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Investigating the murine hepatic immune composition in diet-induced obesity using OMIP-104: Transferring an existing OMIP panel onto the CytoFLEX mosaic Spectral Detection Module

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Introduction

Obesity-induced chronic inflammation, called meta-inflammation, results from an altered immune response in metabolic organs and contributes to the development of fatty liver disease and type 2 diabetes. To study these obesity-induced changes, a 30-color spectral flow cytometry panel was developed to study the immune composition in murine liver and white adipose tissue samples [1]. This panel aims to give a broad overview of the immunological landscape in murine metabolic tissues with a particular focus on metabolically relevant resident and recruited macrophage subsets. This panel differentiates Kupffer cells from recruited macrophages and identifies macrophage subsets in adipose tissue. It also includes markers for macrophage and dendritic cell activation states, enabling detailed analysis of immune cells involved in metabolic disorders.

This study aimed to assess the performance of OMIP-104 using the CytoFLEX mosaic Spectral Detection Module on the CytoFLEX LX Flow Cytometer. This evaluation was conducted without altering the published panel and it assesses the ease of transferring the panel to the CytoFLEX mosaic Spectral Detection Module, panel performance, and the ability to handle autofluorescence on a liver sample.

Materials

Instrument and Supplies

1. CytoFLEX LX flow cytometer (Beckman Coulter, Inc., USA, PN# AD51045) (Table 1)
2. CytoFLEX mosaic 88 Detection Module (Beckman Coulter, Inc., USA, PN# EP1006) (Table 1)
3. CytExpert for Spectral software (Beckman Coulter, Inc., USA).
4. Daily QC Fluorospheres (Beckman Coulter, Inc., USA, PN# 65719).
5. IR QC Daily QC Fluorospheres (Beckman Coulter, Inc., USA, PN# C06147).
6. Cytobank platform (Beckman Coulter, Inc., USA, PN# C47384)
7. For antibodies, please see table 2.
8. For additional materials, please refer to the supplementary section cited in reference 1.

Instrument	Lasers	Detection Module	Detectors
CytoFLEX LX	Deep UV 355 nm	CytoFLEX mosaic 88	U20
	Violet 405 nm		V20
	Blue 488 nm		B16
	Yellow 561 nm		Y12
	Red 638 nm		R10
	IR 808 nm		IR3

Table 1: Instruments and Detection Modules Configuration

Marker	Fluorochrome	Clone	Supplier	Catalog #	Titration	Conc. (µg/ml)
CCR2	PE-Cy5	SA203G11	BioLegend	150638	1:400	0.5
CD3	* BV750	17A2	BioLegend	100249	1:100	2.0
CD4	BV650	RM4-5	BioLegend	100546	1:100	2.0
CD8	BV711	53-6.7	BioLegend	100748	1:200	1.0
CD9	* APC-Fire 750	MZ3	BioLegend	124814	1:400	0.5
CD11b	BUV563	M1/70	BD Biosciences	741242	1:1600	0.125
CD11c	BUV496	HL3	BD Biosciences	750483	1:200	1.0
CD19	eFluor 450	eBio1D3	Invitrogen	48-0193-82	1:100	2.0
CD31	BV605	390	BioLegend	102427	1:3200	0.0625
CD36	Biotin	HM36	BioLegend	102604	1:200	2.5
Biotin	BV570	Streptavidin	BioLegend	405227	1:200	0.5
CD45	APC-Fire 810	30-F11	BioLegend	103174	1:1600	0.125
CD63	* Alexa Fluor 700	NVG-2	BioLegend	143924	1:100	5
CD64	PE-Dazzle 594	X54-5/7.1	BioLegend	139320	1:400	0.5
CD90.2	BV510	30-H12	BioLegend	140326	1:1600	0.125
CD172a	* BUV805	P84	BD Biosciences	741997	1:200	1.0
CD206	BV785	C068C2	BioLegend	141729	1:200	1.0
CLEC2	FITC	17D9	Bio-Rad	MCA5700	1:400	0.25
ESAM	BUV737	1G8	BD Biosciences	752446	1:800	0.25
F4/80	Spark NIR 685	BM8	BioLegend	123168	1:1600	0.3125
Ly6C	PerCP-Cy5.5	HK1.4	BioLegend	128012	1:800	0.25
Ly6G	* Spark Blue 550	1A8	BioLegend	127664	1:800	0.625
MHC II	BUV395	2G9	BD Biosciences	743876	1:1600	0.125
Neutral lipids	* LipidTOX Deep-Red	-	Invitrogen	H34477	1:400	n/a
NK1.1	PE-Fire 700	S17016D	BioLegend	156528	1:1600	0.125
Siglec-F	BV480	E50-2440	BD Biosciences	746668	1:400	0.5
TIM4	PerCP-eFluor 710	RMT4-54	Invitrogen	46-5866-82	1:800	0.25
TREM2	* PE	6E9	BioLegend	824806	1:100	2.0
Viability	* Zombie NIR	-	BioLegend	423106	1:1000	n/a
XCR1	BV421	ZET	BioLegend	148216	1:100	2.0

