#### Supplementary Table 1

Table 1 - Initial Panel Design for 31 colour panel. This panel was optimized to produce the final panel described in the main figures.

Laser Peak channel V1		Marker	Fluorophore	Supplier	Cat	Clone	Ab host	Ab
		CYCRE		Invitragen	101 0195 11		Mourae	
	<u>V1</u>		DV421 Super Dright 426	Invitrogen			Mouse	
	<u>V2</u>			Tamba	12 0002		wouse	IgGTK
	<u>V3</u>		V450		13-0803	N/A	Marraa	1=01 //
	<u>V5</u>		BV480	BD	500184	В-Іуб	Mouse	
	<u>V5</u>		CFIUOF V505	Сутек	R7-20248	SK3	Mouse	
405nm	<u>V/</u>	IgG	BV510	BD	563247	G18-145	Mouse	IgG1 K
	<u>V8</u>	CD45	cFluor V547	Cytek	R7-20012	HI30	Mouse	IgG1 ĸ
	V10	lgк	BV605	BD	752959	G20-193	Mouse	lgG1 к
	V11	CCR7	BV650	BioLegend	353233	G043H7	Mouse	lgG2a к
	V13	CD24	BV711	Biolegend	311135	ML5	Mouse	lgG2a к
	V14	PD-1	BV750	BioLegend	329965	EH12.2H7	Mouse	lgG1 к
	V15	CD45RA	BV785	Biolegend	304139	HI100	Mouse	lgG2b к
	B1	lgM	BB515	BD	564622	G20-127	Mouse	lgG1 к
	B2	CD57	cFluor B532	Cytek	RC-00127	HNK-1	N/A	N/A
	B3	CD14	cFluor B548	Cytek	R7-20116	63D3	Mouse	lgG1 к
	B4	CD21	PE	BD	561768	B-ly4	Mouse	lgG1 к
	B6	CCR6	PE-Dazzle 594	BioLegend	353429	G034E3		
400	B7	CD19	PE-Fire 640	BioLegend	302273	HIB19	Mouse	lgG1 к
488nm	B8	CD8	PerCP	Tonbo	67-0087	SK1	Mouse	lgG1 к
	B9	lgλ	cFluor B690	Cytek	R7-20260	1-155-2	Mouse	lgG1 к
	B10	CD25	cFluor BYG710	Cytek	R7-20585	BC-96	Mouse	lgG1 к
	B12	lgD	cFluor BYG750	Cytek	RC-00521	N/A	N/A	N/A
	B13	CXCR3	PE-Cy7	BioLegend	353719	G025H7	Mouse	lgG1 к
	B14	CD38	PE-Fire 810	BioLegend	397225	S17015F	Mouse	lgG2a к
	R1	IgA	APC	Miltenyi	130-113-998	IS11-8E10	Mouse	lgG1 к
	R2	CD127	AF647	BioLegend	351317	A019D5	Mouse	lgG1 к
	R3	TCRqd	AF660	BioLegend	331239	B1	Mouse	lgG1 к
640nm	R4	CD20	redFluor 710	Tonbo	80-0209	2H7	Mouse	lgG2b к
	R4	CD56	cFluor R720	Cytek	R7-20090	5.1H11	Mouse	lgG1 к
	R7	CD3	APC-Cy7	Tonbo	25-0038	UCHT1	Mouse	lqG1 к
	R8	CD27	APC-Fire 810	BioLegend	302863	O323	Mouse	lgG1 к



Supplementary Figure 1 - CD56 cFluor R720 staining was negatively impacted by spreading contributed by other fluorophores. From left to right, top row shows PBMCs stained with CD3 APC-Cy7, CD20 redFluor710, CD24 BV711 and IgD cFluor BYG750 only. Bottom row shows these same controls (red) overlayed with CD56 cFluor R720 single-stained control (blue). This demonstrates that the spreading contributed by these colours would make true CD56 positive events difficult to resolve.

