

# Analysis of Mixed Microbial Populations and Deep Immunotyping of Peripheral Blood on the ZE5™ Cell Analyzer



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## Cell Analysis

Bulletin 6962

### Abstract

Advancements in cell biology have created a need for flow cytometry instruments to perform accurate high-dimensional multicolor analysis. Users require more analysis parameters, higher sensitivity, and greater size resolution to execute complex multiplex cell analysis assays.

The ZE5 Cell Analyzer is able to successfully perform large panel immunophenotyping with up to five lasers and 30 analysis parameters. The ZE5 Cell Analyzer can also distinguish a wide range of bead sizes and mixed microbial populations.

### Introduction

With up to five lasers and 30 analysis parameters, the ZE5 Cell Analyzer is well equipped for complex multiplex analysis of cells. Excellent fluorescence sensitivity (<100 MESF values in FITC, PE, and APC channels) enables detection of antigens expressed at low levels. The three scatter options — forward scatter (FSC), side scatter (SSC), and forward scatter small particle detector (FSCspd) — provide optimal resolution of particles >0.3 to 50  $\mu\text{m}$  in size. Furthermore, the option to set a dual trigger using any scatter or fluorescence channels allows for optimal background reduction.

The ZE5 Cell Analyzer was used to successfully perform immunophenotyping of multiple cellular subsets in human blood using multicolor panels. Peripheral blood mononuclear cells (PBMCs) from normal healthy donors were stained using a 19-color panel to identify T cell, B cell, NK cell, dendritic cell, and monocyte subpopulations. Results showed that the panel data were compensated accurately on the ZE5 Cell Analyzer and that each cellular subpopulation was well identified.

The ZE5 Cell Analyzer also effectively differentiated size beads and mixed microbial populations. Beads sized  $\geq 0.2 \mu\text{m}$  were discriminated from noise when using scatter or fluorescence as the trigger. Analysis of mixed microbial populations clearly identified the unique populations within the mixture.

### Materials and Methods

#### PBMCs Immunophenotyping

Frozen PBMCs (AllCells) were thawed into PBS + 10% FBS, washed, and counted on the TC20™ Automated Cell Counter (Bio-Rad Laboratories). Cells were washed twice in PBS and plated in a 96-well plate at  $5 \times 10^5$  cells/well. PBMCs were stained using the Zombie UV Fixable Viability Kit (BioLegend) for 30 min at room temperature in the dark. Cells were washed twice with PBS + 1% BSA + 0.1% azide and then incubated with FC-block for 30 min at 4°C in the dark. Antibody staining mixtures were made in BD Horizon Brilliant Stain Buffer at

**Table 1. Lasers, emission filters, and antibodies used in the PBMC immunophenotyping.**

Laser/Ex Filter	Em Filter	Marker	Color/Format
355	700LP	CD25	BUV737
	387/11	CD45RO	BUV395
	447/60	Live/Dead	Zombie UV
	525/50	CD19	BUV496
405	420/10	CD3	Brilliant Violet 421
	460/22	CD4	ef450
	625/24	CD20	Brilliant Violet 605
	525/50	CD27	V500
	750LP	CD16	Brilliant Violet 785
	720/60	CD123	Brilliant Violet 711
488	670/30	CD14	Brilliant Violet 650
	525/35	IgD	Alexa Fluor 488
561	670/30	CD127	PE-Cy5
	615/24	CCR7	PE/Dazzle 594
	577/15	CD11c	PE
	750LP	CD33	PE-Cy7
640	720/60	CD8	Alexa Fluor 700
	670/30	CD56	APC
	775/50	HLA DR	APC-H7

concentrations previously determined via antibody titration. Cells were stained with antibody mixes for 45 min at 4°C in the dark, washed twice, and fixed in 1% PFA prior to analysis. Samples were analyzed on the ZE5 Cell Analyzer using Everest™ Software (Bio-Rad Laboratories). Gating was carried out using FlowJo Software (Tree Star) with gates determined using FMO controls.

**Bead Assay**

Beads — 0.2 µm (Bangs Laboratories), mix of 2, 3, 5, 8, 10, and 16 µm (Spherotech), or 50 µm (Bangs Laboratories) — were diluted in Milli-Q diH<sub>2</sub>O (EMD Millipore) and visualized on the ZE5 Cell Analyzer using either scatter or fluorescence as the trigger.