*Alexa Fluor ("AF") are trademarks or registered trademarks of Thermo Fisher Scientific Inc. *Brilliant Ultraviolet (BUV) trademark or registered trademark of Becton, Dickinson and Company. *Brilliant Violet ("BV") is a trademark or registered trademark of Becton, Dickinson and Company. *Spark Blue is a trademark or registered trademark of BioLegend. *APC-Fire and *Zombie are trademarks or registered trademarks of Biolegend. *LipidTox is a trademark or registered trademark of Invitrogen.

Table 2: Antibody details

Note: Lyve-1 PE-Cy7 was not included in this test, as the data presented here was exclusively acquired on liver tissue samples on which Lyve-1 is not expressed. This marker is used specifically to gate on perivascular macrophages in adipose tissue and does not serve any further purpose in the liver, as demonstrated in figure 1 of reference 1.

Methods

The sample preparation and staining methods described in OMIP-104 were applied to the samples from a different cohort of mice used for this test. Briefly, the samples consist of CD45+ MACS-enriched cells isolated from the livers of male mice fed a western-type diet containing 60%-Kcal high fat diet supplemented with 1% cholesterol. For the test, leukocytes from six individual mice were pooled together.

For a detailed review of the methods used for sample preparation, antibody staining, and preparation of single-stained controls, please refer to the supplementary section cited in reference 1.

Data Acquisition

- Ensure the flow cytometer has been switched on, warmed up, and cleaned appropriately as per manufacturer and institutional instructions.
- Perform Quality Control, create an unmixing template, and an experiment template for a batch of experimental samples.
- Use Assay Settings for acquisition of experimental samples.

Results and Discussions

Spectrum viewer

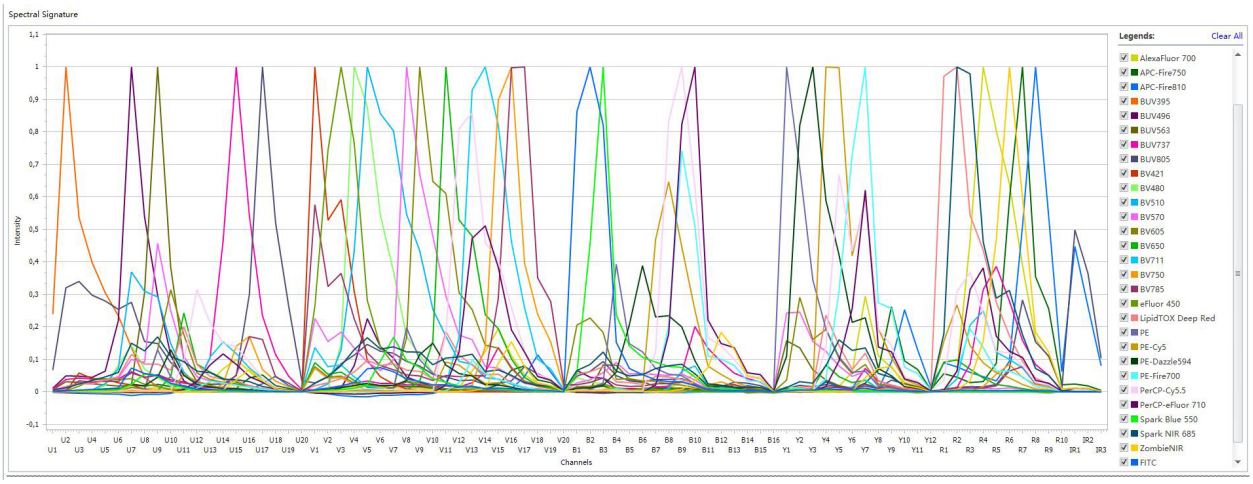
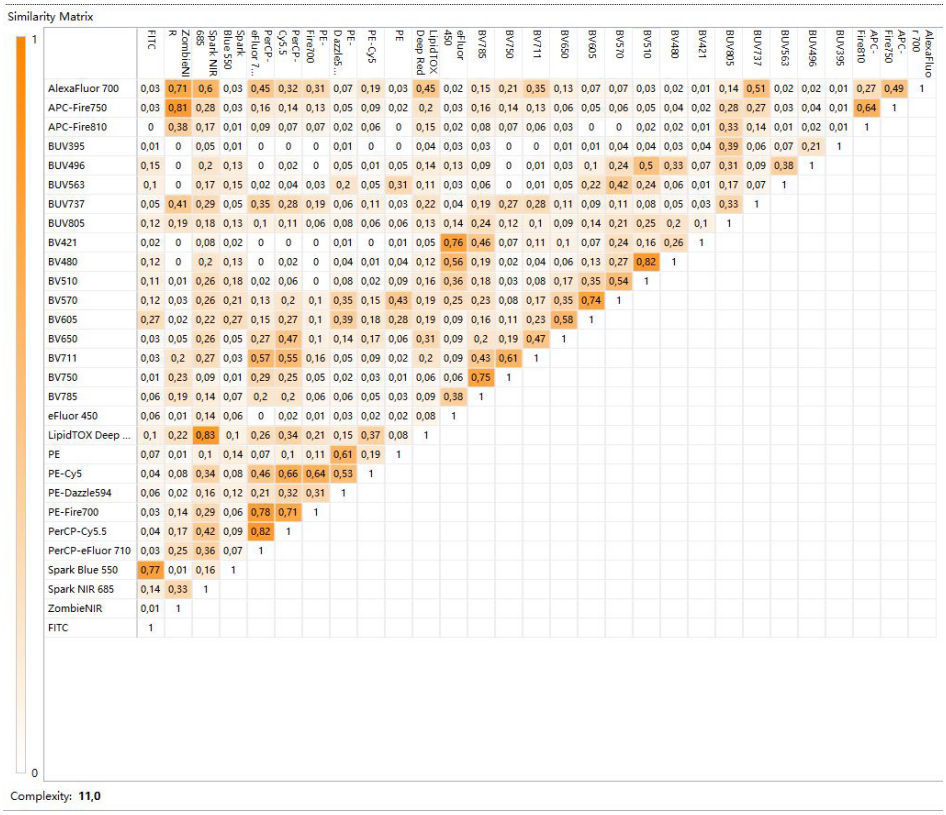