APC-Cy7 ViaDye Red APC-Fire 810	0	0	0	0	0	0	0 0 0	0.01 0 0.01	0.06 0.08 0.05	0.21 0.29 0.15	0.19 0.12 0.13	0.22 0.09 0.2	0 0.01 0	0	0 0.01 0	0	0 0.01 0	0.08 0.15 0.05	0.05 0.11 0.04	0.16 0.29 0.11	0.13 0.29 0.09	0.19 0.27 0.11	0.18 0.18 0.15	0.06 0.08 0.1	0.2 0.38 0.16	0.22 0.45 0.17	0.3 0.64 0.2	1 0.83 0.68	1 0.5	1
Alexa Fluor 660	0	0	0	0	0	0	0.01	0.01	0.16	0.33	0.08	0.04	0	0	0.01	0.01	0.02	0.23	0.21	0.4	0.33	0.12	0.06	0.02	0.63	0.8	1			
Alexa Fluor 647	0	0	0	0	0	0	0	0	0.19	0.19	0.03	0.02	0	0	0	0	0	0.24	0.23	0.29	0.19	0.08	0.03	0.01	0.92	1				
APC	0	0	0	0	0	0.02	0.02	0.06	0.32	0.21	0.08	0.04	0	0	0.01	0.01	0.02	0.29	0.24	0.26	0.17	0.08	0.03	0.01	1					
PE-Fire 810	0	0	0	0.01	0.01	0.02	0.06	0.03	0.02	0.06	0.09	0.13	0.02	0.03	0.1	0.18	0.12	0.13	0.08	0.18	0.23	0.45	0.74	1						
PE-Cy7	0	0	0	0	0	0	0.01	0.02	0.04	0.15	0.22	0.25	0	0	0.03	0.02	0.05	0.16	0.12	0.3	0.36	0.75	1							
cFluor BYG750	0	0	0	0	0	0.01	0.02	0.04	0.08	0.24	0.3	0.21	0	0.01	0.04	0.03	0.09	0.26	0.24	0.46	0.61	1								
cFluor BYG710	0	0	0	0.01	0	0.02	0.04	0.06	0.15	0.36	0.18	0.11	0.01	0.02	0.09	0.1	0.23	0.54	0.45	0.84	1									
cFluor B690	0	0	0	0.01	0	0.04	0.06	0.15	0.36	0.58	0.33	0.22	0	0.01	0.08	0.06	0.29	0.65	0.76	1										
PerCP	0	0	0	0.01	0	0.04	0.08	0.17	0.4	0.3	0.18	0.09	0	0.01	0.09	0.08	0.33	0.68	1											
PE-Fire 640	0	0	0	0.01	0.01	0.03	0.08	0.15	0.27	0.19	0.08	0.05	0.02	0.02	0.15	0.17	0.56	1												
PE-Dazzle 594	0	0	0	0.05	0.04	0.12	0.26	0.35	0.15	0.05	0.03	0.02	0.05	0.08	0.31	0.49	1													
PE	0	0.01	0.01	0.1	0.09	0.21	0.46	0.23	0.05	0.01	0.01	0	0.1	0.17	0.47	1														
cFluor B548	0.02	0.03	0.04	0.21	0.23	0.29	0.24	0.16	0.05	0.02	0.01	0.01	0.53	0.75	1															
cFluor B532	0	0	0	0.06	0.03	0.01	0.02	0	0	0	0	0	0.9	1																
BB515	0	0	0	0.08	0.03	0.01	0.01	0	0	0	0	0	1																	
BV785	0.08	0.08	0.06	0.02	0.01	0.03	0.03	0.08	0.16	0.46	0.82	1																		
BV750	0.06	0.06	0.04	0.02	0.01	0.04	0.04	0.12	0.25	0.68	1																			
BV711	0.08	0.08	0.06	0.03	0.01	0.06	0.08	0.18	0.46	1																				
BV650	0.1	0.09	0.08	0.06	0.04	0.16	0.24	0.53	1																					
BV605	0.08	0.08	0.06	0.16	0.15	0.41	0.7	1																						
BV570	0.18	0.18	0.16	0.27	0.25	0.57	1																							
BV510	0.16	0.21	0.35	0.86	0.92	1																								
cFluor V505	0.15	0.2	0.34	0.9	1																									
BV480	0.27	0.34	0.57	1																										
cFluor V450	0.78	0.86	1																											
Super Bright 436	0.95	1																												
BV421	1																													

