Optimized Twenty-One Color Panel Design Using the ZE5™ Cell Analyzer for Quantification of T Cell Subsets in Stem Cell Transplant Patients

Karl Staser, MD, PhD, Resident, Division of Dermatology*; John F. DiPersio, MD, PhD, Professor of Medicine, Division of Oncology*; William Eades, Associate Professor and Flow Cytometry Director, Department of Medicine*; Angela Goldfain, Scientific Director+; Nathan Trujillo, PhD, Applications Scientist+
*Washington University School of Medicine, St. Louis, Missouri; +Propel Labs, Fort Collins, Colorado

Introduction

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) remains the most effective treatment for patients with high risk and relapsed hematologic malignancies. When donor T cells recognize the host as foreign, they induce an immune response against the host and may cause life-threatening graft-versus-host disease (GvHD), a major complication of allo-HSCT. Monitoring T cell subsets pre- and post-transplantation in correlation with patient outcomes may identify the key cell types mediating engraftment, relapse, GvHD, and drug response. Because there are numerous T cell subset populations, many of which are rare, increasing the types that can be detected simultaneously can provide an advantage in unbiased tracking and quantification.

Using samples from patients enrolled in allo-HSCT clinical trials, researchers at Washington University have designed an optimized 21-color panel (Figure 1) to be used on the ZE5 Cell Analyzer for the purpose of examining changes in T cell populations pre- and post-transplantation, during treatment, and in correlation with patient outcomes.

Flow cytometry is a powerful tool for gathering multiparameter data quickly and efficiently. High-throughput analysis was especially important in this study of more than 150 patient samples. The ZE5 instrument running the 21-color panel analyzed the samples at 2-3 minutes per sample, which preserved the quality of time-sensitive samples and allowed for efficient acquisition of data.

With the recent catalog expansion of fluorochrome-antibody pairings the possibility for more sophisticated experimental design is expanding. The ZE5 Cell Analyzer from Bio-Rad Laboratories Inc. (previously the YETI Cell Analyzer from Propel Labs) provides expanded capacity compared to previous systems. The ZE5 Cell Analyzer allows simultaneous measurement of up to 30 parameters at rates of up to 100,000 cells-per-second. The instrument is equipped with a high-throughput loader and samples can be analyzed from tubes or multi-well plates with options for temperature control and agitation.

Discussion

With the increase of instrumentation capabilities, multi-color experiments can be expanded to gather more information from each sample, thus reducing the quantity and volume of sample required. This is especially relevant in the context of leukopenia following stem cell transplant where the quantity of sample is low and the subset populations within the sample are inherently rare.

However, the increase in instrument capacity can also amplify the complexity of experimental design and therefore necessitate diligent planning. It is imperative to select the right fluorochrome-antibody combinations to optimize population resolution and minimize spectral spillover and plot spreading. Fluorophore brightness and antigen density must be considered, while bearing in mind the optical design and filter configuration of the instrument, which affect detection efficiency and dynamic range. Proper antibody titration and preparation of controls also contribute to the iterative integrity of multi-color experiment design. In this optimized panel, more than 26 well-defined subpopulations can be identified, simultaneously providing quantification of major and minor cell types.

Antigen	Clone	Fluorochrome	Laser	Filter	Purpose
CD14	MΦP9	BUV395	355	387/11	monocytes
viability	Zombie UV	Zombie UV	355	447/60	viability
CD16	3G8	BUV496	355	525/50	monocytes
HLADR	G46-6	BUV661	355	670/30	DCs
CD56	NCAM16.2	BUV737	355	710LP	NKs
CD38	HIT2	BV421	405	420/10	activation
CD20	L27	BV450	405	460/22	B cells
CD4	SK3	BV510	405	525/50	CD4
CD194/CCR4	L291H4	BV605	405	615/24	Th subset
CD8	RPA-T8	BV650	405	670/30	CD8
CD25	2A3	BV711	405	720/60	Treg
CD196/CCR6	G034	BV785	405	750LP	Th subset
CD3	UCHT1	AF488	488	525/35	T cells
CD45RA	HI100	PerCP-Cy5.5	488	692/80	naïve/memory
CD183/CXCR3	1C6	PE	561	577/15	Th subset
CD197/CCR7	150503	PE-CF594	561	615/24	naïve/memory
CD11c	Bly6	PE-Cy5	561	670/30	DCs
CD185/CXCR5	RF8B2	PE-Cy7	561	750LP	Th subset
CCR10	314305	APC	640	670/30	Th subset
CD123	32703	AF700	640	720/60	pDCs
CD127	RDR5	APC-eF780	640	775/50	Treg