**Mixed Microbial Population Analysis**

Aliquots of *Escherichia coli*, *Bacillus subtilis*, and *Enterococcus faecalis* (Sigma-Aldrich) were cultured overnight in Luria broth (LB). One aliquot of *Pseudomonas aeruginosa* (ATCC) was thawed just prior to use. Active dry yeast (*Saccharomyces cerevisiae*) was rehydrated in PBS for 10 min prior to use. All samples were washed twice with PBS and then fixed with 2% paraformaldehyde for 30 min. After fixation, cells were washed twice with PBS and stained for 5 min using ReadIDrop™ Propidium Iodide Cell Viability Dye (Bio-Rad Laboratories). Samples were analyzed on the ZE5 Cell Analyzer and the analysis of populations was carried out using FlowJo Software.

**Results**

**PBMCs Immunophenotyping**

Everest Software, integrated within the ZE5 Cell Analyzer, is able to perform compensation prior to data export. When using the same data file for analysis in FlowJo Software, the compensation matrix is comparable (Figure 1).

**ZE5 Everest Software Compensation Matrix**

	FL03-A	FL07-A	FL08-A	FL11-A	PE/FL13-A	FL14-A	FL15-A	FL16-A	FL17-A	FL18-A	FL19-A	FL20-A	FL21-A	FL23-A	FL24-A	FL25-A	FL26-A	FL27-A	FL29-A
FL03-A :: Alexa Fluor 488-A	100.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.37%	0.64%	0.02%	0.00%	0.04%	0.00%	0.00%	0.00%
FL07-A :: PE-Cy7-A	0.01%	100.00%	1.90%	0.23%	2.86%	0.22%	0.31%	6.82%	0.00%	0.00%	0.41%	0.00%	0.00%	2.70%	0.00%	0.02%	0.54%	10.01%	0.00%
FL08-A :: PE-Cy5-A	0.03%	54.94%	100.00%	0.44%	0.35%	12.45%	8.30%	3.84%	0.00%	0.00%	0.00%	0.00%	0.00%	6.66%	0.00%	0.04%	79.11%	22.06%	62.30%
FL11-A :: PE (R-phycoerythrin)-A	1.33%	13.65%	32.23%	100.00%	64.25%	4.81%	2.78%	1.03%	0.00%	0.00%	11.93%	0.00%	0.02%	2.48%	0.00%	0.11%	0.07%	0.10%	0.00%
FL13-A :: PE-Dazzle 594-A	0.15%	35.60%	68.05%	5.08%	100.00%	8.66%	5.04%	2.01%	0.00%	0.00%	16.17%	0.00%	0.00%	4.14%	0.00%	0.05%	0.33%	0.05%	0.30%
FL14-A :: Brilliant Violet (BV) 650-A	0.01%	5.50%	9.31%	0.00%	0.85%	100.00%	63.55%	24.57%	2.71%	15.42%	9.80%	0.24%	0.02%	21.64%	0.21%	0.00%	22.51%	5.54%	15.89%
FL15-A :: Brilliant Violet (BV) 711-A	0.01%	5.82%	0.09%	0.00%	0.00%	3.95%	100.00%	60.27%	2.02%	12.28%	0.00%	0.12%	0.01%	48.78%	0.14%	0.07%	63.56%	23.12%	1.35%
FL16-A :: Brilliant Violet (BV) 786-A	0.75%	3.91%	0.11%	0.09%	0.13%	0.16%	4.66%	100.00%	1.51%	8.93%	0.00%	0.09%	0.01%	17.41%	0.10%	0.07%	1.17%	11.94%	0.10%
FL17-A :: eFluor 450-A	0.03%	0.02%	0.01%	0.01%	0.01%	0.10%	0.01%	0.01%	100.00%	41.46%	0.68%	12.21%	0.72%	0.01%	2.58%	0.19%	0.02%	0.02%	0.00%
FL18-A :: Brilliant Violet (BV) 421-A	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	0.01%	17.31%	100.00%	0.06%	1.48%	0.28%	0.00%	2.18%	0.02%	0.00%	0.00%	0.00%
FL19-A :: Brilliant Violet (BV) 605-A	0.01%	13.17%	20.87%	0.60%	20.01%	76.26%	46.44%	22.62%	4.11%	24.27%	100.00%	0.27%	0.01%	18.95%	0.32%	0.09%	0.23%	0.04%	0.27%
FL20-A :: V500-A	7.99%	0.05%	0.02%	0.00%	0.02%	14.24%	7.61%	2.87%	14.21%	0.00%	33.35%	100.00%	7.38%	0.91%	0.31%	0.58%	0.03%	0.00%	0.00%
FL21-A :: Brilliant Ultraviolet (BUV) 496-A	8.19%	0.19%	0.06%	0.08%	0.05%	0.60%	0.16%	0.10%	3.65%	1.61%	2.59%	18.82%	100.00%	6.93%	18.73%	31.30%	0.17%	0.15%	0.00%
FL23-A :: Brilliant Ultraviolet (BUV) 737-A	0.02%	0.04%	0.02%	0.01%	0.01%	0.00%	5.46%	6.58%	0.00%	0.04%	0.00%	0.03%	100.00%	0.78%	1.95%	55.65%	35.35%	0.41%	
FL24-A :: Zombie UV-A	0.00%	1.22%	0.60%	1.01%	1.07%	4.25%	0.59%	0.00%	46.38%	58.27%	4.77%	17.50%	74.58%	20.36%	100.00%	9.57%	0.00%	0.52%	1.85%
FL25-A :: Brilliant Ultraviolet (BUV) 395-A	0.02%	0.00%	0.01%	0.00%	0.01%	0.00%	0.00%	0.00%	0.35%	2.82%	0.00%	0.00%	0.16%	51.06%	100.00%	0.01%	0.01%	0.00%	
FL26-A :: Alexa Fluor 700-A	0.03%	4.47%	0.03%	0.00%	0.01%	4.65%	2.45%	0.00%	0.00%	0.00%	0.00%	0.00%	3.90%	0.00%	0.05%	100.00%	32.47%	0.92%	
FL27-A :: APC-Cy7-A	0.01%	20.80%	0.06%	0.02%	0.01%	0.00%	0.09%	7.03%	0.00%	0.00%	0.00%	0.00%	0.00%	3.57%	0.00%	0.04%	3.40%	100.00%	50.00%
FL29-A :: APC (Allophycocyanin)-A	0.01%	7.11%	14.07%	0.00%	0.05%	7.06%	3.59%	1.72%	0.00%	0.00%	0.00%	0.00%	0.00%	4.53%	0.00%	0.05%	101.05%	29.99%	100.00%

