Designing multi-color panels for T/Treg populations using FITMaN guidelines: a case study.

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Abstract. Multi-parametric analysis by flow cytometry has become an essential tool to characterize complex lymphocyte populations. The combination of multiple fluorescent markers has enabled strategies to define functional cell subsets, especially important in characterizing disease etiology and progression. Following guidelines provided by the FITMaN initiative on immunophenotyping (sponsored by FOCIS Centers of Excellence), two 10-color panels were developed using a "backbone" strategy: 8-color panels sharing common reagents with allowance for 2 elective reagents. This approach allows modification of the panels with relative ease. The cocktail development process took into account the selected antigenic immunophenotypes, the characteristics of available fluorochromes, and the use of well-defined control populations.

Results. Data from case studies are presented here with special emphasis on optimization of flow analysis and compensation visualization. A standard set of gating strategy can be applied to both panels while generating reproducible and optimal characterization of CD4+CD25+ Treg subsets with respect to additional differentiation/functional surface markers on 5 normal donors.

Conclusions. This FOCIS FITMaN design approach aims to create a standardized testing condition for optimized resolution of Treg populations. Our data demonstrates the ability of our designed panels to achieve our aims and can be incorporated to enhance capabilities for clinical studies and to render assays compatible across platforms for correlative analyses.

Materials and Methods

Antibody conjugates were obtained and titered for optimal test dosages, which spanned from 1.25 µL to 20 µL. All dye-conjugated antibodies were acquired from Beckman Coulter (Brea, CA), with the exceptions of: CD39-FITC, CCR4-PE and CCR7-PE, which were obtained from a third party.
100 µL of normal, whole blood was stained with the appropriate volumes for single colors and nine color cocktails. Samples were prepared using VersaLyseTM Lysing Solution after 20 minute incubation at room temperature in the dark. Samples were then centrifuged, washed, recentrifuged, and resupsended in 7-Aminoactinomycin D (*7-AAD)*. After a 20-minute incubation at room temperature, cells were fixed in 750 µL of buffer + 0.5% formaldehyde. Samples were assessed on the GalliosTM cytometer (Beckman Coulter), and analyzed using Kaluza software (Beckman Coulter).

Target	Fluorophore	Clone
CD39	FITC dye	A1
CCR4	R-PE	205410
CCR7	R-PE	150503
CD3	ECD	UCHT1
CD25	PE-Cy7	B1.49.9
CD38	APC	LS.198
CD127	APC	R34.34
CD8	APCAlexaFluor700	B9.11
CD45RA	APCAlexaFluor750	2H4LDH11LD89
HLADR	Pacific Blue	IMMU.357
CD4	Krome Orange	13B8.2

Table1. Antibody conjugates used in study

Results

	FITC	PE	ECD	PC5.5	PC7	APC	APCA700	APCA750	PacBlu	KrOr
FOCIS panel 1	CD39	CCR7	CD3	7AAD	CD25	CD127	CD8	CD45RA		CD4
FOCIS panel 2	CD39	CCR7	CD3	7AAD	CD25	CD38	CD8	CD45RA	HLA-DR	CD4

Requirement

- **2 panels**
- **Live/Dead**
- **CCR4/CCR7 on PE**
- **CD38 on APC**

Additional design

Backbone
Ease of compensation

backbone
required
optional

Table 2. Rationale for FOCIS Panel.
Final design is shown above, which fulfills all given requirements. In addition, this design incorporates a backbone strategy that would only require one compensation matrix for both panels. As a result, one gating strategy for CD4+/CD4+CD25+ is applicable for both panels. CD39 is placed in the FITC channel as an option to enhance finer resolution in differentiating T/ T-regulatory cells. The compensation matrix for the two panels is derived from single color controls and is shown here.

