Application Note

Cell Analysis

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A 30-color Immunophenotyping Panel of Mice Infected with Influenza Using the Agilent Novocyte Penteon Flow Cytometer

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Abstract

Use of flow cytometry has become routine across scientific disciplines, encompassing both the basic and clinical research spaces. It has proven to be a powerful tool to analyze different subpopulations of immune cells and gather a comprehensive overview of the immune system. Due to great advancements within the field, newer flow cytometers can support multiple colors, enabling identification of a variety of cells simultaneously. In this application note, a 30-color immunophenotyping panel was designed for the five-laser Agilent NovoCyte Penteon flow cytometer to see the distribution of immune cells in the lung, spleen, and mediastinal lymph nodes of mice infected with influenza.

Introduction

Complex and sophisticated multicolor flow cytometry experiments capable of evaluating multiple immune cells simultaneously are increasingly accessible thanks to key improvements in the field. Keeping pace with advancements, the Novocyte Penteon is equipped with five lasers (UV, 349 nm; violet, 405 nm; blue, 488 nm; yellow-green, 561 nm; and red, 637 nm) and 30 fluorescence detectors to gain the most information possible from one biological sample. With 30 colors, it is possible to identify multiple subpopulations in one tube with a single acquisition.

In this panel designed at the Centre d'Immunologie Marseille Luminy (CIML), all detectors are used to delve into the immune system of an influenza infected mouse and obtain a comprehensive overview of immune cell subpopulations post-infection. The 30-color immunophenotyping panel was designed through diligent fluorophore selection considering expression markers, fluorescence intensity, spectral overlap, and Agilent NovoCyte Penteon configuration. Resulting data was analyzed using NovoExpress software and revealed a difference in cell distribution across three lymphoid organs.

Experimental

Materials

- RPMI-1640 Medium (GIBCO, 21875034)
- Fetal Calf Serum, FCS (Biowest, S1810-500)
- DNAse I (Sigma-aldrich, 10104159001)
- Collagenase II (Sigma-aldrich, C5138)
- UltraPure 0.5 M EDTA, pH 8 (Invitrogen, 15575-038)
- 1X RBC Lysis Buffer (eBioscience, 00-4333-57)
- FACS Buffer (PBS 1x, 0.5% BSA, 2 mM EDTA)
- Brilliant stain buffer (Becton Dickinson, 563794)
- Flow cytometry antibodies (Table 1)
- Agilent NovoExpress Software
- Agilent Novocyte Penteon flow cytometer

All work was conducted on a C57Bl6 mouse, infected at 8 weeks by 5 PFU of influenza (PR8, H1N1). The mouse was euthanized 35 days post infection. Spleen, lungs, and mediastinal lymph nodes were extracted. A pulmonary lavage was conducted with 10 mL of PBS 1x.

Organ preparation

Lung and spleen:

1. Add the organ to 3 mL of RPMI 5% FCS containing 45 µL of DNase I (150 µg/mL) and 30 µL of collagenase II (500 µg/mL).
2. Dissociate the organ tissue with OctoGentleMacs (Miltenyi) using the "m_lung-01_02" program.
3. Wait 30 minutes at 37 °C. Run the "m_lung-01_02" program again on OctoGentleMacs (Miltenyi).
4. Add 30 µL of EDTA (0.5 M) and wait 2 minutes.
5. Filter with a cell strainer (70 µm).
6. Add media until the total volume is 15 mL.
7. Centrifuge for 7 minutes at 4 °C, 400 g.
8. Aspirate the supernatant.
9. Resuspend the pellet with 3 mL of RBC and wait 5 minutes.
10. Add media until total volume is 15 mL.
11. Centrifuge for 7 minutes at 4 °C, 400 g.
12. Resuspend in 500 µL of FACS Buffer containing 10% Fc Block + 20% Brilliant Stain Buffer. Keep on ice.

Lymph nodes

1. Dissociate manually.
2. Filter with a cell strainer (70 µm).
3. Add media until the total volume is 15 mL.
4. Pellet the cells by centrifugation for 7 minutes (400 g at 4 °C).
5. Resuspend the pellet in 200 µL of FACS Buffer containing 10% Fc Block + 20% Brilliant Stain Buffer. Keep on ice.