Supplementary Figure 2 - Similarity index of 30 colours in theory. Matrix generated on CYTEK Cloud (cloud.cytekbio.com/). Numbers in each cell indicate the similarity index of the two fluorophores in that row/column pair. Theoretical calculated complexity index is 19.59.

BV421	1																															
Super Bright 436	0.96	1																														
cFluor V450	0.77	0.87	1																													
BV480	0.29	0.37	0.6	1																												
cFluor V505	0.15	0.21	0.36	0.9	1																											
BV510	0.17	0.23	0.37	0.85	0.93	1																										
BV570	0.17	0.18	0.16	0.27	0.26	0.56	1																									
BV605	0.06	0.07	0.07	0.16	0.15	0.4	0.69	1																								
BV650	0.1	0.1	0.08	0.07	0.04	0.15	0.23	0.52	1																							
BV711	0.07	0.07	0.06	0.04	0.02	0.06	0.08	0.19	0.46	1																						
BV750	0.06	0.06	0.05	0.03	0.02	0.04	0.05	0.12	0.25	0.67	1																					
BV785	0.15	0.16	0.15	0.12	0.09	0.11	0.08	0.11	0.17	0.47	0.81	1																				
BB515	0	0	0.01	0.07	0.05	0.02	0.02	0.01	0	0	0	0	1														S	imi	arit	y Ir	nde	X
cFluor B532	0	0	0.01	0.07	0.06	0.03	0.03	0.01	0	0	0	0	0.9	1															1.0	U		
cFluor B548	0.03	0.04	0.07	0.28	0.31	0.38	0.3	0.2	0.07	0.03	0.02	0.04	0.54	0.75	1												-	-	0.7	5		
PE	0	0.01	0.02	0.11	0.11	0.23	0.49	0.25	0.05	0.01	0.01	0.02	0.12	0.21	0.51	1											_	-	0.5	0		
PE-Dazzle594	0.01	0.01	0.01	0.07	0.06	0.15	0.29	0.41	0.18	0.06	0.03	0.03	0.06	0.1	0.34	0.47	1												0.2	5		
PE-Fire 640	0	0	0	0.02	0.01	0.04	0.08	0.16	0.31	0.21	0.09	0.06	0.02	0.04	0.16	0.16	0.54	1											0.2	0		
PerCP	0	0	0	0.02	0.01	0.06	0.1	0.25	0.61	0.44	0.27	0.15	0.01	0.01	0.09	0.07	0.3	0.63	1										0.0	0		
cFluor B690	0	0	0	0.02	0.01	0.05	0.07	0.18	0.47	0.71	0.4	0.27	0	0.01	0.08	0.05	0.25	0.6	0.78	1												
cFluor BYG710	0	0	0	0.02	0.01	0.04	0.09	0.09	0.15	0.35	0.19	0.11	0.03	0.04	0.15	0.21	0.29	0.55	0.44	0.77	1											
PE-Fire 810	0	0	0	0.01	0.01	0.03	0.06	0.04	0.02	0.07	0.1	0.15	0.02	0.04	0.1	0.17	0.11	0.13	0.08	0.17	0.25	1										
cFluor BYG750	0	0	0	0	0	0.01	0.02	0.04	0.08	0.25	0.31	0.22	0	0.01	0.05	0.03	0.09	0.26	0.23	0.42	0.57	0.45	1									
PE-Cy7	0	0	0	0.01	0.01	0.01	0.02	0.03	0.04	0.15	0.24	0.27	0.01	0.01	0.04	0.03	0.06	0.15	0.12	0.27	0.33	0.77 (	).74	1								
APC	0	0	0	0.01	0	0.02	0.03	0.08	0.35	0.22	0.07	0.04	0	0	0.01	0.01	0.03	0.37	0.33	0.33	0.13	0.01 0	0.08	0.03	1							
Alexa Fluor 647	0	0	0	0	0	0	0	0	0.19	0.18	0.03	0.01	0	0	0	0	0.01	0.32	0.27	0.32	0.13	0.01 0	0.07	0.03	).93	1						
Alexa Fluor 660	0.01	0.01	0.01	0.02	0.02	0.03	0.02	0.03	0.18	0.29	0.05	0.04	0.01	0.01	0.03	0.02	0.04	0.32	0.27	0.45 (	0.24	0.02 0	).11 (	0.05	).69 (	0.85	1					
APC-Cy7	0	0	0	0	0	0	0	0.01	0.06	0.19	0.17	0.21	0	0	0	0	0	0.09	0.06	0.17	0.09	0.07 0	).19 (	0.21 (	).19	0.2	0.25	1				
ViaDye Red	0	0	0	0	0	0	0	0.01	0.09	0.27	0.1	0.08	0	0	0.01	0.01	0.02	0.2	0.13	0.31 (	0.21 (	0.08	).27 (	0.19 (	).41 (	).45	0.59	0.8	1			
APC-Fire 810	0	0	0	0	0	0	0	0.01	0.05	0.14	0.12	0.18	0	0	0	0	0	0.07	0.05	0.13	0.06	0.12 (	).12 (	0.16 (	).17 (	0.16	0.18	0.72 <b>C</b>	).52	1		
	RV421	Super Bright 436	cFluor V450	BV480	cFluor V505	BV510	BV570	BV605	BV650	BV711	BV750	BV785	BB515	cFluor B532	cFluor B548	ΡE	PE-Dazzle594	PE-Fire 640	PerCP	cFluor B690	cFluor BYG710	PE-Fire 810	cFluor BYG750	PE-Cy7	APC	Alexa Fluor 647	Alexa Fluor 660	APC-Cy7	ViaDye Red	APC-Fire 810		