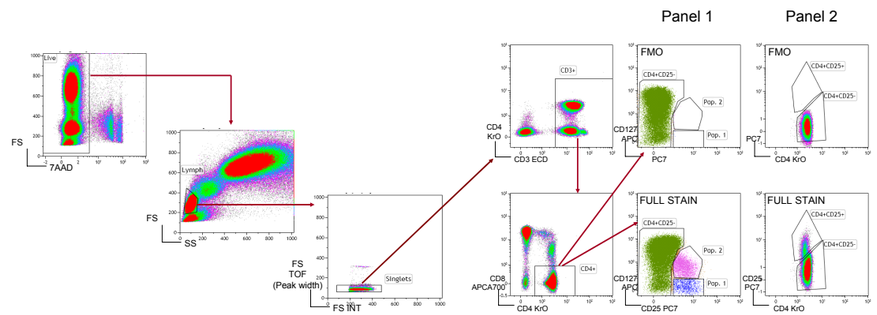


Figure 1. Preliminary Gating Strategy. Live cells are gated from dead cells using 7AAD staining. From this gate, the lymphocyte subpopulation is identified and from it singlets are selected. CD3 positive cells are gated on from the live/lymph/singlets and then further discriminated into CD4 and CD8 subsets. CD4 positive cells in panel 1 are further resolved by plotting CD127 versus CD25 to identify two CD4+CD25+ subsets. For panel 2, CD4 positive cells are plotted against CD25 to identify the T-regulatory and non T-regulatory cells. A FMO (fluorescence- minus-one) stain is also performed without CD25 to verify that the CD25 positive cells are correctly gated.

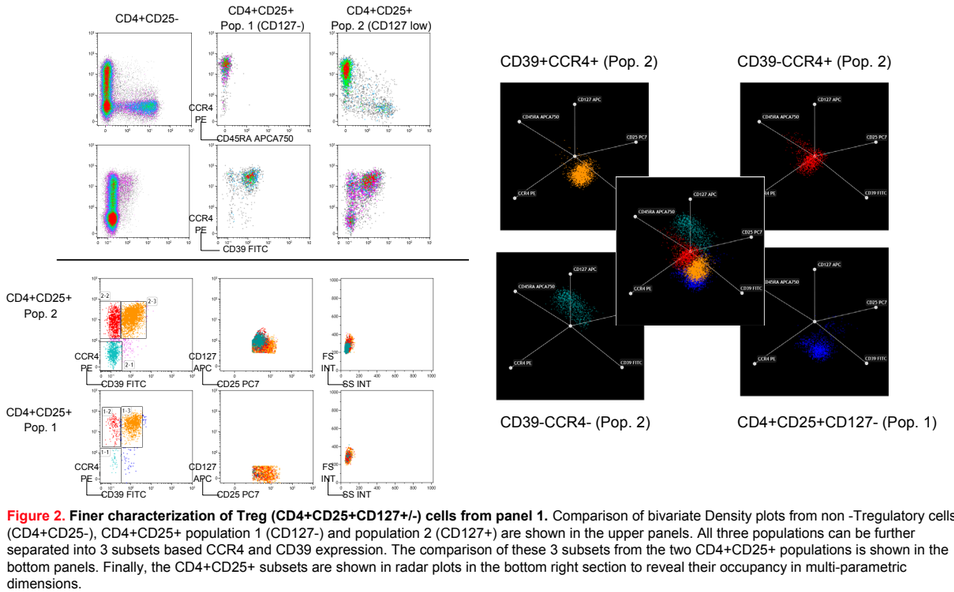
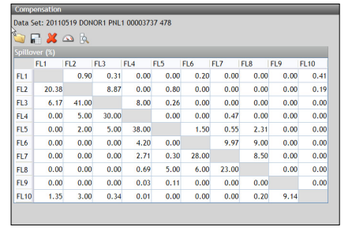
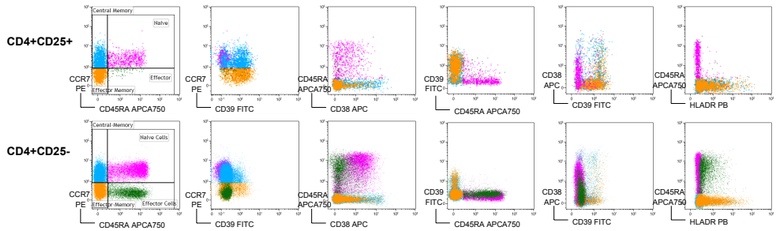
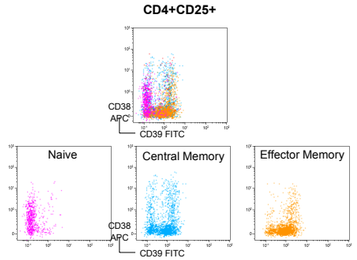


Figure 2. Finer characterization of Treg (CD4+CD25+CD127+/-) cells from panel 1. Comparison of bivariate Density plots from non -Tregulatory cells (CD4+CD25-), CD4+CD25+ population 1 (CD127-) and population 2 (CD127+) are shown in the upper panels. All three populations can be further separated into 3 subsets based CCR4 and CD39 expression. The comparison of these 3 subsets from the two CD4+CD25+ populations is shown in the bottom panels. Finally, the CD4+CD25+ subsets are shown in radar plots in the bottom right section to reveal their occupancy in multi-parametric dimensions.