Sample preparation

1. After 10 minutes on ice, count 3 million cells.
2. Pellet cells by centrifugation for 7 minutes (400 g at 4 °C).
3. Resuspend each sample with 50 µL of antibody cocktail (Table 1).
4. Incubate for 30 minutes at 37 °C in the dark.
5. Add 150 µL of FACS Buffer.
6. Pellet the cells by centrifugation for 7 minutes (400 g at 4 °C).
7. Rinse the cells with 200 µL of PBS.

8. Pellet the cells by centrifugation for 7 minutes (400 g at 4 °C).
9. Resuspend the cells in 100 µL of Live Dead Fixable blue (diluate at 1/1,000 in PBS) for 15 minutes at room temperature (RT) in the dark.
10. Add 150 µL of FACS Buffer.
11. Pellet the cells by centrifugation for 7 minutes (400 g at 4 °C).
12. Acquire the samples on a NovoCyte Penteon flow cytometer.

Table 1. Antibodies used in 30-color immunophenotyping panel on the Agilent NovoCyte Penteon Flow Cytometer, ordered by channels. The laser and filter used for detection for each antibody can be found in the table, as well as concentration and product details for each antibody.

Laser	Filter	Specificity	Fluorochrome	Clone	Vendor	Part Number	Dilution	Amount Added to 3×10^6 Cells (50 µL)
349	445/45	Live/Dead Fixable Blue	BUV395		Invitrogen	L23105	1/1000	0.05
	525/45	MHC-II	BUV496	M5/114.15.2	Becton Dickinson	750281	1/800	0.0625
	586/20	CD3	BUV563	17A2	Becton Dickinson	741319	1/400	0.125
	615/20	gdT	BUV615	GL3	Becton Dickinson	751183	1/200	0.25
	667/30	CD88	BUV661	20/70	Becton Dickinson	750080	1/400	0.125
	725/40	CD43	BUV737	S7	Becton Dickinson	612840	1/200	0.25
	780/60	NKp46	BUV805	29A1.4	Becton Dickinson	742066	1/100	0.5
405	445/45	XCR1	BV421	ZET	BioLegend	148216	1/400	0.125
	525/45	CD103	BV480	M290	Becton Dickinson	566118	1/200	0.25
	586/20	TCRb	BV570	H57-597	BioLegend	109231	1/200	0.25
	615/20	Siglec H	sb600	eBio440c	Thermo Fisher Scientific	63-0333-82	1/200	0.25
	667/30	CD11b	BV650	M1/70	BioLegend	101259	1/400	0.125
	725/40	CD8b	sb702	53-6.7	Thermo Fisher Scientific	67-0081-82	1/400	0.125
	780/60	CD69	BV786	H1.2F3	Becton Dickinson	564683	1/200	0.25
488	525/45	SiglecF	BB515	E50-2440	Becton Dickinson	564514	1/400	0.125
	586/20	CD45	Spark 574	30F11	BioLegend	103184	1/200	0.25
	615/20	CD4	BB630-P	GK1.5	Becton Dickinson	Prototype	1/400	0.125
	667/30	CD90.2	PerCP	30H12	BioLegend	105322	1/200	0.25
	695/40	Ly6G	BB700	1A8	Becton Dickinson	566435	1/800	0.0625
	725/40	CD62L	BB755	MEL-14	Becton Dickinson	Prototype	1/400	0.125
561	586/20	B220	SYG570	RA36B2	BioLegend	103286	1/400	0.125
	615/20	CD172a	AF594	P84	BioLegend	144020	1/400	0.125
	667/30	CD25	PE Cy5	PC61	BioLegend	102010	1/200	0.25
	695/40	CD11c	PE Cy5.5	N418	Thermo Fisher Scientific	35-0114-82	1/400	0.125
	725/40	CD19	PE-AF700	1D3	AAT-bioquest	ATB10194100	1/400	0.125
	780/60	NK1.1	PE-Cy7	PK136	BioLegend	108714	1/100	0.5
637	667/30	CD64	AF647	X54-5/7.1	BioLegend	139322	1/200	0.25
	695/40	IgD	SPARK 685	11-26c.2a	BioLegend	405750	1/1000	0.05
	725/40	CD44	APC-R700	IM7	Becton Dickinson	565480	1/400	0.125
	780/60	Ly6C	APC-Cy7	HK1.4	BioLegend	128026	1/800	0.0625