Figure 1: Spectrum viewer. Combination of fluorochromes generated using CytExpert for Spectral software.

Similarity Index



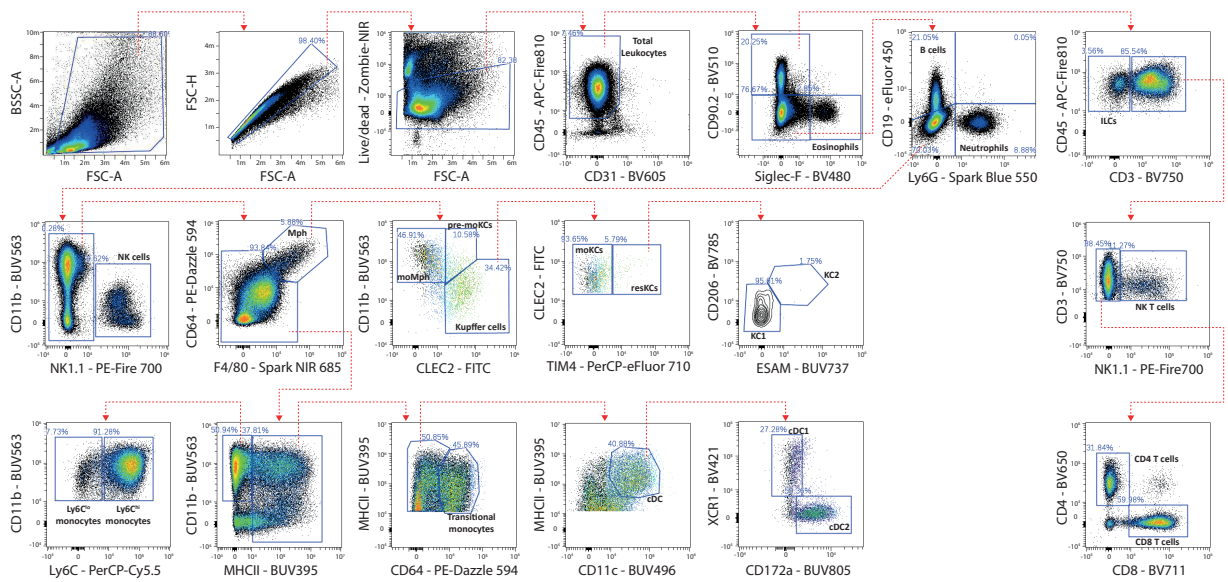
	FITC	R ZombieNI	685 Spark NIR	Spark Blue 550	PerCP-eFluor 7...	PerCP-Cy5.5	PerCP-Fire700	PE-Dazzle5...	PE-Cy5	PE	LipidTOX Deep Red	eFluor 450	BV785	BV750	BV711	BV650	BV605	BV570	BV510	BV480	BV421	BUV805	BUV737	BUV563	BUV496	BUV395	APC-Fire810	APC-Fire750	AlexaFluo 700
AlexaFluor 700	0,03	0,71	0,6	0,03	0,45	0,32	0,31	0,07	0,19	0,03	0,45	0,02	0,15	0,21	0,35	0,13	0,07	0,07	0,03	0,02	0,01	0,14	0,51	0,02	0,02	0,01	0,27	0,49	1
APC-Fire750	0,03	0,81	0,28	0,03	0,16	0,14	0,13	0,05	0,09	0,02	0,2	0,03	0,16	0,14	0,13	0,06	0,05	0,06	0,05	0,04	0,02	0,28	0,27	0,03	0,04	0,01	0,64	1	
APC-Fire810	0	0,38	0,17	0,01	0,09	0,07	0,07	0,02	0,06	0	0,15	0,02	0,08	0,07	0,06	0,03	0	0	0,02	0,02	0,01	0,33	0,14	0,01	0,02	0,01	1		
BUV395	0,01	0	0,05	0,01	0	0	0	0,01	0	0	0,04	0,03	0,03	0	0	0,01	0,01	0,04	0,04	0,03	0,04	0,39	0,06	0,07	0,21	1			
BUV496	0,15	0	0,2	0,13	0	0,02	0	0,05	0,01	0,05	0,14	0,13	0,09	0	0,01	0,03	0,1	0,24	0,5	0,33	0,07	0,31	0,09	0,38	1				
BUV563	0,1	0	0,17	0,15	0,02	0,04	0,03	0,2	0,05	0,31	0,11	0,03	0,06	0	0,01	0,05	0,22	0,42	0,24	0,06	0,01	0,17	0,07	1					
BUV737	0,05	0,41	0,29	0,05	0,35	0,28	0,19	0,06	0,11	0,03	0,22	0,04	0,19	0,27	0,28	0,11	0,09	0,11	0,08	0,05	0,03	0,33	1						
BUV805	0,12	0,19	0,18	0,13	0,1	0,11	0,06	0,08	0,06	0,06	0,13	0,14	0,24	0,12	0,1	0,09	0,14	0,21	0,25	0,2	0,1	1							
BV421	0,02	0	0,08	0,02	0	0	0	0,01	0	0,01	0,05	0,76	0,46	0,07	0,11	0,1	0,07	0,24	0,16	0,26	1								
BV480	0,12	0	0,2	0,13	0	0,02	0	0,04	0,01	0,04	0,12	0,56	0,19	0,02	0,04	0,06	0,13	0,27	0,82	1									