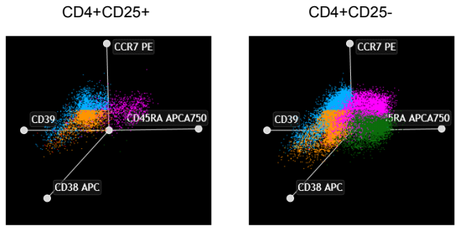


Figure 3. Finer characterization of Treg (CD4+CD25+CD127+/-) cells from panel 2. CD4+CD25+ and CD4+CD25- cells are further divided into 4 distinct subsets: Naïve, Central memory, Effector memory, and Effector cells. Each of these subsets is color-coded and can be followed when these cells are analyzed in other parameters, see upper section. Shown in the bottom left section, the three main subsets in CD4+CD25+ Treg cells have distinct CD39 and CD38 patterns in their phenotypic progression.
Both CD4+CD25+ and CD4+CD25- populations are shown in radar plots in the bottom right section to reveal their occupancy in multi-parametric dimensions.

Panel 1: Hierarchal Gating and Population Statistics-Donor 1

Gate	Number	%Total	%Gated	Logic
All	597,292	100.00	100.00	Ungated
Live	578,771	96.90	96.90	Live
Lymph	159,130	26.64	27.49	Lymph AND Live
Singlets	158,767	26.58	99.77	Singlets AND Lymph AND Live
CD3+	119,792	20.06	75.45	CD3+ AND Singlets AND Lymph AND Live
CD4+	61,178	10.24	51.07	CD4+ AND CD3+ AND Singlets AND Lymph AND Live
CD4+CD25-	54,787	9.17	89.55	CD4+CD25- AND CD4+ AND CD3+ AND Singlets AND Lymph AND Live
Pop. 1	1,288	0.22	2.11	"Pop. 1" AND CD4+ AND CD3+ AND Singlets AND Lymph AND Live
1-1	37	0.01	2.87	1-1 AND "Pop. 1" AND CD4+ AND CD3+ AND Singlets AND Lymph AND Live
1-2	160	0.03	12.42	1-2 AND "Pop. 1" AND CD4+ AND CD3+ AND Singlets AND Lymph AND Live
1-3	1,018	0.17	79.04	1-3 AND "Pop. 1" AND CD4+ AND CD3+ AND Singlets AND Lymph AND Live
Pop. 2	4,473	0.75	7.31	"Pop. 2" AND CD4+ AND CD3+ AND Singlets AND Lymph AND Live
2-1	777	0.13	17.37	2-1 AND "Pop. 2" AND CD4+ AND CD3+ AND Singlets AND Lymph AND Live
2-2	928	0.16	20.75	2-2 AND "Pop. 2" AND CD4+ AND CD3+ AND Singlets AND Lymph AND Live
2-3	2,526	0.42	56.47	2-3 AND "Pop. 2" AND CD4+ AND CD3+ AND Singlets AND Lymph AND Live