Results and discussion

Flow cytometry has long been established as the gold standard to analyze heterogenous cell mixtures in both basic research and clinical applications. Newer, more sophisticated instruments with increased channels and lasers build upon their predecessors and expand the breadth of capabilities in lab and the clinic. By increasing the number of lasers and detectors, more antibodies can be included in a panel to identify target populations more precisely by their phenotypic markers. Immunophenotyping labels cells with fluorescent antibodies directed against cell surface markers, enabling visualization by flow cytometry. Design of a 30-color immunophenotyping panel maximizes throughput on a 30-detector instrument—to date the maximum number of detectors on a single flow cytometer.

The main objective of the panel design described in this study was to evaluate the distribution of immune cells in an influenza-infected mouse. Specifically, the distribution of immune cell types across three lymphoid organs was analyzed: lung, spleen, and lymph nodes. The subpopulations of T cell types were also closely monitored to compare differences in distribution across the three organs during influenza infection. The same gating strategy was used across all three organs to easily compare the data. Antibody clone, detection channel, vendor information, and the amount of each antibody added are described in Table 1.

The 30-color flow cytometry panel used in this application note identified several immune cell types across three organs by expression of cell surface receptors, as well as T cell activation status. To generate the panel, 50 µL of the antibody cocktail was added to 3 million cells. After staining, the sample data was acquired and analyzed using the NovoCyte Penteon flow cytometer and NovoExpress software. The gating hierarchy used to identify immune cell populations and subsets is described in the following paragraphs.

Initial gating with forward scatter (FSC) and side scatter (SSC) removed debris to identify the cell population of interest. Doublets were removed following FSC-A and FSC-H gating, and live cells were then identified by Live/Dead Fixed Blue Dead Cell Stain kit and carried forward for further analysis. Hematopoietic cells were identified by expression of CD45+ and analyzed further, while low expression indicated the presence of basophils. CD45+ cells were gated using CD19 and MHC II, which revealed a subset of B cells at high expressions of both. B cells were gated further with IgD and B220, which indicated approximately 61.39% of the B cells were naïve.

Cells lacking expression of CD19 were segmented with markers for CD11b and Ly6G. High expression of CD11b and Ly6G determined that 6.23% of cells identified were neutrophils, while the rest were not. By maintaining gating for CD11b, but changing the Y axis to Siglec F, alveolar macrophages (high Siglec F, low CD11b) and eosinophils (high Siglec F, high CD11b) were identified. The population of cells with little to no expression of Siglec F were pushed forward for further dissection, as T cells contain very low levels of Siglec F.

To differentiate between T cells and others, gating for CD3+ and CD90+ was applied. CD3+CD90+ cells were then used to closely analyze T cell subsets. CD3-CD90- cells were further characterized to determine the remaining immune cell composition