Fig. 1 ZE5 Cell Analyzer 21-color panel for quantifying major and minor cell types in human peripheral blood.

Visit bio-rad.com/ze5 for more information.

Materials and Methods

To design a panel with this complexity, the Fluorophore Selector in Everest™ Software along with other fluorescence viewers were used to refine fluorescent marker selection. Antibodies colocalized to the same cell (e.g. CD3 and CD4) were given additional consideration when they were balanced for antigen density to dye brightness. All antibodies were titrated and tested on four normal human peripheral blood samples.

After initial photo multiplier tube (PMT) standardization, detector voltages were fine tuned to minimize spillover while keeping CVs to lowest values. This approach carefully maintained the experiment populations within usable dynamic range boundaries determined by 8-peak bead references for each parameter. The highest compensation value was just over 70%, with most being under 10%. Digital plot spreading was minimal after compensation. Fluorescence-minus-one (FMO) controls were included for most fluorophores during the experimental design. Later, some FMO controls were excluded if they were not required for gating.

During acquisition on the ZE5 instrument, the 96-well plate input was used exclusively with 4ºC temperature control enabled because the cells were unfixed. The instrument was set to agitate for 5 seconds per sample to maintain cell suspension. With an approximate event rate of 10,000 cells per second and a cell concentration of $1x10^6$ cells / 200 µL in each well, gate limits were set to collect 100-200K white blood cells. Unused sample was returned to the well. Everest Software was used for set up and acquisition, at which point the data files were exported as .fcs to FlowJo v10.2 for compensation, analysis and presentation.

Gating Strategy

Normal human peripheral blood mononuclear cells (PBMCs) were plotted on a forward scatter versus side scatter pulse height density plot with a polygon region placed around the white blood cell (WBC) population to exclude red cell debris (Figure 2). Following doublet discrimination (Figure 3), the live singlet cells were isolated using the Zombie UV Viability Dye (Figure 4). Major T cell subsets, regulatory T cells, T helpers, and myeloid cells were gated from the live singlet cells for further analysis.

Live singlet cells were separated into T cell, myeloid cell, and B cell populations (Figures 5 and 17) using a plot of CD3 versus CD20. For basic T cell analysis, $CD4^+$ T cells (Figures 6 and 8) were gated to isolate central memory T cells, naïve T cells, and effector memory T cells using a plot of CD45RA versus CD197 (Figures 7 and 11). For regulatory T cell (Treg) analysis, $CD4^+$ T cells were plotted against CD25 versus CD127, with Tregs defined as $CD25^+$ and $CD127^{lo/-}$. Activated T regs were detected using CD38 versus Human Leukocyte Antigen D Related (HLADR) (Figure 10). Further T helper cell (Th) subset analysis was performed by gating $CD45RA^-$ memory $CD4^+$ T cells on CCR10 versus CD185 (Figure 12) to discriminate T follicular helper (Tfh) cells distinctly from other Th subsets. These non-Tfh Th cells were then identified as T helper type 1 (Th1), T helper type 2 (Th2), T helper type 9 (Th9), T helper type 17 (Th17), T helper type 22 (Th22), and T helper GM-CSF-secreting (ThG) cells using CD196 versus CD194 (Figure 13) and CD183 versus CCR10 (Figures 15 and 16).