Panel 2: Hierarchal Gating and Population Statistics-Donor 1

Gate	Number	%Total	%Gated	Logic
All	561,460	100.00	100.00	Ungated
Live	536,686	95.59	95.59	Live
Lymph	143,783	25.61	26.79	Lymph AND Live
Singlets	143,467	25.55	99.78	Singlets AND Lymph AND Live
CD3+	109,283	19.46	76.17	CD3+ AND Singlets AND Lymph AND Live
CD4+	55,902	9.96	51.15	CD4+ AND CD3+ AND Singlets AND Lymph AND Live
CD4+CD25-	49,991	8.90	89.43	CD4+CD25- AND CD4+ AND CD3+ AND Singlets AND Lymph AND Live
Central-Memory	20,390	3.63	40.79	Central-Memory AND CD4+CD25- AND CD4+ AND CD3+ AND Singlets AND Lymph AND Live
Effector cells	4,300	0.77	8.60	"Effector cells" AND CD4+CD25- AND CD4+ AND CD3+ AND Singlets AND Lymph AND Live
Effector-Memory	16,453	2.93	32.91	Effector-Memory AND CD4+CD25- AND CD4+ AND CD3+ AND Singlets AND Lymph AND Live
Naive cells	7,614	1.36	15.23	"Naive-cells" AND CD4+CD25- AND CD4+ AND CD3+ AND Singlets AND Lymph AND Live
CD4+CD25+	4,786	0.85	8.56	CD4+CD25+ AND CD4+ AND CD3+ AND Singlets AND Lymph AND Live
Central-Memory	1,877	0.33	39.22	"Central-Memory" AND CD4+CD25+ AND CD4+ AND CD3+ AND Singlets AND Lymph AND Live
Effector	53	0.01	1.11	Effector AND CD4+CD25+ AND CD4+ AND CD3+ AND Singlets AND Lymph AND Live
Effector-Memory	2,095	0.37	43.77	"Effector-Memory" AND CD4+CD25+ AND CD4+ AND CD3+ AND Singlets AND Lymph AND Live
Naive	601	0.11	12.56	Naive AND CD4+CD25+ AND CD4+ AND CD3+ AND Singlets AND Lymph AND Live

Percentages of Singlets

		Donor1	Donor 2	Donor 3	Donor 4	Donor 5	Mean	STDEV
Panel 1								
Singlet		100.00	100.00	100.00	100.00	100.00	100.00	N/A
CD3+		75.40	61.99	75.40	67.04	76.83	71.33	5.81
CD4+		38.46	36.61	57.55	29.77	31.65	38.81	9.89
CD4+CD25+CD127-		1.02	0.41	0.76	0.60	0.32	0.62	0.25
	CD39-CCR4-	0.08	0.03	0.00	0.00	0.00	0.02	0.03
	CD39-CCR4+	0.15	0.07	0.06	0.11	0.05	0.09	0.04
	CD39+CCR4+	0.72	0.31	0.65	0.46	0.28	0.48	0.18
CD4+CD25+CD127low		3.20	3.57	3.17	1.82	1.88	2.73	0.73
	CD39-CCR4-	0.72	2.21	1.16	0.42	1.19	1.14	0.61
	CD39-CCR4+	0.83	0.34	0.45	0.35	0.16	0.43	0.22
	CD39+CCR4+	1.47	0.78	1.22	0.95	0.44	0.97	0.36
		Donor 1	**Donor 2**	**Donor 3**	**Donor 4**	**Donor 5**	**Mean**	**STDEV**
Panel 2								
Singlet		100.00	100.00	100.00	100.00	100.00	100.00	N/A
CD3+		76.16	62.32	75.93	67.42	81.66	72.70	6.91
CD4+		38.98	37.12	57.82	30.10	31.82	39.17	9.88
CD4+CD25+		3.33	3.01	2.68	1.99	1.49	2.50	0.67
	Naïve	0.51	1.57	0.76	0.21	1.49	0.91	0.54
	Central Memory	1.37	0.63	0.85	0.77	0.23	0.77	0.37
	Effector Memory	1.33	0.63	0.88	0.91	0.50	0.85	0.28
	Effector	0.04	0.10	1.13	0.03	0.07	0.28	0.43
CD4+CD25-		34.83	33.48	54.21	27.44	56.48	41.29	11.76
	Naïve	5.76	17.00	17.18	5.94	14.06	11.99	5.14
	Central Memory	14.91	7.31	19.35	10.34	7.42	11.87	4.65
	Effector Memory	10.33	7.03	12.43	10.27	7.33	9.48	2.03
	Effector	3.21	1.64	4.18	0.17	0.34	1.91	1.57

Table 3. Gating hierarchy and statistics for FOCIS FITMan panels. The hierarchal scheme and population percentages for donor 1 in both panels are shown in the left section. The statistical analysis of all five donors normalized to singlets is shown in the right section.

Summary

- We have created a set of panel proposals that fulfills the FOCIS FITMan design requirements.
- The panel proposal is designed in a manner that one compensation matrix will satisfy both panels and also an initial gating strategy that can be applied to both panels.
- We have demonstrated the feasibility of these panels to resolve T cell populations and more specifically, to generate detailed characterization of CD4+CD25+ T-regulatory subsets.
- We have shown the applicability of these panels in the analysis of five normal donors.