Development of a 40+ colour panel for the characterization of conventional & unconventional T cell subsets, B cells, NK cells and dentritic cells using spectral flowcytometry

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BACKGROUND

Even though many flow cytometers can analyze 30-50 parameters, it is still challenging to develop a 40+ colour panel for the phenotyping of immune cells using fluorochrome conjugated antibodies due to limitations in the availability of spectrally unique fluorochromes that can be excited by the commonly used laser lines (UV, Violet, Blue, Green/Yellow-green and Red). Spectral flowcytometry is capable of differentiating fluorochromes with significant overlap in the emission spectra, enabling the use of spectrally similar fluorochrome pairs such as BD Horizon Brilliant™ Blue 515 and FITC in a single panel.

We have developed and optimized a 43 colour panel to characterize the subsets of conventional T cells, unconventional T cells such as invariant natural killer T cells (iNKT), γδ T cell subsets (TCR Vδ2, TCR Vγ9) and mucosal-associated invariant T cells (MAIT), B cells, NK cells, dendritic cells and innate lymphoid cells (ILC). The panel includes surface markers to analyze activation (CD38, HLA-DR, ICOS/CD278, LAMP-1/CD107a), differentiation (CCR7, CD45RA, CD27, CD28, CD57), expression of cytokine & chemokine receptors (CD25, CD127, CCR10, CCR6, CXCR3, CXCR5, CRTH2/CD294) and co-inhibitory molecules & exhaustion (PD-1, CD223/LAG-3, CTLA-4, TIGIT, TIM3/CD366) which enables a deep characterization of PBMCs from peripheral blood. Cells were analyzed on a 5-laser Cytek Aurora and data analysis was done using FlowJo (for selecting population of interest) and R (FlowAI, FlowVS, CATALYST, FlowSOM, FastPG, UMAP from uwot, optSNE from Rtsne).

METHODS

Peripheral Blood Mononuclear Cells (PBMC) from healthy donor was purchased from iQBiosciences (Berkeley, California, USA) and used as samples. Veri-Cells PBMC from Biolegend was used for titrations, when applicable. AbC Total Antibody Compensation Bead Kit from Invitrogen was used for creating fluorochrome reference spectrum in Cytek SpectroFlo software for all the antibodies used in the study. For each staining, $1x10^6$ PBMC were used. PBMCs were thawed as per standard protocols just before the staining and initially incubated with Human TruStain FcX Fc Receptor Blocking Solution (Biolegend) and True-Stain Monocyte Blocker (Biolegend) for 10 minutes. Titrated volumes of antibodies was prepared as a master mix and mixed with PBMCs in the wells of a Laminar Wash 96-well plate (Curiox Biosystems) and incubated for 40 minutes at room temperature in dark. The cells were then washed by laminar flow using the Curiox Laminar Wash System HT1000 for 9 cycles. The cells were then transferred to regular 96-well U bottom plated for acquisition in Cytek Aurora flow cytometer using the SpectroFlo Software v2.2.0.2. The cells were unmixed using reference controls setup earlier and then unmixed files were used for further data analysis.

The antibodies used is listed below.

Sl. No	Antibody/Reagent	Clone	Sl. No	Antibody/Reagent	Clone
1	CD45RA	HI100	23	CD366 (TIM3)	F38-2E2
2	CCR10	564769	24	CD38	HIT2
3	CCR6 (CD196)	11A9	25	CD4	C4/206
4	CD10	HI10a	26	CD56	NCAM16.2
5	CD107a (LAMP-1)	H43A	27	CD57	555619
6	CD11b	OF-5A12	28	CD8	RPA-T8
7	CD11c	B-ly6	29	CRTH2	BM16
8	CD123	6H6	30	CTLA-4	RM0199-6G10
9	CD127 (IL-7Rα)	HIL-7R-M21	31	CXCR3 (CD183)	1C6/CXCR3
10	CD14	63D3	32	CXCR5 (CD185)	RF8B2
11	CD141	M80	33	HLA DR	L243
12	CD16	3G8	34	ICOS (CD278)	DX29
13	CD161	HP-3G10	35	IgD	IA6-2
14	CD19	SJ25-C1	36	PD1 (CD279)	EH12
15	CD1c	L161	37	SYTOX Blue	Viability dye
16	CD223 (LAG-3)	3DS223H	38	TCR Vα24-Jα18	6B11
17	CD24	ML5	39	TCR Vβ11	REA559
18	CD25 (IL-2Rα)	M-A251	40	TCR Vδ2	B6
19	CD26	BA5b	41	TCR Vγ9	B3
20	CD27	M-T271	42	TCR γδ	B1.1
21	CD28	L293	43	TIGIT	741182
22	CD3	UCHT1			

