Analytical Performance Characteristics of a 25-Parameter Spectral Cytometry Panel in Peripheral Blood for Immune Monitoring Biomarker Applications

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Abstract

We previously developed and optimized a 25-paramater spectral cytometry immune monitoring panel in whole blood and PBMC using a 3-laser Cytek Aurora system [Poster, CYTO 2019]. The assay has since been evaluated on a 5-laser system.

Herein we describe the analytical performance characteristics of the panel in whole blood and PBMC which includes assessment of specimen stability, sample precision, donor variability, and reagent stability.

We conclude by exploring manual vs. advanced data analysis strategies in healthy volunteer samples.

Panel Development

Using OMIPs and other references, we designed a flow cytometry panel for the Cytek Aurora, applying 25 immune markers (24 fluorophores) to a 3-laser system.

Panels utilizing major immune biomarkers

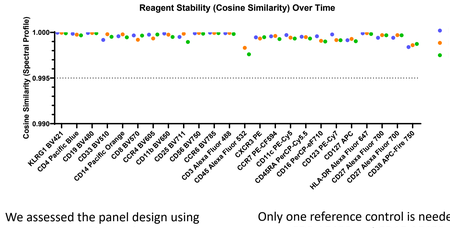
OMIP-042	
Marker	Fluorophore
CD14	BUV395
viability	Zombie UV
CD16	BUV496
HLA-DR	BUV661
CD56	BUV737
CD38	BV421
CD20	V450
CD4	BV510
CCR4	BV605
CD8	BV650
CD25	BV711
CCR6	BV785
CD3	AF488
CD45RA	PerCP-Cy5.5
CXCR3	PE
CCR7	PE-CF594
CD11c	PE-Cy5
CXCR5	PE-Cy7
CCR10	APC
CD123	AF700
CD127	APC-eF780

Maxpar Kit (CyTOF)	
Marker	
CD3	CD56
CD4	CD57
CD8	CD66b
CD11c	CD123
CD14	CD127
CD16	CD161
CD19	CD294
CD20	CCR4
CD25	CCR6
CD27	CCR7
CD28	CXCR3
CD38	CXCR5
CD45	HLA-DR
CD45RA	IgD
CD45RO	TCRγδ

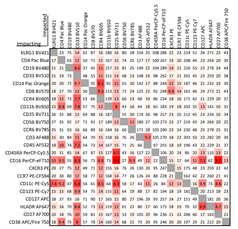
This Panel
25 Immune Markers on a 3-laser system

Cytek Aurora		Antibody Panel Reagents				
Laser	Chan.	Marker	Clone	Fluorophore	Vendor	Cat. No.
405 nm	V1	KLRG1	SA231A2	BV421	BL	367705
405 nm	V3	CD4	SK3	Pacific Blue	BL	344620
405 nm	V4	CD19	S25C1	BV480	BD	566164
405 nm	V5	CD33	WM53	BV510	BL	303421
405 nm	V7	CD14	Tuk4	Pacific Orange	Thermo	MHCD1430
405 nm	V8	CD8	RPA-T8	BV570	BL	301037
405 nm	V10	CCR4	L291H4	BV605	BL	359418
405 nm	V11	CD11b	ICRF44	BV650	BL	301336
405 nm	V13	CD25	2A3	BV711	BD	563159
405 nm	V14	CD56	5.1H11	BV750	BL	362555
405 nm	V15	CCR6	G034	BV785	BL	353422
488 nm	B1	CD3	UCHT1	Alexa Fluor 488	BD	557694
488 nm	B1	CD15	HI98	Alexa Fluor 488	BL	301910
488 nm	B3	CD45	HI30	Alexa Fluor 532	Thermo	58-0459-41
488 nm	B4	CXCR3	G025H7	PE	BL	353705
488 nm	B6	CCR7	150503	PE-CF594	BD	562381
488 nm	B8	CD11c	Bly6	PE-Cy5	BD	551077
488 nm	B9	CD45RA	HI100	PerCP-Cy5.5	BD	563429
488 nm	B10	CD16	3G8	PerCP-eF710	Thermo	46-0168-42
488 nm	B13	CD123	6H6	PE-Cy7	BL	306009
640 nm	R1	CD127	A019D5	APC	BL	351315
640 nm	R2	HLA-DR	L243	Alexa Fluor 647	BL	307621
640 nm	R4	CD27	O323	Alexa Fluor 700	BL	302813
640 nm	R6	Live/Dead	--	Zombie NIR	BL	423105
640 nm	R7	CD38	HIT2	APC-Fire 750	BL	303545

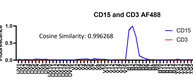
We assessed the stability of fluorophore reagents by calculating Cosine Similarity (i.e. Similarity Index) over time. All reagents are highly stable with proper storage and handling.