**FlowJo Software Compensation Matrix**

	FL03-A	FL07-A	FL08-A	FL11-A	PE/FL13-A	FL14-A	FL15-A	FL16-A	FL17-A	FL18-A	FL19-A	FL20-A	FL21-A	FL23-A	FL24-A	FL25-A	FL26-A	FL27-A	FL29-A
FL03-A :: Alexa Fluor 488-A	100.00%	0.01%	0.00%	0.01%	0.00%	-0.02%	-0.01%	-0.01%	-0.04%	-0.03%	-0.01%	0.36%	0.63%	0.01%	-0.01%	0.04%	0.00%	0.00%	-0.02%
FL07-A :: PE-Cy7-A	0.01%	100.00%	1.86%	0.24%	2.78%	0.22%	0.31%	6.58%	-0.01%	-0.01%	0.43%	-0.03%	0.00%	2.69%	0.00%	0.03%	0.53%	10.05%	0.02%
FL08-A :: PE-Cy5-A	0.03%	54.46%	100.00%	0.45%	0.35%	11.72%	7.76%	3.50%	-0.01%	0.00%	0.02%	-0.04%	-0.01%	6.79%	0.00%	0.05%	75.49%	20.93%	59.52%
FL11-A :: PE (R-phycoerythrin)-A	1.31%	13.50%	33.11%	100.00%	65.54%	4.68%	2.77%	1.07%	0.00%	0.00%	11.64%	-0.01%	0.03%	2.40%	0.01%	0.13%	0.10%	0.13%	0.00%
FL13-A :: PE-Dazzle 594-A	0.15%	33.50%	66.95%	4.97%	100.00%	8.06%	4.75%	1.95%	-0.02%	-0.01%	15.28%	-0.06%	-0.01%	4.18%	0.00%	0.06%	0.34%	0.05%	0.31%
FL14-A :: Brilliant Violet (BV) 650-A	0.01%	5.73%	9.98%	0.02%	0.95%	100.00%	64.95%	25.17%	2.82%	16.11%	10.25%	0.27%	0.03%	22.67%	0.25%	0.09%	22.81%	5.66%	16.62%
FL15-A :: Brilliant Violet (BV) 711-A	0.00%	6.03%	0.12%	0.01%	0.00%	4.08%	100.00%	59.14%	2.04%	12.31%	-0.09%	0.14%	0.01%	48.97%	0.17%	0.09%	61.01%	23.14%	1.46%
FL16-A :: Brilliant Violet (BV) 786-A	0.82%	4.09%	0.15%	0.11%	0.16%	0.20%	4.68%	100.00%	1.55%	8.97%	0.04%	0.12%	0.02%	17.78%	0.13%	0.08%	1.23%	11.91%	0.14%
FL17-A :: eFluor 450-A	0.03%	0.02%	0.02%	0.04%	0.00%	0.20%	0.06%	0.02%	100.00%	40.87%	0.83%	11.99%	0.87%	0.02%	2.67%	0.03%	0.03%	0.03%	0.09%
FL18-A :: Brilliant Violet (BV) 421-A	0.00%	0.00%	0.00%	0.00%	0.00%	0.00%	0.01%	0.01%	17.44%	100.00%	0.07%	1.48%	0.30%	0.01%	2.36%	0.00%	0.00%	0.00%	0.01%
FL19-A :: Brilliant Violet (BV) 605-A	0.01%	13.86%	22.03%	0.65%	20.87%	76.54%	47.64%	22.85%	4.12%	24.21%	100.00%	0.30%	0.03%	19.48%	0.37%	0.11%	0.28%	0.05%	0.32%