BV510	0,11	0,01	0,26	0,18	0,02	0,06	0	0,08	0,02	0,09	0,16	0,36	0,18	0,03	0,08	0,17	0,35	0,54	1										
BV570	0,12	0,03	0,26	0,21	0,13	0,2	0,1	0,35	0,15	0,43	0,19	0,25	0,23	0,08	0,17	0,35	0,74	1											
BV605	0,27	0,02	0,22	0,27	0,15	0,27	0,1	0,39	0,18	0,28	0,19	0,09	0,16	0,11	0,23	0,58	1												
BV650	0,03	0,05	0,26	0,05	0,27	0,47	0,1	0,14	0,17	0,06	0,31	0,09	0,2	0,19	0,47	1													
BV711	0,03	0,2	0,27	0,03	0,57	0,55	0,16	0,05	0,09	0,02	0,2	0,09	0,43	0,61	1														
BV750	0,01	0,23	0,09	0,01	0,29	0,25	0,05	0,02	0,03	0,01	0,06	0,06	0,75	1															
BV785	0,06	0,19	0,14	0,07	0,2	0,2	0,06	0,06	0,05	0,03	0,09	0,38	1																
eFluor 450	0,06	0,01	0,14	0,06	0	0,02	0,01	0,03	0,02	0,02	0,08	1																	
LipidTOX Deep ...	0,1	0,22	0,83	0,1	0,26	0,34	0,21	0,15	0,37	0,08	1																		
PE	0,07	0,01	0,1	0,14	0,07	0,1	0,11	0,61	0,19	1																			
PE-Cy5	0,04	0,08	0,34	0,08	0,46	0,66	0,64	0,53	1																				
PE-Dazzle594	0,06	0,02	0,16	0,12	0,21	0,32	0,31	1																					
PE-Fire700	0,03	0,14	0,29	0,06	0,78	0,71	1																						
PerCP-Cy5.5	0,04	0,17	0,42	0,09	0,82	1																							
PerCP-eFluor 710	0,03	0,25	0,36	0,07	1																								
Spark Blue 550	0,77	0,01	0,16	1																									
Spark NIR 685	0,14	0,33	1																										
ZombieNIR	0,01	1																											
FITC	1																												

Figure 2: Similarity Index. Similarity index of selected fluorochromes used in this OMIP with Complexity score of 11.0, generated using CytExpert for Spectral software.