Spectroflo software provides an option to extract the cellular autofluorescence of the samples during unmixing. Autofluorescence extraction has been known to improve marker resolution in single colour and 2-3 marker staining experiments, but its effect on complex panels were unknown. To evaluate this, we unmixed the 43 colour stained sample with and without autofluorescence extraction. The data was transformed to asinh scale using same cofactors for both files and the flowset was used to run clustering (FastPG) and dimensionality reduction (UMAP and optSNE).

RESULTS

Our data demonstrates that 40+ colour panels are possible with fluorochrome based flow cytometry even though the marker and fluorochrome selection and pairing is a tedious, but feasible task.

Autofluorescence extraction does not help in complex panels and it increases spread in some channels.

CONCLUSION

The optSNE and UMAP plots shows that the dimensionality reduction visualizations are able to separate relatively minor populations such as DCs and γδ T cells, but their subsets (ex. CD141+ DCs, CD1c+ DCs, CD16+ DCs, TCR Vδ2 cells, TCR Vγ9 cells, etc) can not be visualized. Even clustering tools such as FlowSOM (with 60 Meta Clusters) and FastPG failed to identify those smaller subsets as a pure cluster (Data not shown). This shows some of the limitations of current automated analysis methodologies.

It might be possible to add 3 more fluorochromes to the panel and we are waiting for the necessary reagents. If successful, it will enable the panel to identify subsets on ILCs and reach 46 markers.

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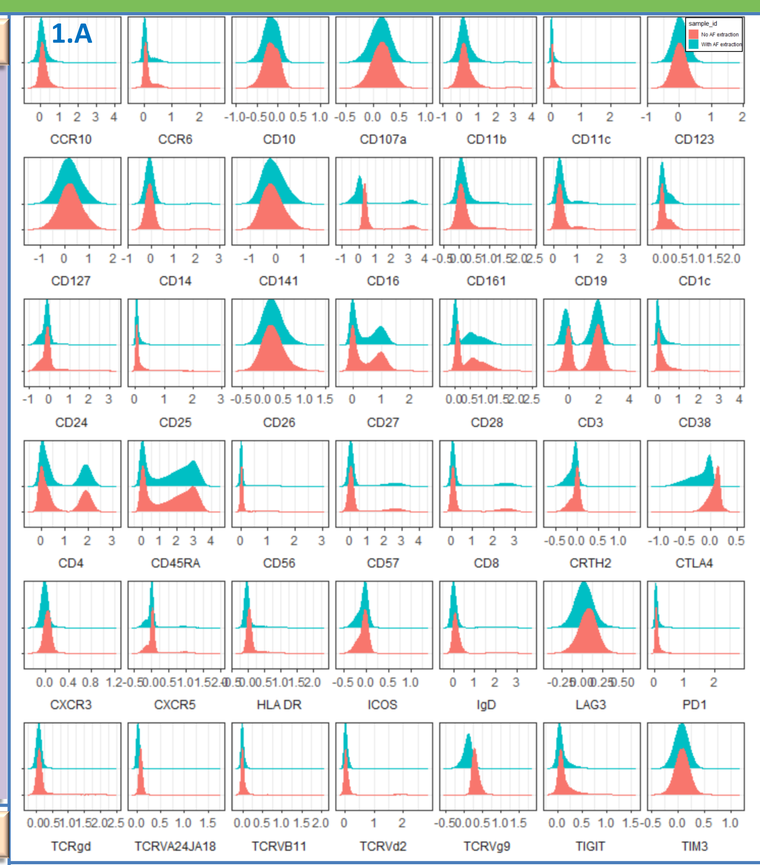