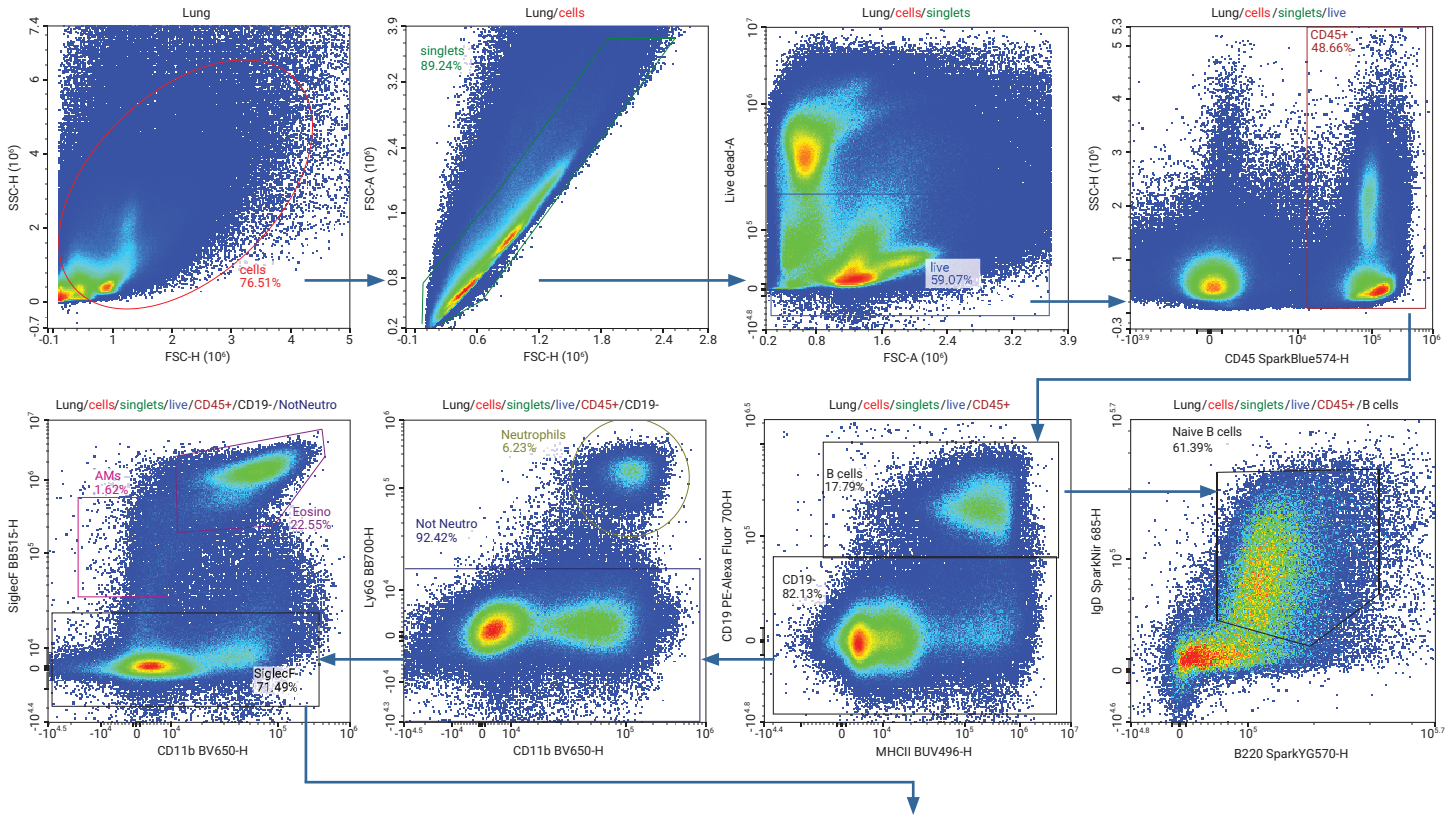
NK cells were identified by gating for expression of NK1.1 and NKp46. Cells with low expression were not considered NK cells and gated against Siglec H and B220 to identify plasmacytoid dendritic cells. The rest of the cells were plotted against CD43 and Ly6C to identify monocytes. Cells not categorized as monocytes were then gated for CD88+ and CD64+ to find macrophages, which were further confirmed by gating against MHC II and CD11c. CD8-CD64- cells were similarly gated to identify dendritic cells, which populated the upper right corner of the plot. It was possible to distinguish between the two types of conventional dendritic cells, cDC1 and cDC2 by gating for XCR1, a chemokine receptor exclusively on cDC1.

T cells (CD3+CD90+ cells) were explored in further depth than the other immune cell populations, as the objective was to gather insight to their activation status and distribution across the three lymphoid organs during infection. Approximately 3.3% of the identified T cells expressed the gamma delta receptor. The rest were gated for expression of CD4 and CD8, then divided into two groups: CD4+CD8– and CD8+CD4–. Gating CD4+CD8– cells for CD25 determined less than 2% were regulatory T cells (1.66%). The rest were gated for CD62L+ and CD44+ to distinguish between naïve CD4+ memory cells (CD62L+) and activated CD4+ cells (CD62L–). Resident memory T cells (Trm) were then identified by expression of CD103+CD69. CD8+CD4- cells followed a similar gating strategy to distinguish between CD8+ memory (CD44+CD62L+), activated (CD44+CD62L–), and Trm (CD103+CD69+) cells.