FL20-A :: V500-A	8.02%	0.04%	0.01%	0.02%	0.00%	14.54%	7.60%	3.06%	14.65%	0.31%	32.32%	100.00%	7.57%	0.96%	0.48%	0.66%	0.08%	0.04%	0.18%
FL21-A :: Brilliant Ultraviolet (BUV) 496-A	7.86%	0.24%	0.11%	0.42%	0.03%	1.17%	0.50%	0.19%	4.59%	2.75%	3.62%	18.34%	100.00%	6.93%	19.87%	32.86%	0.21%	0.14%	1.60%
FL23-A :: Brilliant Ultraviolet (BUV) 737-A	0.01%	0.14%	0.01%	0.00%	0.00%	-0.07%	3.40%	6.58%	-0.02%	0.13%	-0.25%	0.07%	100.00%	0.07%	0.88%	2.01%	55.24%	36.05%	0.56%
FL24-A :: Zombie UV-A	1.48%	5.29%	0.64%	0.47%	0.31%	5.39%	3.47%	1.86%	60.68%	113.36%	9.58%	22.39%	65.64%	18.92%	100.00%	16.78%	2.68%	1.09%	3.87%
FL25-A :: Brilliant Ultraviolet (BUV) 395-A	0.01%	0.01%	0.01%	0.03%	0.00%	-0.15%	-0.05%	-0.01%	0.46%	2.72%	-0.48%	-0.23%	5.06%	0.21%	50.75%	100.00%	0.03%	0.02%	0.10%
FL26-A :: Alexa Fluor 700-A	0.03%	4.60%	0.04%	0.01%	0.00%	0.03%	4.45%	2.38%	-0.02%	-0.01%	-0.07%	-0.07%	-0.01%	4.12%	0.00%	0.06%	100.00%	33.05%	1.03%
FL27-A :: APC-Cy7-A	0.01%	21.36%	0.07%	0.03%	0.00%	0.00%	0.10%	6.47%	-0.01%	0.01%	-0.07%	-0.04%	-0.01%	3.63%	0.00%	0.05%	3.39%	100.00%	0.58%
FL29-A :: APC (Allophycocyanin)-A	0.01%	7.48%	14.91%	0.01%	0.07%	6.98%	3.46%	1.68%	-0.01%	0.00%	-0.05%	-0.01%	4.65%	0.00%	0.05%	101.38%	29.97%	100.00%	

Fig. 1. Everest and FlowJo Software perform comparable compensation.

**T Cell Subset Analysis**

Lymphocytes were identified based on live cell gating and then discriminated by size and granularity. CD3<sup>+</sup> T cells are separated into CD3<sup>+</sup>CD4<sup>+</sup> or CD3<sup>+</sup>CD8<sup>+</sup> subpopulations (Figure 2). Regulatory T cells (Treg) are identified as the CD25<sup>hi</sup>CD127<sup>lo</sup> of CD4<sup>+</sup> T cells. Naïve phenotype, central memory phenotype (Tcm), or effector memory phenotype (Tem) CD4<sup>+</sup> and CD8<sup>+</sup> T cells can be characterized based on expression of CCR7 and/or CD45RO lineage markers.