Supplementary Figure 3 - Similarity Index of 30 colours in practice. Matrix generated using ggplot2 in R-studio using similarity indices reported by SpectroFlo after acquiring 30 single-stained controls. The calculated complexity index (SpectroFlo) was 23.25.



16hr resolution better than 30 mins and

Comp-Alexa Fluor 660-A :: TCR gd Supplementary Figure 4 - Overnight staining to improve population resolution. PBMCs were stained with antibody cocktail for 30 minutes (orange) or overnight/16 hours (green), stained with one antibody (black), or unstained (grey).





16hr resolution better than 30 mins This fluorophore not used in final panel



Comp-redFluor 710-A :: CD20

16hr no different to 30 mins



16hr resolution slightly better than 30 mins



Comp-Alexa Fluor 647-A :: CD127

Comp-APC-A :: IgA

16hr resolution better than 30 mins

and single colour







Comp-cFluor BYG750-A :: laD

More negative spreading due to higher MFI in other channels



16hr no different to 30 mins











Comp-PE-Cy7-A :: CD183 (CXCR3)



Supplementary Figure 5 - Comparison of two fixation methods. Top row: Single Live cells. Bottom row: Lymphocytes (SSC-A low FSC-A low/mid). Left column: cells fixed with eBioscience <sup>™</sup> Foxp3 / Transcription Factor Staining Buffer Set (ThermoFisher 00-5523-00) as per kit instructions. Right column: cells fixed with 1% paraformaldehyde in 1X PBS (ThermoFisher J19943.K2).



Supplementary Figure 6 - Comparison of staining temperature to improve population resolution. PBMCs were stained with antibody cocktail for 30 minutes. Cells were incubated at 37°C (dark green), room temperature between 21°C and 23°C (orange) or at 4°C (blue). Controls were stained for 30 minutes at 4°C with one antibody (black) or unstained (grey).