While Figure 1 shows data pertaining to the lung sample specifically, it is relevant across all organs, given the same strategy was used. This was done so it was easy to compare immune cell distribution and activation across all three organs. Comparison of immune cell subpopulation frequencies in the three organs revealed marked differences in distribution in some instances (Figure 2). In this study, the frequency value was the percentage parent, which refers to the percentage of the subpopulation within the population from which it comes.

A direct comparison T cell distribution across the three organs showed there was increased T cell expression in the lymph nodes (90%) compared to the lungs (62%) and the spleen (54%). CD8 T cells followed a similar distribution pattern, though much less drastic (54% vs 43%). Interestingly, the pattern changes remarkably. Neutrophils were also greatly reduced in the lymph nodes relative to the lungs and spleen (1:10:20 ratio, respectively.) Meanwhile, pDC cells had increased distribution in the lymph nodes with minimal presence in the lungs and none in the spleen. With 30 parameters, it is easy to have a quick overview of the organs, with a single acquisition.

The absolute count (on event/µL) of each sample was also determined by NovoExpress Software during acquisition. Figure 3 shows the absolute count of T cell subpopulations in the lung of the infected mouse. A close look at the distribution of T cells in the lung revealed the highest concentration—measured as events per microliter—consisted of activated CD4+ and CD8+ cells. Though not quite 1:1, the distribution between the two did not differ greatly. The next set of most common T cells were much less frequent. Interestingly, CD8+ Trm cells were an order of magnitude more frequent than CD4+ Trm cells.