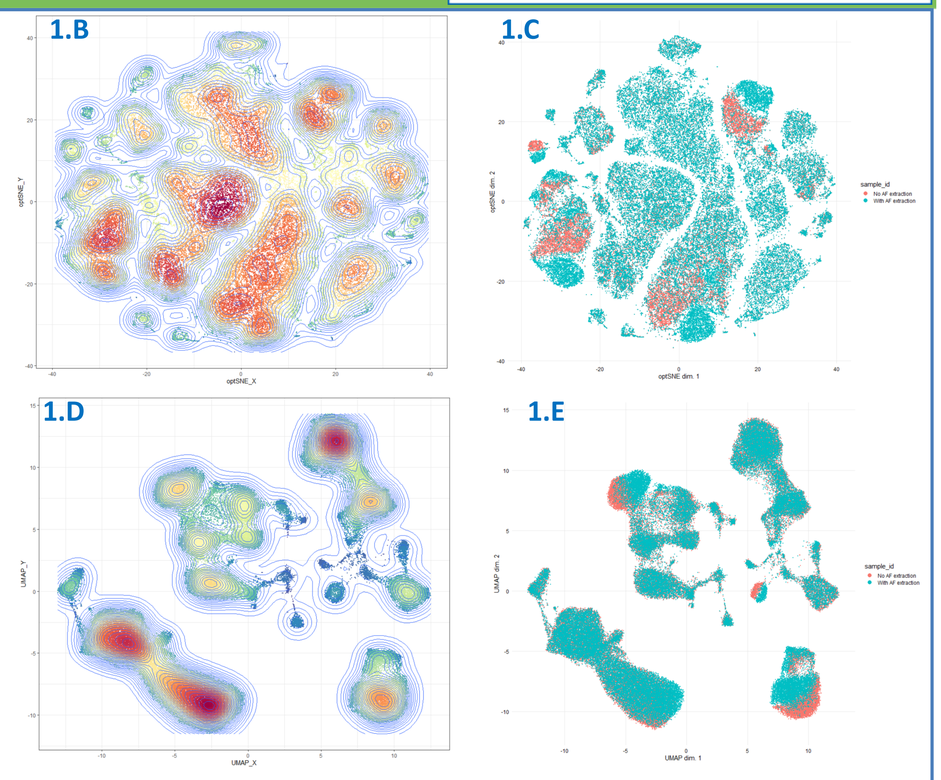

Figure 1. A – Histogram plots of all markers. B – optSNE plot with density. C - optSNE plot coloured by samples. D – UMAP plot with density. E - UMAP plot coloured by samples.

