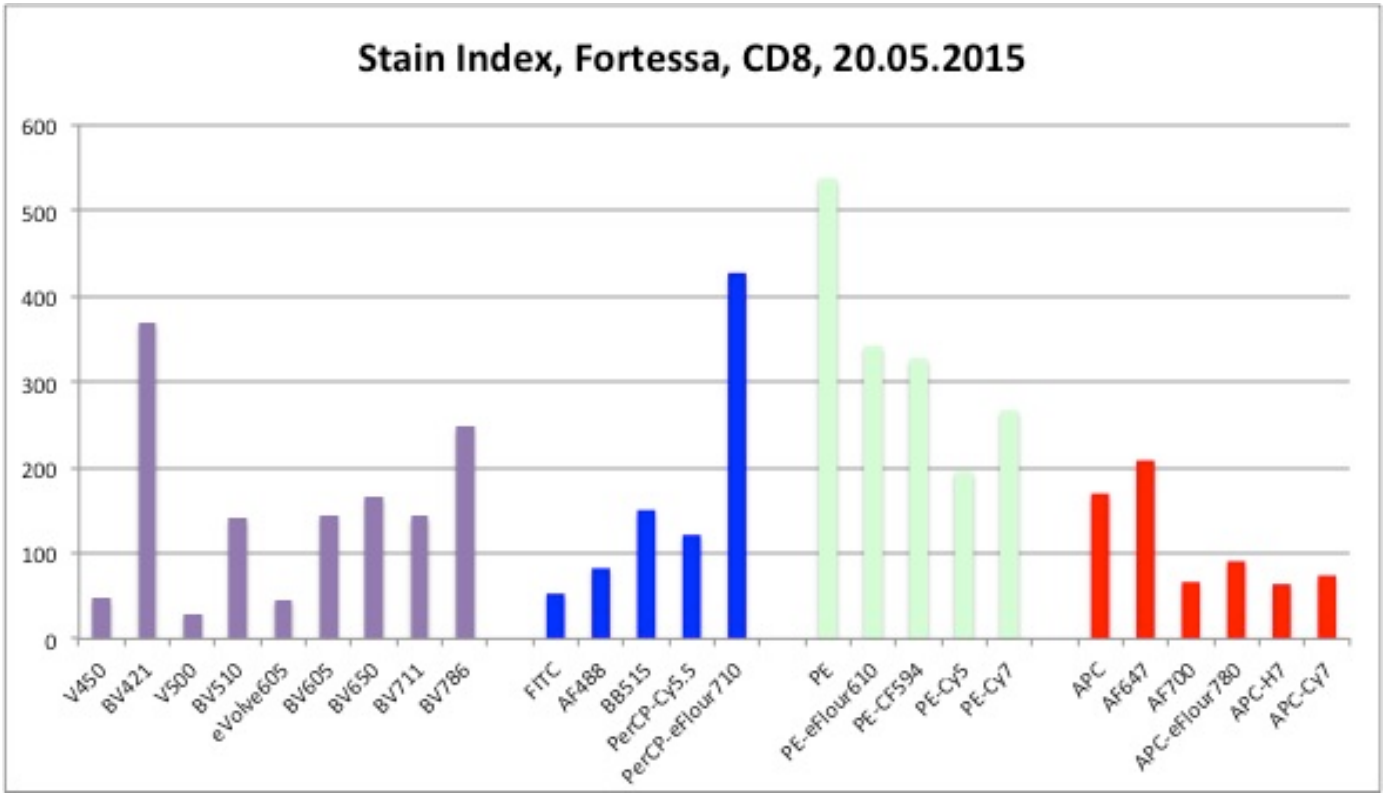
Spill over from row to column

PMT	V1	V2	V3	V4	V5	V6	B1	B2	YG1	YG2	YG3	YG5	R1	R2	R3	% comp.
Band Pass filter	V 442_46	V 525_50	V 605_15	V 660_20	V 711_25	V 785_60	Blue 530_30	Blue 710_50	YG 575_15	YG 615_20	YG 660_20	YG 780_60	Red 670_30	Red 730_45	Red 780_60	color key
V450		25,6%	4,0%	0,8%	0,3%	0,1%	0,0%	0,0%	0,0%	0,0%	-0,1%	0,0%	-0,3%	-0,1%	0,0%	0-5
BV421		11,3%	1,6%	0,4%	0,1%	0,1%	0,1%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	5-10
V500	3,3%		53,7%	14,7%	4,9%	1,8%	5,9%	0,3%	0,0%	0,0%	0,0%	0,0%	-0,2%	-0,1%	0,0%	10-20
BV510	7,5%		99,0%	36,3%	15,2%	7,3%	0,7%	0,1%	0,0%	0,0%	0,0%	0,0%	-0,1%	0,0%	0,0%	20-30
eVolve605	0,0%	0,0%		1,4%	0,1%	0,1%	0,0%	0,0%	1,5%	5,0%	0,2%	0,0%	-0,1%	0,0%	0,0%	30-40
BV605	1,5%	0,2%		49,4%	18,0%	7,8%	0,0%	1,2%	5,1%	8,5%	12,4%	1,2%	0,0%	0,1%	0,0%	40-60
BV650	1,7%	0,3%	17,4%		40,1%	13,5%	0,0%	0,8%	0,1%	0,9%	7,1%	0,6%	8,4%	8,3%	1,9%	60-80
BV711	4,4%	0,7%	0,5%	2,6%		53,5%	0,0%	4,2%	0,0%	0,0%	0,1%	0,4%	1,0%	25,4%	7,7%	80-100
BV786	3,9%	0,8%	0,7%	1,1%	2,2%		0,0%	0,1%	0,0%	0,0%	0,0%	0,5%	0,2%	2,0%	7,1%	
FITC	0,0%	3,8%	1,5%	0,4%	0,2%	0,1%		1,8%	0,0%	0,0%	0,0%	0,0%	-0,1%	0,0%	0,0%	
AF488	-0,6%	0,9%	0,3%	0,1%	0,0%	0,0%		1,1%	0,0%	0,0%	0,0%	0,0%	-0,1%	0,0%	0,0%	
BB515	-0,2%	0,9%	0,2%	0,0%	0,0%	0,0%		0,9%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	0,0%	