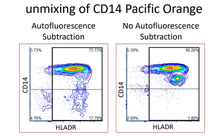
We assessed the panel design using Cross Stain Index



Only one reference control is needed for CD3 AF488 and CD15 AF488



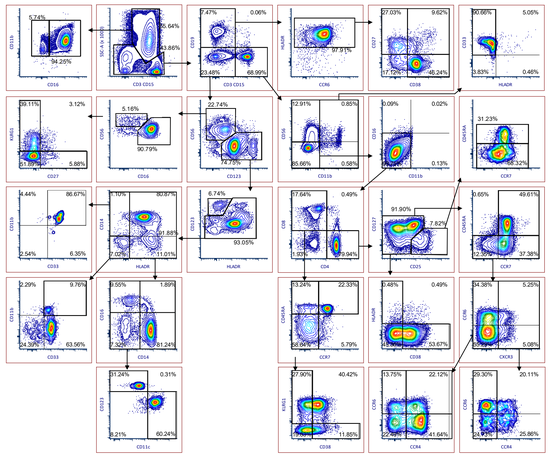
Autofluorescence subtraction is required for unmixing of CD14 Pacific Orange



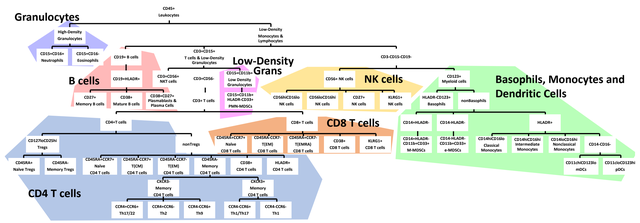
Traditional Gating & Clustering Analysis Comparison

The hierarchical gating strategy employs 59 gates and 27 bivariate plots (after debris elimination, doublet discrimination, Live/Dead and CD45+ gate). A RBC-lysed peripheral blood sample is shown. The same gating scheme can be applied to PBMCs, excluding the high-density granulocyte gate.

Optimized Hierarchical Gating

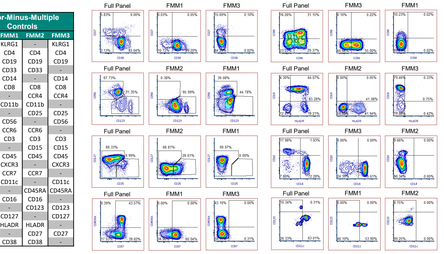


Tree chart showing the applied hierarchical gating scheme

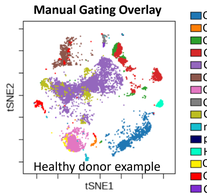


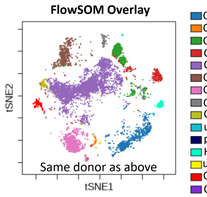
Only three FMM (Fluor-Minus-Multiple) controls are needed to assess gating placement

Fluor-Minus-Multiple Controls			
Panel	FMM1	FMM2	FMM3
KLRG1	KLRG1	--	KLRG1
CD4	CD4	CD4	CD4
CD19	CD19	CD19	CD19
CD33	CD33	CD33	--
CD14	CD14	--	CD14
CD8	CD8	CD8	CD8
CCR4	--	CCR4	CCR4
CD11b	CD11b	--	CD11b
CD25	--	CD25	CD25
CD56	CD56	--	CD56
CCR6	CCR6	CCR6	--
CD3	CD3	CD3	CD3
CD15	--	CD15	CD15
CD45	CD45	CD45	CD45
CXCR3	CXCR3	--	CXCR3
CCR7	CCR7	CCR7	--
CD11c	CD11c	--	CD11c
CD45RA	--	CD45RA	CD45RA
CD16	CD16	CD16	--
CD123	--	CD123	CD123
CD127	CD127	--	CD127
HLADR	HLADR	HLADR	--
CD27	--	CD27	CD27
CD38	CD38	CD38	--



In Cytobank, we can use viSNE to quickly visualize our manual gating and compare to automated clustering algorithms.

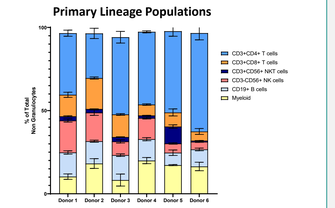


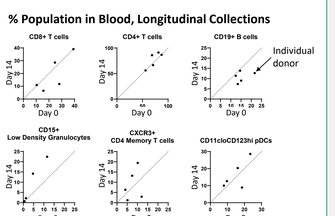


Using 15 metaclusters, some differences in Tregs and NK cell gating can be seen. FlowSOM did not separate mDCs from classical monocytes, nor intermediate monocytes from nonclassical monocytes. However FlowSOM identifies some less frequent populations that were previously assigned to other gates.