Gating Strategy

Figure 3A shows a representative manual gating strategy used in this panel to delineate the major leukocyte subsets on a liver sample with relevant fluorescence minus one (FMO) control displayed in figure 3B. For an extensive overview of immune subsets, and their respective characterizing markers, please refer to Table 3.

A



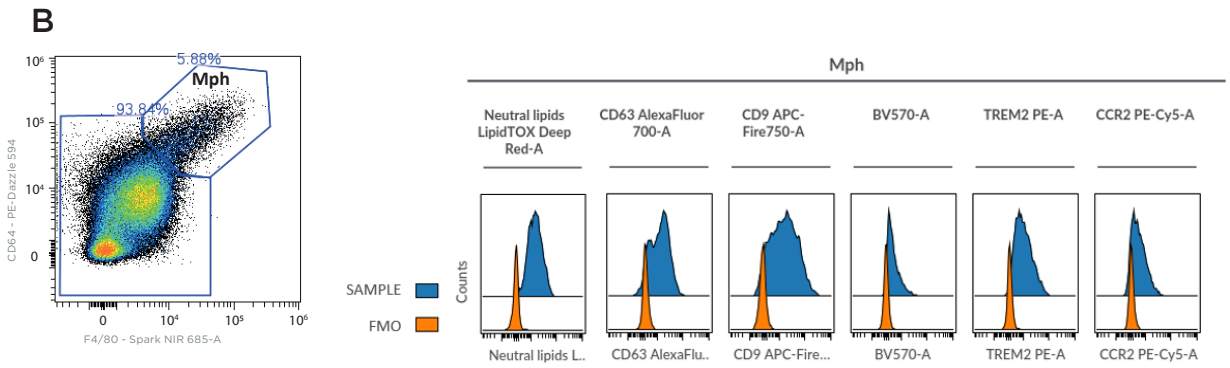


Figure 3: Gating strategy. A) A time gate was set to validate the stability of acquisition (not shown) after which debris, doublets and non-viable cells were first excluded, and total tissue leukocytes were next identified as CD45$^+$CD31$^-$. Subsequently, all the major immune cell populations, and their relevant subsets, were identified as displayed. B) Fluorescence minus one control. FMOs measured for activation markers LipidTOX Deep Red, CD63–AlexaFluor 700, CD9–APC-Fire750, CD36–BV570, CCR2–PE-Cy5 and TREM2–PE. Samples were pre-gated on total Mph as shown in (A). FMOs are displayed in orange, stained samples are displayed in blue. Plots were generated using the Cytobank data analysis platform.

Note: In the original OMIP-104 gating strategy, the process involved an initial exclusion of B cells and neutrophils, followed by gating on CD64 and F4/80 to identify macrophages. From the CD64 and F4/80 double negatives, NK cells were then identified by gating on CD11b by NK1.1. However, the approach was revised here, and the gating strategy was adjusted. In the revised approach, after the initial exclusion of B cells and neutrophils, NK cells are first excluded by gating on CD11b by NK1.1. Subsequently, macrophages are identified from the NK1.1 negatives by gating on CD64 and F4/80. This adjustment in the gating strategy does not affect the final data. However, it is noted here to maintain transparency and provide an exact replication of the OMIP-104 gating strategy, should it be required for comparative studies or consistency in methodology.