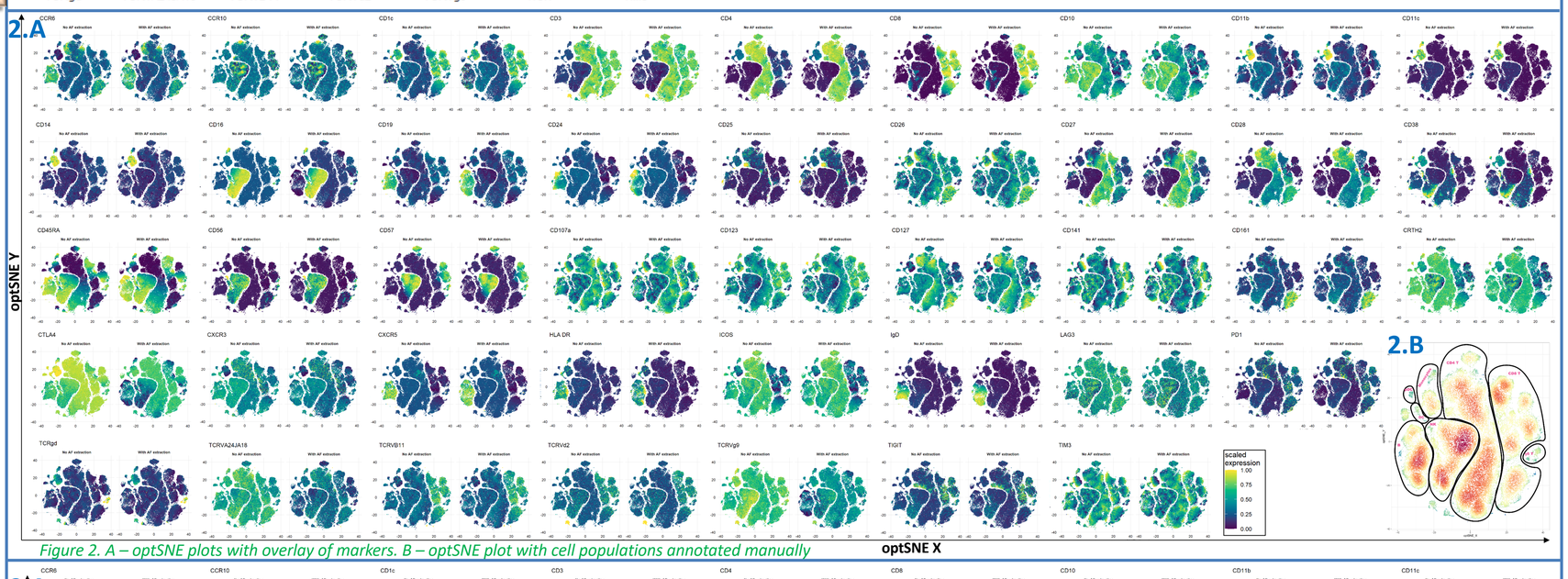

Figure 2. A – optSNE plots with overlay of markers. B – optSNE plot with cell populations annotated manually

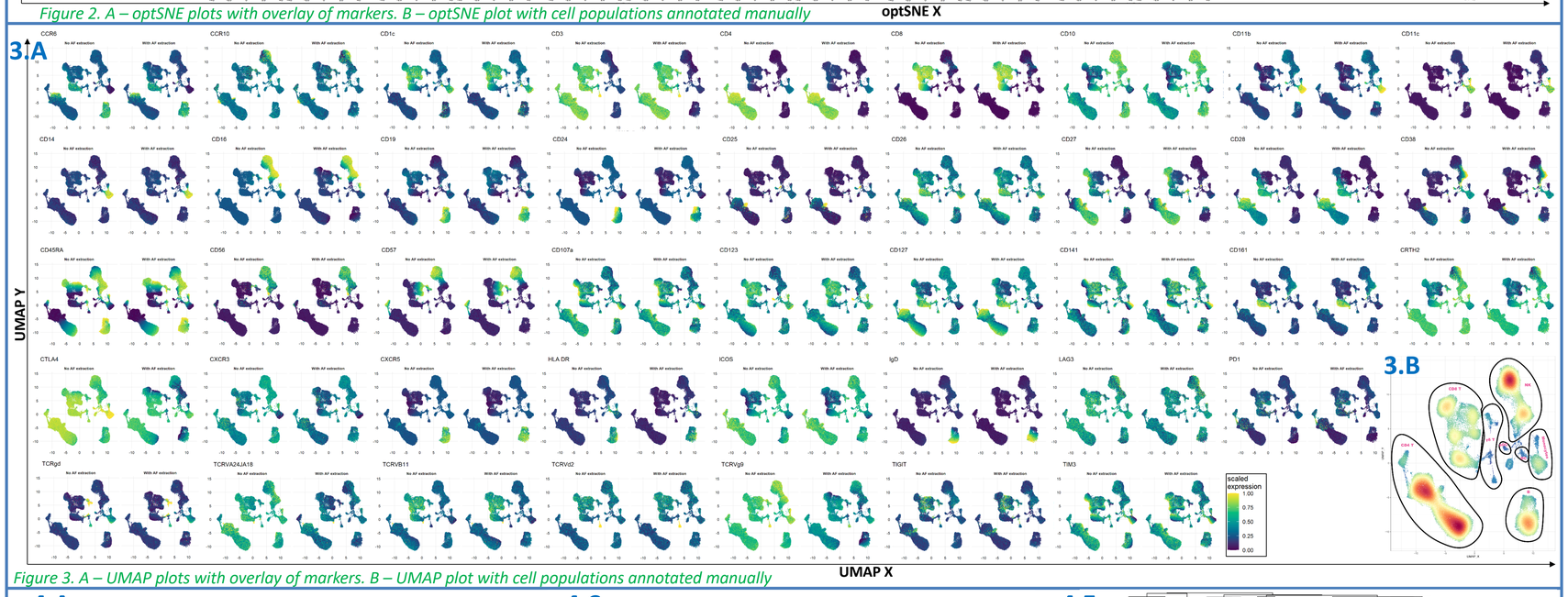

Figure 3. A – UMAP plots with overlay of markers. B – UMAP plot with cell populations annotated manually