Donor Variability

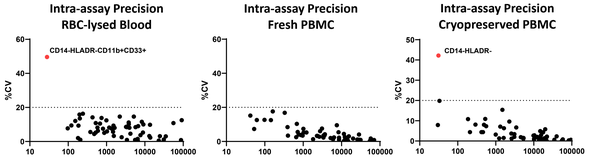
Healthy donors are compared to show inter-donor variability in major immune cell populations. Bottom, longitudinal intra-donor variability is shown for select populations.



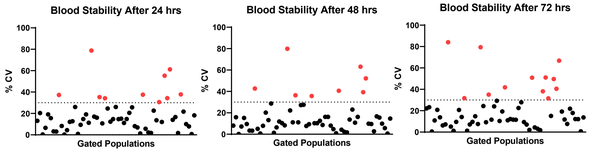


Assay Precision & Biomarker Stability

At the applied application settings, only populations CD14-HLADR-CD11b+CD33+ (blood analysis) and CD14-HLADR- (frozen PBMC analysis) failed intra-assay precision (%CV > 20%)

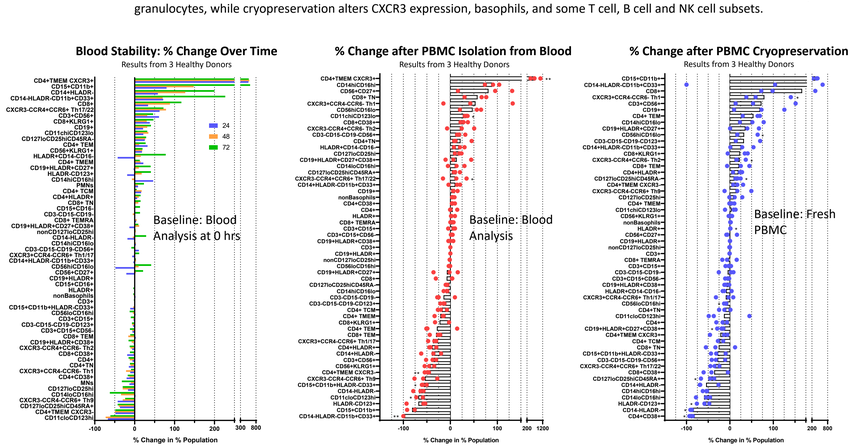


Peripheral blood samples we analyzed at 0, 24, 48 and 72 hrs post collection. Samples were stored at 4ºC during the interim. Assay precision was assessed across 0 hrs and either 24, 48 or 72 hrs.



- CD15+CD11b+, CD4+ TMEM CXCR3+, CD4+ TMEM CXCR3-, CXCR3-CCR4-CCR6+, CD14+HLADR-, CD14-HLADR-, CD14-HLADR-CD33+CD11b+, HLADR+CD14-CD16, CD11cloCD123hi, CD56hiCD16lo
- CD15+CD11b+, CD4+ TMEM CXCR3+, CD4+ TMEM CXCR3-, CD8+, CD14+HLADR-, CD14-HLADR-, CD14-HLADR-CD33+CD11b+, CD11cloCD123hi
- CD15+CD11b+, CD4+ TMEM CXCR3+, CD4+ TMEM CXCR3, CD8+, CD127loCD25hiCD45+, CD14+HLADR-, CD14-HLADR-, CD14-HLADR-CD33+CD11b+, HLADR+CD14-CD16-, CD11cloCD123hi, CD14loCD16hi, CD14hiCd16hi

We assessed the stability of immune markers within peripheral blood at 0, 24, 48 and 72 hours post collection in Na-Hep tubes. We also compared populations for fresh PBMC vs blood and cryopreserved PBMC vs fresh PBMC. Most populations exhibit less than 50% change across comparisons. CXCR3 expression increased significantly in blood after 24 hrs. PMBC isolation alters CXCR3 expression, dendritic cells and low-density granulocytes, while cryopreservation alters CXCR3 expression, basophils, and some T cell, B cell and NK cell subsets.



Conclusions

- We previously developed a 25-immune biomarker assay on the Cytek Aurora platform.
- Data can be analyzed manually using 27 bivariate plots, and only three FMMs are needed to assist with gating.
- We achieve %CV < 20% intra-assay sample precision with exception to low event count reportables like CD14-HLADR-CD11b+CD33+.
- On average, 49 out of 59 (80-86%) gated populations were stable in whole blood collected in Na-Hep tubes and analyzed at 24, 48 or 72 hrs post collection after interim storage at 4ºC. Exceptions include populations with low event counts or very particular increased marker expression (CXCR3).
- Both PBMC isolation and subsequent cryopreservation can induce additional immune changes; this must be taken into account when interrogating a population of interest.
- Our next goals include advanced analysis workflows and expansion of the panel for a 5-laser Cytek Aurora system.

Acknowledgements

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