Population	Identification
Total leukocytes	CD45$^+$CD31$^-$
Eosinophils	CD45$^+$Siglec-F$^+$
ILCs	CD45$^+$Siglec-F$^-$ CD90.2$^+$CD3-
NK T cells	CD45+Siglec-F$^-$ CD90.2$^+$CD3$^-$ NK1.1$^+$
CD4 T cells	CD45$^+$Siglec-F$^-$ CD90.2+CD3- NK1.1- CD4$^+$
CD8 T cells	CD45$^+$Siglec-F$^-$ CD90.2$^+$CD3$^-$ NK1.1$^-$ CD8$^+$
Neutrophils	CD45$^+$Siglec-F$^-$CD90.2$^-$ Ly6G$^+$
B cells	CD45$^+$Siglec-F$^-$ CD90.2$^-$ CD19$^+$
Total macrophages (Mph)	CD45$^+$ Siglec-F$^-$CD90.2$^-$Ly6G$^-$CD19$^-$ CD64$^+$ F4/80$^+$
Kupffer cells	CD45$^+$Siglec-F$^-$CD90.2$^-$Ly6G$^-$CD19$^-$CD64$^+$ F4/80$^+$CD11b^{low}CLEC2$^+$
Pre-monocyte-derived Kupffer cells	CD45$^+$Siglec-F$^-$CD90.2$^-$Ly6G$^-$CD19$^-$CD64$^+$F4/80$^+$CD11b$^+$ CLEC2^{intermediate}
Monocyte-derived macrophages	CD45$^+$Siglec-F$^-$CD90.2$^-$Ly6G$^-$CD19$^-$CD64$^+$F4/80$^+$CD11b$^+$ CLEC2$^-$
Monocyte-derived Kupffer cells	CD45$^+$Siglec-F$^-$CD90.2$^-$Ly6G$^-$CD19$^-$CD64$^+$F4/80$^+$CD11b^{low}CLEC2$^+$ TIM4$^-$
Resident Kupffer cells	CD45$^+$Siglec-F$^-$CD90.2$^-$Ly6G$^-$CD19$^-$CD64$^+$F4/80$^+$ CD11b^{low}CLEC2$^+$ TIM4$^+$
NK cells	CD45$^+$Siglec-F$^-$ CD90.2$^-$ CD19$^-$CD11b$^-$NK1.1$^+$
Ly6C^{high}monocytes	CD45$^+$Siglec-F$^-$CD90.2$^-$Ly6G$^-$CD19$^-$CD64$^-$F4/80$^-$NK1.1$^-$ CD11b$^+$ MHC-II$^-$ Ly6C^{high}
Ly6C^{low}monocytes	CD45$^+$Siglec-F$^-$CD90.2$^-$Ly6G$^-$CD19$^-$CD64$^-$F4/80$^-$NK1.1$^-$ CD11b$^+$ MHC-II$^-$ Ly6C^{low}
Transitional monocytes	CD45$^+$Siglec-F$^-$CD90.2$^-$Ly6G$^-$CD19$^-$CD64$^-$F4/80$^-$NK1.1$^-$CD11b$^+$ MHC-II$^+$CD64^{intermediate}
Dendritic cells	CD45$^+$Siglec-F$^-$CD90.2$^-$Ly6G$^-$CD19$^-$CD64$^-$F4/80$^-$NK1.1$^-$CD11b$^+$MHC-II$^+$ CD64$^-$CD11c$^+$
cDC1s	CD45$^+$Siglec-F$^-$CD90.2$^-$Ly6G$^-$CD19$^-$CD64$^-$F4/80$^-$NK1.1$^-$CD11b$^+$MHC-II$^+$CD64$^-$CD11c$^+$XCR1$^+$ CD172a$^-$
cDC2	CD45$^+$Siglec-F$^-$CD90.2$^-$Ly6G$^-$CD19$^-$CD64$^-$F4/80$^-$NK1.1$^-$CD11b$^+$MHC-II$^+$CD64$^-$CD11c$^+$XCR1$^-$ CD172a$^+$

Table 3: List of immune cell populations in liver tissue

Autofluorescence (AF) Subtraction and Unmixing

Most of the single-stained controls for spectral unmixing were generated using cells to match the fluorescent spectra of the fully stained panel. However, for some markers, cells did not provide a solid spectrum. For example, beads were used for Siglec-F – BV480 because this marker is expressed only on eosinophils, which have a unique autofluorescence spectrum and are difficult to separate using FSC/SSC alone. Therefore, the BV480 spectrum was easier to determine using beads. Four unique autofluorescent populations were identified from a pool of unstained samples (Figure 4).

Our observations indicate that subtracting the spectra of four unique autofluorescent signatures during the unmixing process reduces unmixing errors and thereby improves resolution compared to using a single autofluorescent signature (Figure 5 A-B). The data presented in Figure 5 highlights the importance of extracting multiple AF signatures for improved unmixing accuracy.