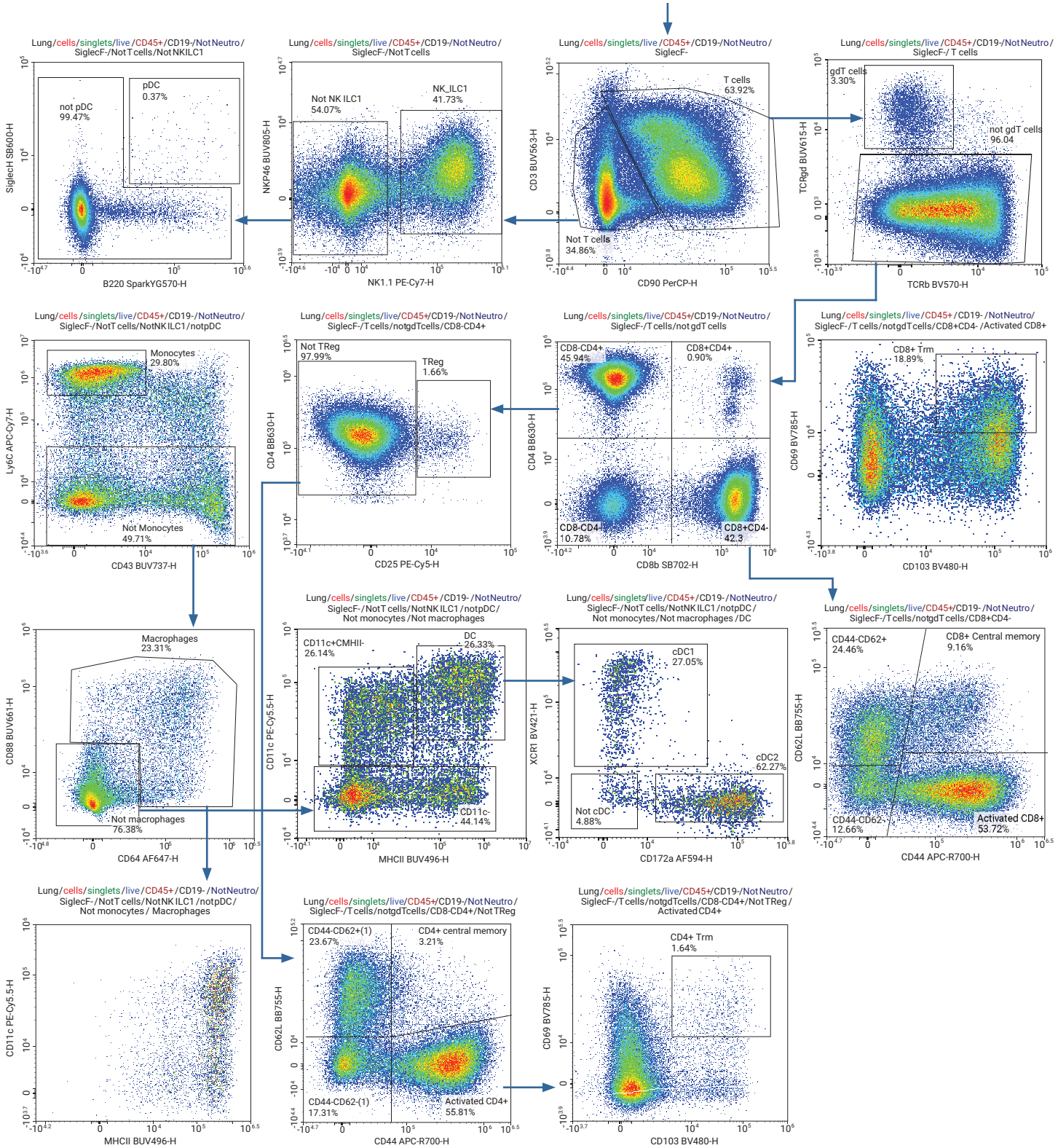


Figure 1. Identification strategy utilized to define cell subpopulations in 30-color immunophenotyping panel on the Agilent Novocyte Penteon Flow Cytometer. Figure is specific to lung cells but is also representative of spleen and lymph node samples, as the same strategy was applied. After staining, samples were acquired on the NovoCyte Penteon and analyzed with Agilent NovoExpress software. Hierarchical gating was used to identify all major cell subsets.