Supplementary Figure 7



Supplementary Figure 7 - Cell viability after staining at different temperatures. PBMCs were stained with antibody cocktail for 30 minutes at either 4°C, room temperature between 21°C and 23°C, or at 37°C. After staining, cells were incubated with viability dye, followed by fixative. Percentage of live cells were measured among B cells (CD3<sup>-</sup> CD19<sup>+</sup> CD20<sup>+</sup>), CD4 T cells (CD3<sup>+</sup> TCR $\gamma\delta^-$  CD4<sup>+</sup> CD8<sup>-</sup>), CD8 T cells (CD3<sup>+</sup> TCR $\gamma\delta^-$  CD4<sup>-</sup> CD8<sup>+</sup>),  $\gamma\delta$  T cells (CD3<sup>+</sup> TCR $\gamma\delta^+$ ), Tregs (CD3<sup>+</sup> TCR $\gamma\delta^-$  CD4<sup>+</sup> CD8<sup>-</sup> CD4<sup>+</sup> CD8<sup>-</sup> CD127<sup>-</sup> CD25<sup>+</sup>).

Pre-gated on Single Live CD45+ CD3+ CD4+ T cells



Supplementary Figure 8 - Sequential staining resulted in small improvements in signal for CCR6, TCR $\gamma\delta$  and CXCR5. In contour plots or histograms, red lines represent sample where the antibody was stained in a primary layer and all other colours were stained in a secondary layer. Black lines represent sample where all 30 colours stained together in one layer.

The two right histograms show frequency of positive and negative events as percent of parent (e.g. CXCR5- 93.6%, CXCR5+ 6.45%), the MFI of the positive population (e.g. Median BV421 9532) and the Stain index (MFI-positive – MFI-negative / 2 x SD-negative) for that sample (e.g. Stain index = 3.30).

Pre-gated on Single Live CD45+





SSC-Ahi PE-Cy7+: Median : PE-Cy7 18777 SSC-Ahi PE-Cy7low-neg: Median : PE-Cy7 1189



SSC-Ahi cFluorB690+: Median : cFluor B690 61737 SSC-Ahi cFluor B690low-neg : Median : cFluor B690 2493



SSC-Ahi cFluorB690+: Median : cFluor B690 51671 SSC-Ahi cFluor B690low-neg : Median : cFluor B690 2255





SSC-Ahi APC-Cy7+: Median : APC-Cy7 19522 SSC-Ahi APC-Cy7low-neg: Median : APC-Cy7 257.6

Supplementary Figure 9 - True-Stain Monocyte Blocker (Biolegend) slightly reduces the background staining of cyanine dyes on monocytes. Median MFI of the flurophore for positive and negative SSC-A high cells are shown under each plot.



Supplementary Figure 10 - Comparison of Fc blocking to improve population resolution. PBMCs were incubated with either Human TruStain FcX (Biolegend #422301, green line), Human FC Block Pure Fc1 (BD #564220, purple line), purified Human IgG (Merck, #I4506) at 100µg/mL (orange line) or no blocking (only diluent, 1X PBS, black line) before proceeding with panel staining. Unstained control shown in grey.



Supplementary Figure 11 - Fluoresence Minus One (FMO) controls. PBMC samples were stained with a cocktail of all antibodies in the panel, except one. Plots show FMO of the Y-axis antibody in the left column and the FMO of the X-axis antibody in the right column. Plots are pre-gated with the relevant population written underneath the plot.



Supplementary Figure 12 - Preliminary gating of FCS files. First, all fluorescent markers vs time are analysed to identify pressure disturbances that may have affected the acquisition of that sample. A time gate is set on CD24 BV711 vs time. A series of doublet exclusion gates are applied (FCS-A vs FSC-H, SSC-A vs SSC-H), followed by a red blood cell exclusion gate (SSC-Blue-H vs SSC-H) which is useful if the PBMC sample had considerable RBC contamination. Two gates are applied to remove any antibody aggregates (hyper-fluorescent events). Finally, small FSC-A events are excluded in a "debris" exclusion gate. This data is saved as a new FCS file which is used for further analysis.