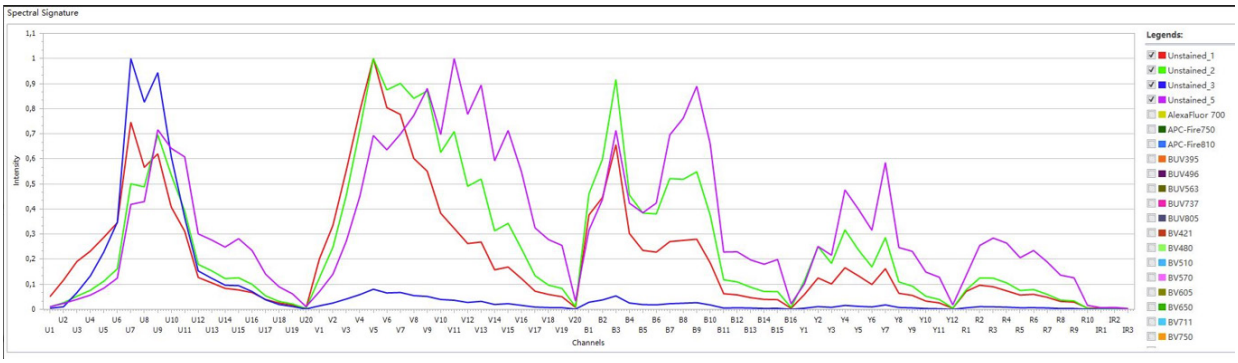


Figure 4: Fluorescent spectra of unique autofluorescent populations identified from unstained controls. Spectra were generated using CytExpert for Spectral software.

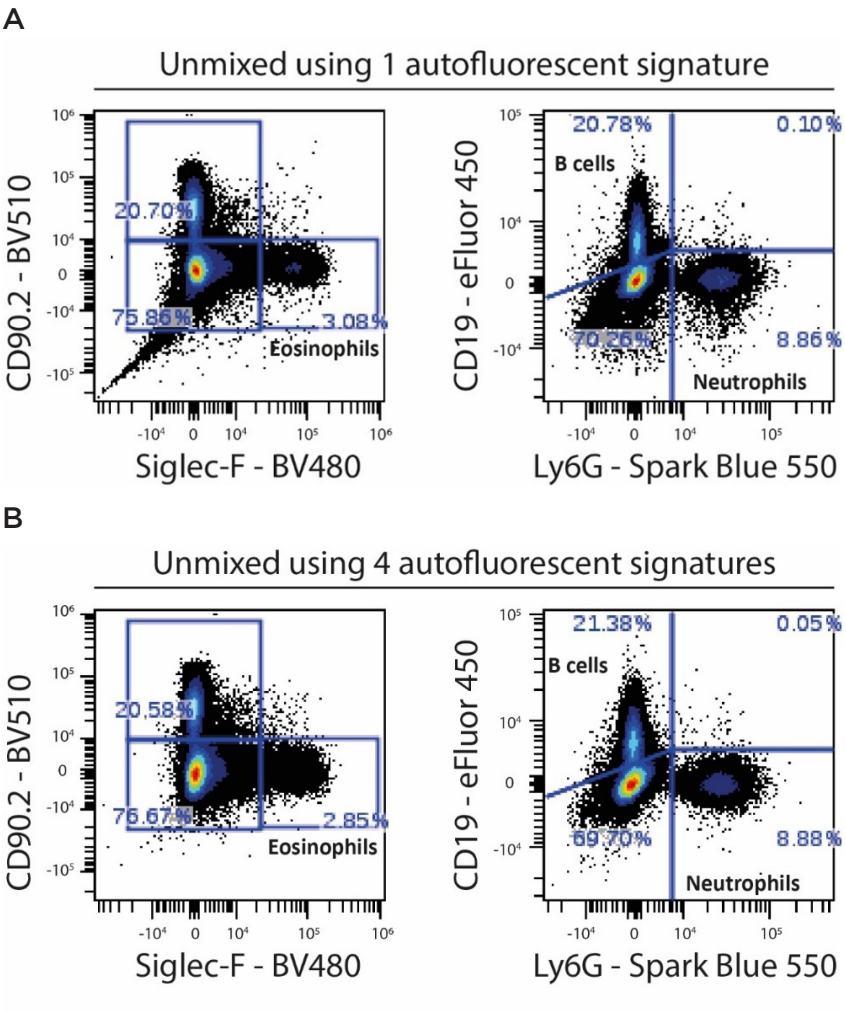


Figure 5: Spectral unmixing with multiple autofluorescent signatures. A) Example of the same sample unmixed using 1 unique autofluorescent signature. B) Using 4 autofluorescent signatures. Data was pre-gated on total leukocytes as displayed in Figure 3A. Plots were generated using the Cytobank analysis platform.

Unmixing Algorithms in CytoFLEX mosaic Spectral Detection Module

The CytoFLEX mosaic Spectral Detection Module offers two distinct unmixing algorithms: the commonly used Least Squares Method (LSM) and a proprietary modification of the Poisson algorithm designed to improve resolution in complex panels with significant spread error concerns. Users are encouraged to alternate between these two unmixing algorithms to determine which one performs best for their specific panels.