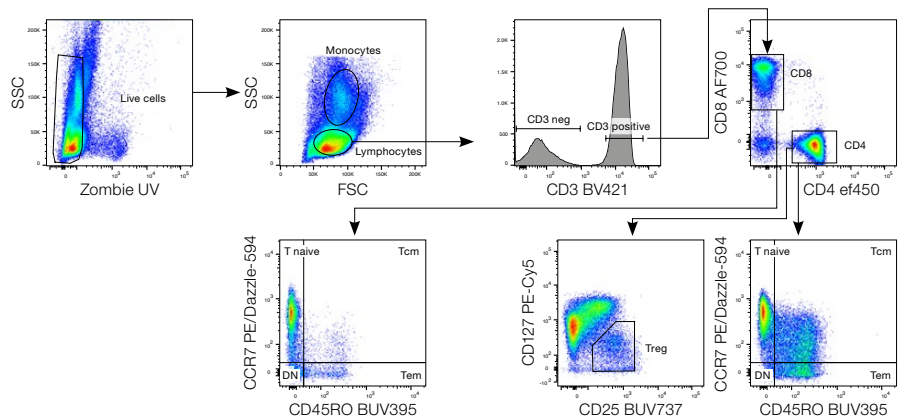


Fig. 2. Analysis of T cell subsets.

### B/NK/DC Cell Subset Analysis

Lymphocytes were identified based on live cell gating and then discriminated by size and granularity. T cells were excluded by gating on CD3<sup>-</sup> (non-T cells) (Figure 3). B cells are identified as the CD19<sup>+</sup>CD20<sup>+</sup> population. NK cells are discriminated by taking the CD3<sup>-</sup>CD19<sup>-</sup>CD20<sup>-</sup> population and examining expression of CD56. CD56<sup>dim</sup>CD16<sup>+</sup> and CD56<sup>bright</sup>CD16<sup>-</sup> subpopulations are clearly identified. To identify dendritic cells (DCs), the CD3<sup>-</sup>CD19<sup>-</sup>CD20<sup>-</sup>CD56<sup>-</sup> population was gated for HLA-DR<sup>+</sup> expression. Plasmacytoid DCs are HLA-DR<sup>+</sup>CD123<sup>+</sup> while myeloid DCs are HLA-DR<sup>+</sup>CD11c<sup>+</sup>.

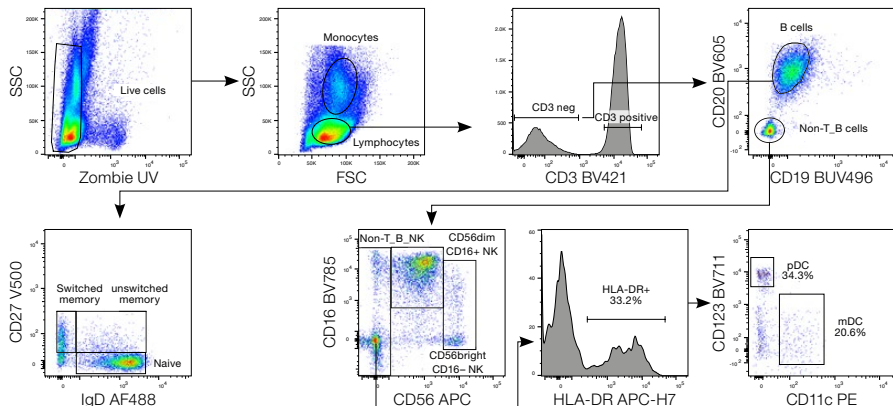


Fig. 3. Analysis of B, NK, and DC cell subsets.

### Monocyte Analysis

Monocytes were identified based on live cell gating and then discriminated by size and granularity. T cells, B cells, and NK cells were removed by selecting the CD3<sup>-</sup>CD19<sup>-</sup>CD56<sup>-</sup> population (Figure 4). CD14<sup>+</sup>CD33<sup>+</sup> double positive populations classify monocytes, which can be further discriminated into CD16<sup>hi</sup>HLA-DR<sup>hi</sup> proinflammatory monocytes.