PerCP-Cy5.5	0,0%	0,0%	0,0%	13,8%	83,2%	39,2%	0,0%		0,0%	0,0%	8,2%	11,5%	17,7%	74,5%	23,7%	
PerCP-eFlour710	0,0%	0,0%	0,0%	2,0%	77,6%	35,7%	0,0%		0,0%	0,0%	1,1%	9,2%	2,9%	93,8%	24,7%	
PE	0,0%	0,1%	12,8%	3,2%	1,1%	0,3%	0,9%	9,9%		46,6%	31,0%	2,0%	0,0%	0,0%	0,0%	
PE-eFlour610	0,0%	0,0%	23,7%	6,6%	1,8%	0,6%	0,1%	15,7%	40,3%		81,9%	4,6%	0,1%	0,1%	0,0%	
PE-CF594	0,0%	0,0%	24,1%	7,8%	3,6%	1,5%	0,1%	33,2%	19,1%		87,1%	10,1%	0,3%	0,4%	0,1%	
PE-Cy5	0,0%	0,0%	0,2%	8,3%	4,0%	1,7%	0,0%	40,1%	1,5%	0,8%		13,0%	33,7%	31,9%	8,6%	
PE-Cy7	0,0%	0,0%	0,1%	0,0%	0,1%	17,0%	0,1%	1,0%	0,7%	0,4%	0,3%		0,0%	1,3%	16,9%	
APC	0,0%	0,0%	0,2%	11,6%	3,1%	1,4%	0,0%	0,7%	0,0%	0,6%	44,7%	3,3%		82,9%	20,8%	
AF647	0,0%	0,0%	0,0%	0,3%	0,1%	0,1%	0,0%	0,3%	0,0%	0,0%	8,0%	1,2%		97,8%	26,8%	
AF700	0,0%	0,0%	0,0%	0,0%	4,0%	2,2%	0,0%	1,1%	0,0%	0,0%	0,1%	2,1%	1,1%		25,9%	
APC-eFlour780	0,0%	0,0%	0,0%	0,6%	0,2%	8,3%	0,0%	0,1%	0,0%	0,0%	2,3%	10,8%	6,7%	11,1%		
APC-H7	0,0%	0,0%	0,0%	0,1%	0,0%	8,6%	0,0%	0,0%	0,0%	0,0%	0,2%	9,8%	0,7%	5,0%		
APC-Cy7	0,0%	0,0%	0,0%	0,4%	0,1%	7,8%	0,0%	0,0%	0,0%	0,0%	1,4%	10,6%	3,9%	11,5%		

"Lymfocyte settings":

Short name	V1	V2	V3	V4	V5	V6	B1	B2	YG1	YG2	YG3	YG5	R1	R2	R3
Band Pass filter	V 442_46	V 525_50	V 605_15	V 660_20	V 711_25	V 785_60	Blue 530_30	Blue 710_50	YG 575_15	YG 615_20	YG 660_20	YG 780_60	Red 670_30	Red 730_45	Red 780_60
PMT Voltage	460	480	620	630	680	710	500	600	520	540	660	660	700	720	720