In this carefully designed panel, no fluorochrome combinations exceeded a similarity index of 0.84, indicating a minimal risk of excessive spread errors (Figure 2). To compare the impact of these algorithms on cell resolution, we generated opt-SNE plots of the flow data using the Cytobank analysis platform. The data presented in Figure 6 reveals that both unmixing algorithms perform similarly in defining and resolving cell populations for this panel.

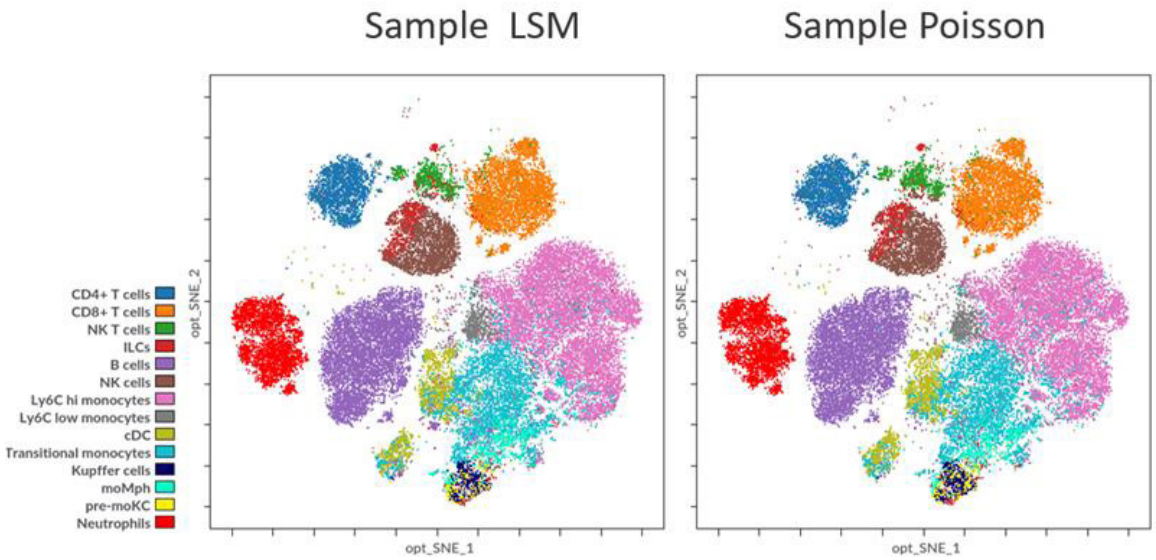


Figure 6: Manual Gating Overlay on optSNE. Overlay of manual population gating on optSNE – total Leukocyte population from the same sample unmixed with either LSM (left) or Poisson (right) unmixing. optSNEs were generated on each file separately.

Discussion and Conclusion

OMIP-104 serves as a robust tool for analyzing immune cell subsets in mouse metabolic organs and can be easily adapted for studying various tissues or specific leukocyte subsets. The panel transitions seamlessly to the CytoFLEX mosaic Spectral Detection Module, where we have successfully identified all key leukocyte populations and their subsets, as demonstrated in Figure 3. Since this panel was not initially designed for the CytoFLEX mosaic Spectral Detection Module, titration and optimization of experimental settings could further enhance its performance. For example, no fluorochromes designed for the 808 nm near-infrared laser were included in the current panel. These fluorochromes offer an easy solution to expand the panel or replace fluorochromes that result in spread errors. This establishes a reliable foundation for users to confidently apply antibody panels from other instruments onto the CytoFLEX mosaic Spectral Detection Module.

Abbreviations

Abbreviation	Full Form
APC	Allophycocyanin
BUV	Brilliant Ultraviolet
BV	Brilliant Violet
Cy	Cyanine
FITC	Fluorescein isothiocyanate
IR	Infrared
ILCs	Innate Lymphoid Cells
Mph	Macrophages
NIR	Near-Infrared
NK	Natural Killer
PE	Phycoerythrin
PN	Part Number
QC	Quality Control
TIM4	T-cell Immunoglobulin and Mucin domain-containing protein 4
TREM2	Triggering Receptor Expressed on Myeloid cells 2
XCR1	X-C Motif Chemokine Receptor 1

References

1. Lambooij JM, Tak T, Zaldumbide A, Guigas B. OMIP-104: A 30-color spectral flow cytometry panel for comprehensive analysis of immune cell composition and macrophage subsets in mouse metabolic organs. Cytometry. 2024;105(7):493–500

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