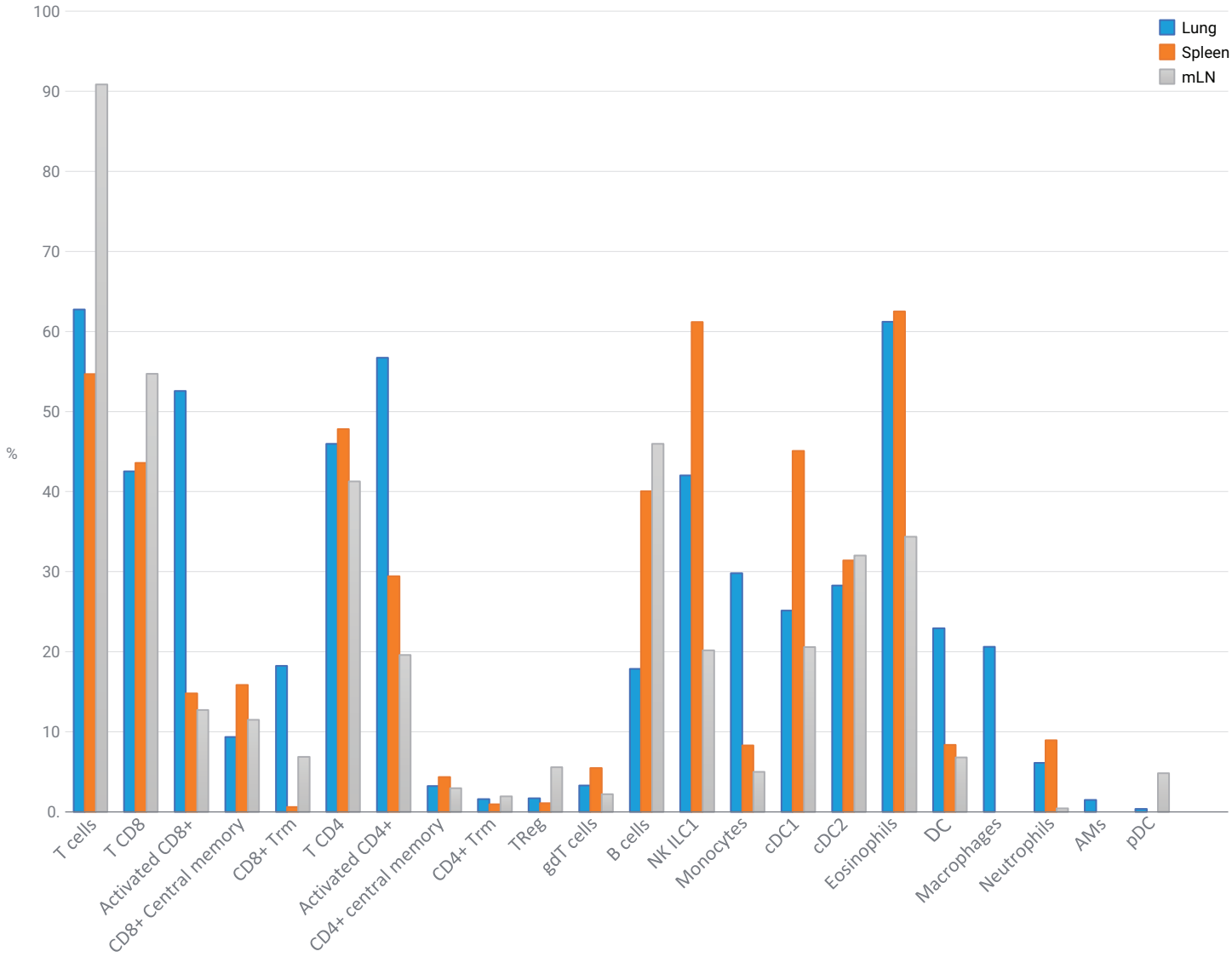


Figure 2. Frequencies (%parents) for main subpopulations defined on the 30-color panel, on each organ: lung, spleen, and mediastinal lymph node.

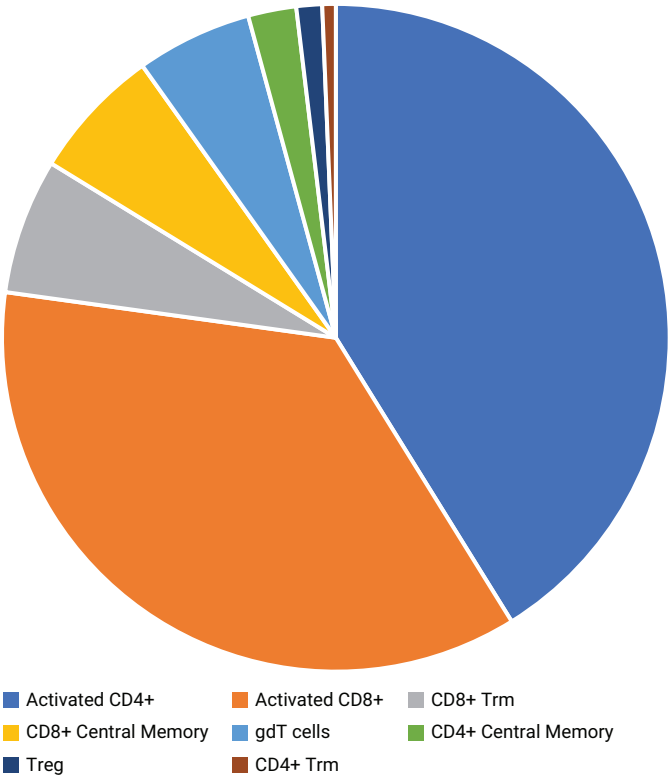


Figure 3. T cell subpopulation distribution according to their concentration (events/µL) in lung.

Conclusion

The Agilent NovoCyte Penteon flow cytometer enables researchers to study multiple important parameters in one acquisition—a great asset in immunophenotyping. With five lasers and 30 detectors, it is possible to extract more information from a single tube, saving precious biological material. Absolute count provides even more information about the sample. In this application note, the same gating strategy was applied on three lymphoid organs, permitting comparison of T cell distribution and allowing insight into the immune system during influenza infection. The high detection capabilities of the NovoCyte Penteon also allowed identification of other immune cell subsets in the lung, spleen, and lymph nodes in the same experiment.

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