Short names for the PMTs: The PMTs are named from 1 and forthgoing for each laser, where V is violet laser and YG is yellow-green laser
The PMT YG4 (710/40) has not been used in this experiment


Stain Index, Fortessa, CD8, 20.05.2015
600
500
400
300
200
100
0
V450
BV421
V500
BV510
eVolve605
BV605
BV650
BV711
BV786
FITC
AF488
BB515
PerCP-Cy5.5
PerCP-eFlour710
PE
PE-eFlour610
PE-CF594
PE-Cy5
PE-Cy7
APC
AF647
AF700
APC-eFlour780
APC-H7
APC-Cy7

Fluorochrome/PMT suggestions for 4-8 color panel

Based on our overview "Lasers and Filters at the LSRFortessa" (page 11) and a glance to the Compensation Matrix from this experiment, we will set up suggestions for choosing fluorochromes/PMTs for multicolor panels.

Theese suggestions are NOT the only way to do it!!

You should still rememter to take into account the abundance of the target antigen, when you choose fluorochrome. Choose a fluorochrome with a high Stain Index for a weakly expressed antigen and vice versa.
An example: If you look for a fluorochrome for V1, you could choose BV421 for a weakly expressed antigen and V450 for a higly expressed antigen.
You can use more colors, if you have subpopulations, which do not express all antigens in your panel.

In stead of proposing fluorochromes for the panels, we will propose PMTs to use.
(see page3 or 5 to understand the abrevations)