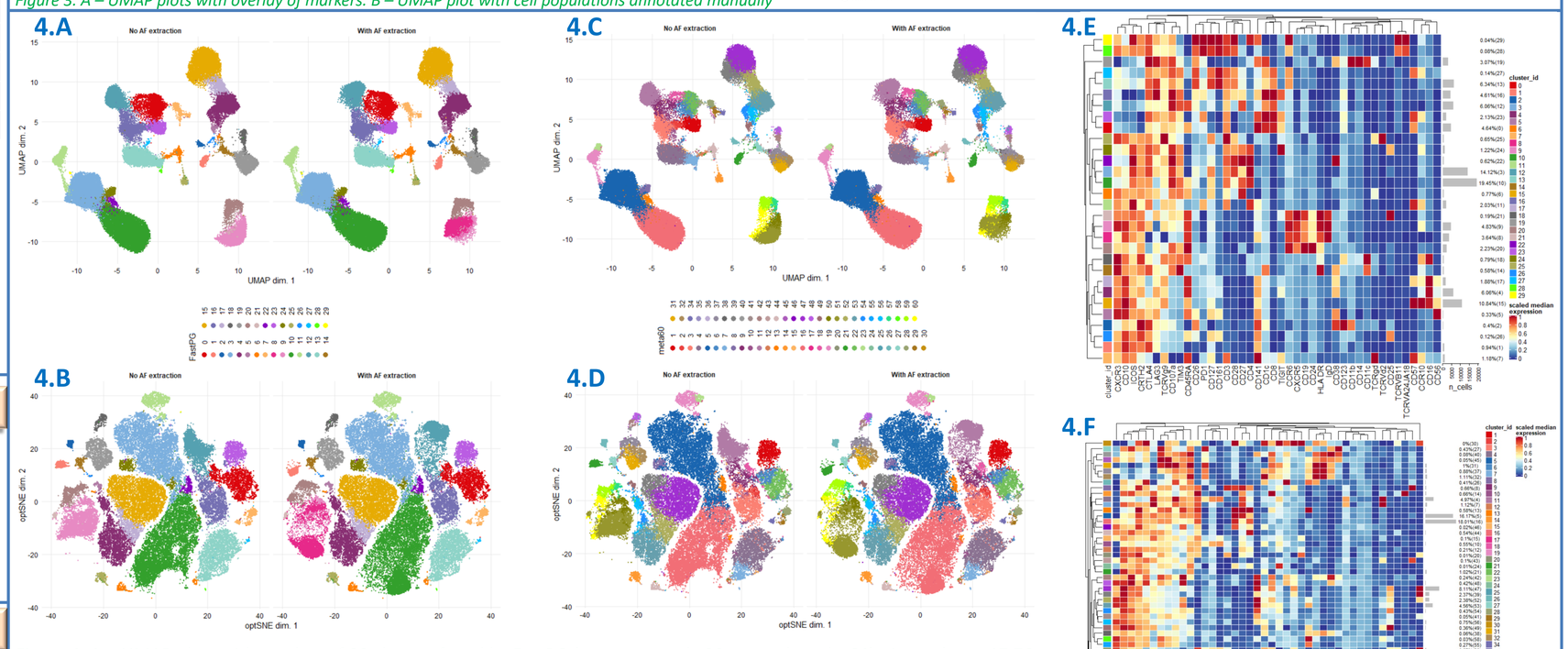

Figure 4. A – UMAP plots with overlay of 30 clusters found by FastPG (parameters - k=30, num_threads=64), time taken – 17.76 seconds for 100,000 events dataset. B – optSNE plots with overlay of 30 clusters found by FastPG. C – UMAP plots with overlay of 60 metaclusters found by FlowSOM (parameters - xdim = 40, ydim = 40, maxK = 60), time taken – 2759.05 seconds for 100,000 events. D – UMAP plots with overlay of 60 metaclusters found by FlowSOM. E – Heatmap of all markers expression in the 30 clusters found by FastPG. F – Heatmap of all markers expression in the 60 metaclusters found by FlowSOM.

"FastPG was 162 times faster than FlowSOM for this dataset when analyzed on a desktop with 64 threads"

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