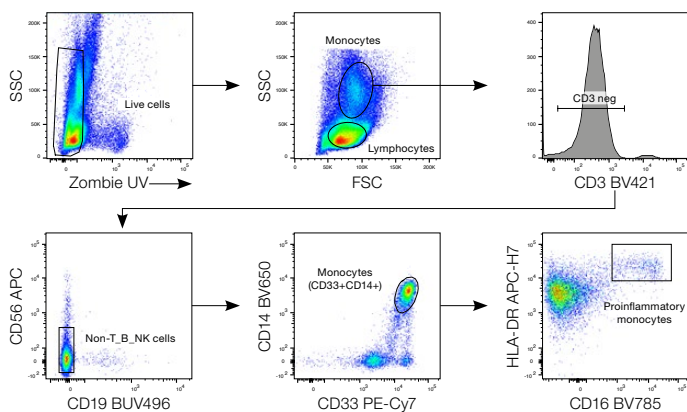


Fig. 4. Analysis of monocytes.

### Bead Assay

The ZE5 Cell Analyzer clearly resolves polystyrene beads of various sizes based on their forward and side scatter properties (Figure 5). Specifically, beads as small as 0.2  $\mu\text{m}$  and as large as 50  $\mu\text{m}$  can be optimally resolved, and 2, 3, 5, 8, 10, and 16  $\mu\text{m}$  beads in a mixed-bead population can be distinguished from each other.

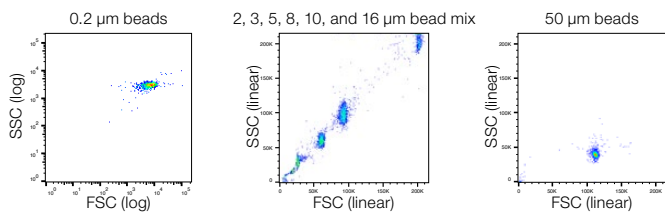
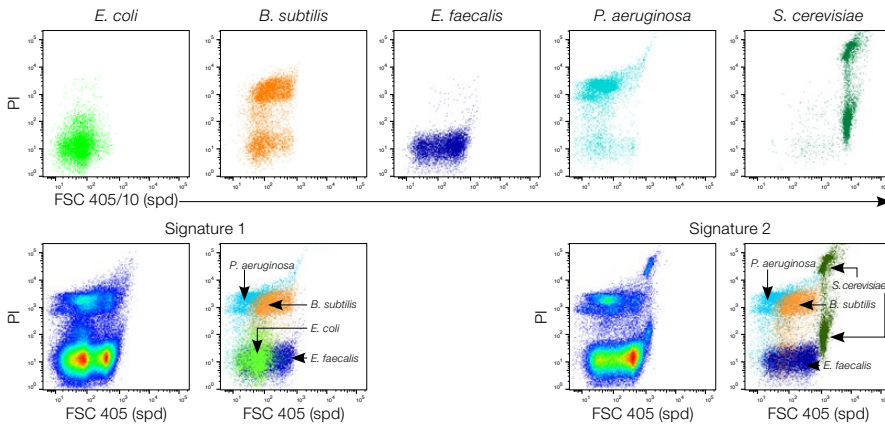


Fig. 5. Resolution of polystyrene beads.

**Mixed Microbial Populations**

The ZE5 Cell Analyzer resolves distinct signatures from mixed microbial populations (Figure 6). *E. coli*, *B. subtilis*, *E. faecalis*, *P. aeruginosa*, and *S. cerevisiae* were fixed with 2% paraformaldehyde, washed, and then stained with ReadIDrop Propidium Iodide (PI) Dye prior to analysis on the ZE5 Cell Analyzer. Microbes were analyzed individually and as mixed populations containing the indicated strains.



**Fig. 6. Resolution of distinct signatures from mixed microbial populations.**

**Conclusions**

- The excellent fluorescence sensitivity and multiple analysis parameters on the ZE5 Cell Analyzer allow for clear discrimination of multiple immune cell populations from a single PBMC sample with a 19-color staining panel
- The analyzer’s three scatter options and dual trigger provide resolution of particles 0.2 to 50 µm in size, including identification of a microbial signature from mixed microbial populations

Visit [bio-rad.com/ZE5](http://bio-rad.com/ZE5) for more information.

Data were originally presented as Abstract #81.23 at the 2017 American Association of Immunologists (AAI) meeting (Washington D.C.).

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