PMT suggestions

4 colors: V1, B1, YG1, R1

5 colors: + YG5

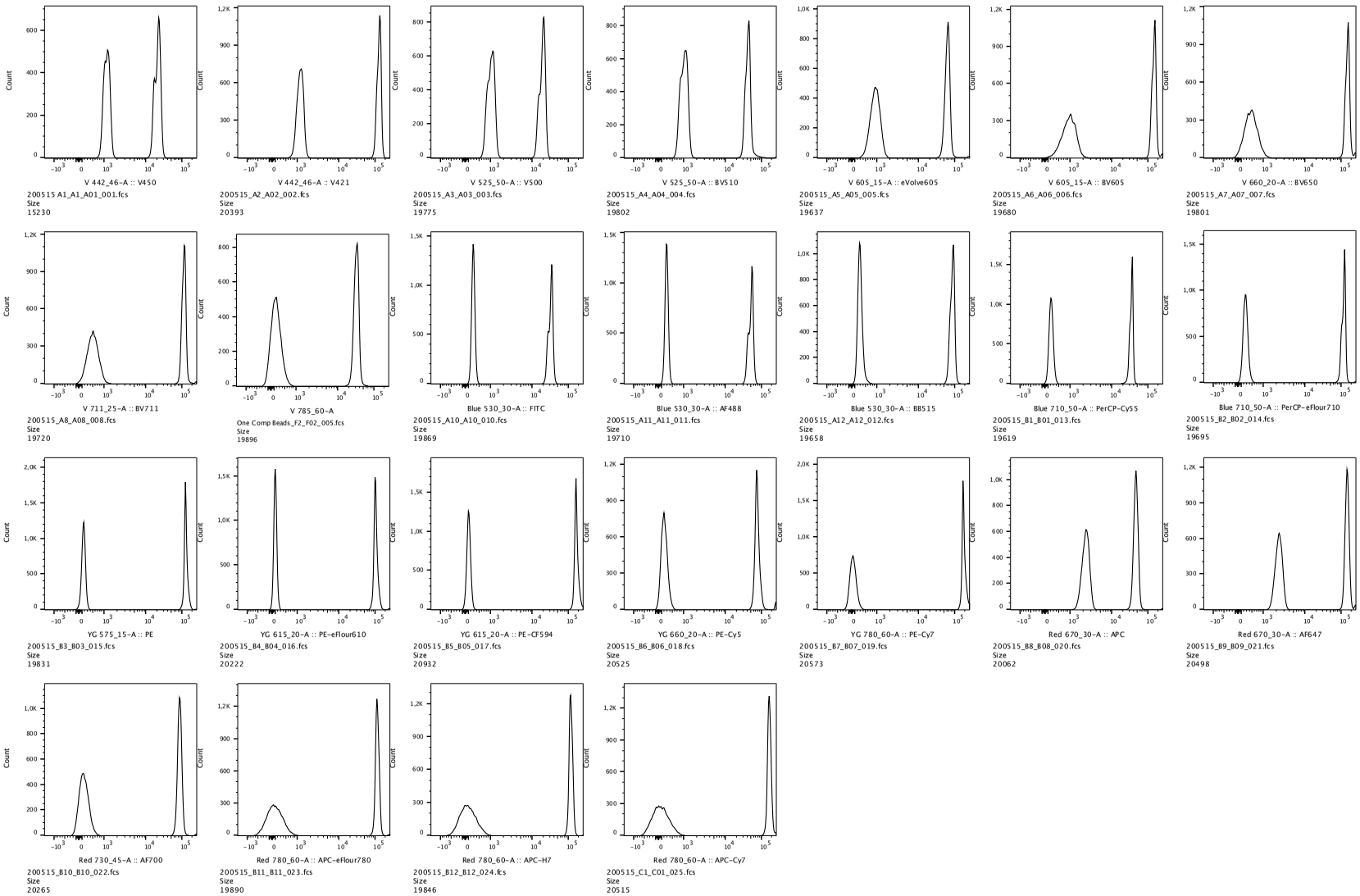
6 colors: +V6

7 colors: +V3

8 colors: +R3

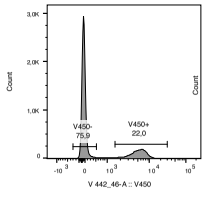
+8 colors: Come to FACS Core Facility (you are welcome, also for fewer colors ☺)

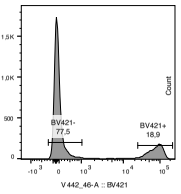
Compensation Beads Positivity for each Fluorochrome. Analyzed on LSRFortessa 2015.05.20 BV586 analyzed 2016.02.08

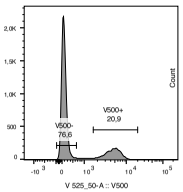


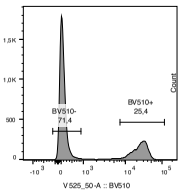
Lymfocytes. Gating for calculating Stain Index

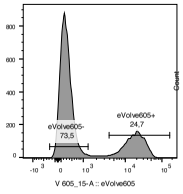
Analyzed on LSR Fortessa 2015.05.20 BV786 analyzed 2016.02.08

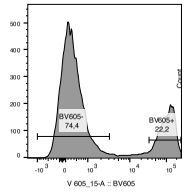


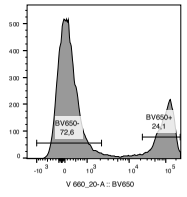












200515_A1_A01_001.fcs
Lymphocytes
27904

200515_A2_A02_002.fcs
Lymphocytes
26864

200515_A3_A03_003.fcs
Lymphocytes
27041

200515_A4_A04_004.fcs
Lymphocytes
26882

200515_A5_A05_005.fcs
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Lymphocytes
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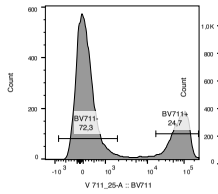

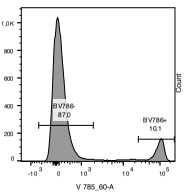

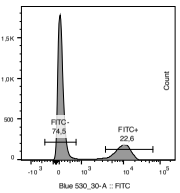

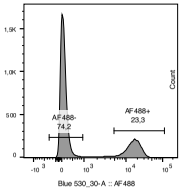

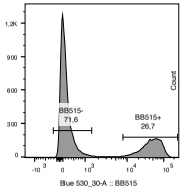

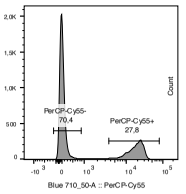

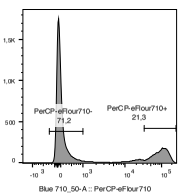

200515_A8_A08_008.fcs
Lymphocytes
26818

MNC_E3_E03_002.fcs
Lymphocytes
28511

200515_A10_A10_010.fcs
Lymphocytes
26784

200515_A11_A11_011.fcs
Lymphocytes
26477

200515_A12_A12_012.fcs
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200515_B1_B01_013.fcs
Lymphocytes
26815

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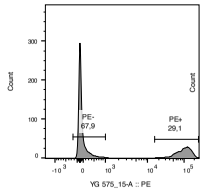
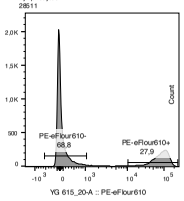
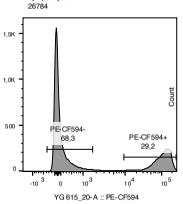
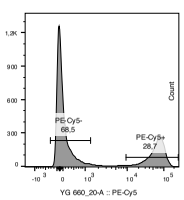
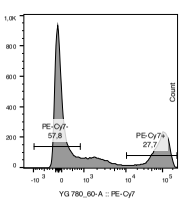
Lymphocytes
26652

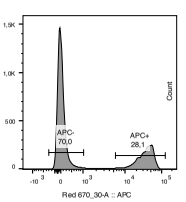


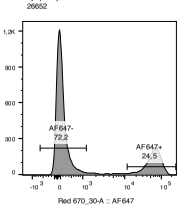












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Lymphocytes
3087

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Lymphocytes
26864

200515_B5_B05_017.fcs
Lymphocytes
26831

200515_B6_B06_018.fcs
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26591

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Lymphocytes
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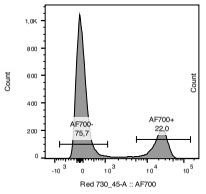
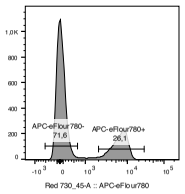
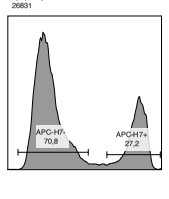
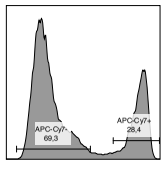
Lymphocytes
27109









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Lymphocytes
27147

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Lymphocytes
26862

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Lymphocytes
27312

200515_C1_C01_025.fcs
Lymphocytes
26984

Stain Index for 25 different CD8 fluorochrome conjugates

Fortessa, 200515, CD8	Laser	Filter	**Fluorochrom**	**Stain Index**	Median pos	Median neg	rSD neg
200515_A1_A01_001.fcs	VIOLET	442/46	**V450**	**46**	5404	79,6	57,3
200515_A2_A02_002.fcs	VIOLET	442/46	**BV421**	**370**	80682	61,6	109
200515_A3_A03_003.fcs	VIOLET	525/50	**V500**	**29**	4682	186	77,9
200515_A4_A04_004.fcs	VIOLET	525/51	**BV510**	**141**	26259	166	92,8
200515_A5_A05_005.fcs	VIOLET	610/20	**eVolve605**	**46**	18458	277	198
200515_A6_A06_006.fcs	VIOLET	610/20	**BV605**	**145**	110959	310	382
200515_A7_A07_007.fcs	VIOLET	660/20	**BV650**	**166**	108268	198	326
200515_A8_A08_008.fcs	VIOLET	711/25	**BV711**	**143**	81675	162	286
MNC_E3_E03_002.fcs	VIOLET	785/60	**BV786**	**247**	101078	123	204
200515_A10_A10_010.fcs	BLÅ	530/30	**FITC**	**53**	9795	170	91,4
200515_A11_A11_011.fcs	BLÅ	530/30	**AF488**	**82**	16344	177	98,2
200515_A12_A12_012.fcs	BLÅ	530/30	**BB515**	**150**	48255	174	160
200515_B1_B01_013.fcs	BLÅ	710/50	**PerCP-Cy5.5**	**121**	18986	123	77,8
200515_B2_B02_014.fcs	BLÅ	710/50	**PerCP-eFlour710**	**427**	100831	82,2	118
200515_B3_B03_015.fcs	GUL-GRØN	586/15	**PE**	**537**	102577	30,8	95,4
200515_B4_B04_016.fcs	GUL-GRØN	615/20	**PE-eFlour610**	**342**	86829	7,7	127
200515_B5_B05_017.fcs	GUL-GRØN	615/20	**PE-CF594**	**327**	113161	8,98	173
200515_B6_B06_018.fcs	GUL-GRØN	660/20	**PE-Cy5**	**195**	69002	47,5	177
200515_B7_B07_019.fcs	GUL-GRØN	780/60	**PE-Cy7**	**266**	94835	35,9	178
200515_B8_B08_020.fcs	RØD	670/30	**APC**	**169**	38075	123	112
200515_B9_B09_021.fcs	RØD	670/30	**AF647**	**208**	63821	123	153
200515_B10_B10_022.fcs	RØD	730/45	**AF700**	**66**	22716	62,9	171
200515_B11_B11_023.fcs	RØD	780/60	**APC-eFlour780**	**90**	61082	87,4	337
200515_B12_B12_024.fcs	RØD	780/60	**APC-H7**	**64**	58606	146	458
200515_C1_C01_025.fcs	RØD	780/60	**APC-Cy7**	**74**	76083	130	514

Lasers and filters at the LSRFortessa

May 2016. Facs Core Facility, Aarhus University