For myeloid sub-population analysis, $CD20^-$ and $CD3^-$ cells (Figure 17) were gated on CD14 versus HLADR to isolate monocytes and dendritic cells (Figure 18). Using HLADR versus CD16, monocytes were separated into classical, non-classical and myeloid derived suppressor cells (MDSC) (Figure 19). Dendritic cells were separated into plasmacytoid dendritic cells (pDCs) and monocytic dendritic cells (mDCs) using CD123 versus CD11c (Figure 20). Natural killer (NK) cells were isolated from the $CD14^-$/$HLADR^-$ population using CD16 versus CD56 (Figure 21). Finally, $CD20^+$ cells were gated to identify activated B cells using CD38 versus HLADR (Figure 22).

Validated on Normal Human Cryopreserved PBMCs $1x10^6$ Per Test

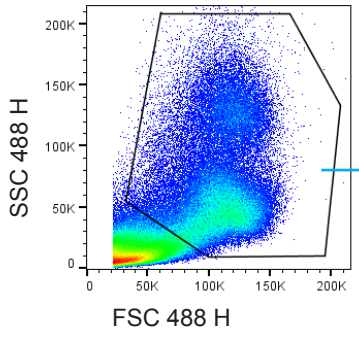

Fig. 2 PBMC plotted on a FSC vs. SSC density plot with a region drawn around the WBC population.

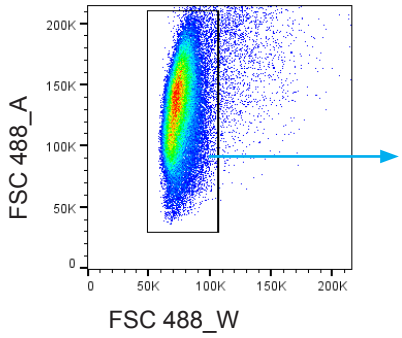

Fig. 3 Gated WBC population with a region drawn to include single cells and exclude doublets.

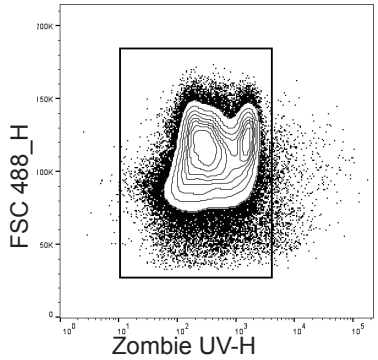

Fig. 4 Gated single cells with a region drawn to include live cells and exclude dead cells.

T Cell Basic Analysis Gated on Live Single Cells

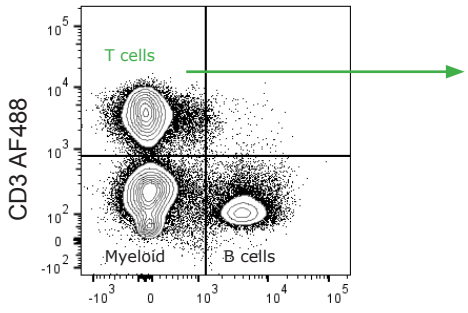

Fig. 5 Gated single, live WBCs with regions identifying T cells, B cells, and Myeloid cells.

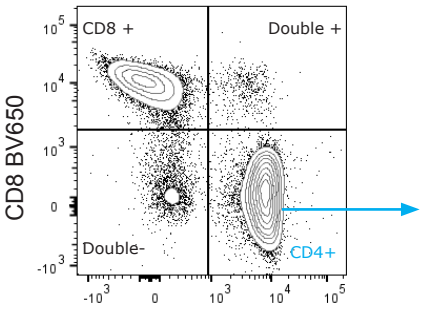

Fig. 6 Gated T cells identifying CD8 and CD4 populations.

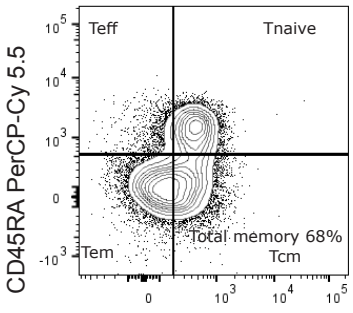

Fig. 7 Gated CD4+ T cells identifying Teff, Tnaive, Tem, and Tcm cells.

T Regulatory Cell Analysis

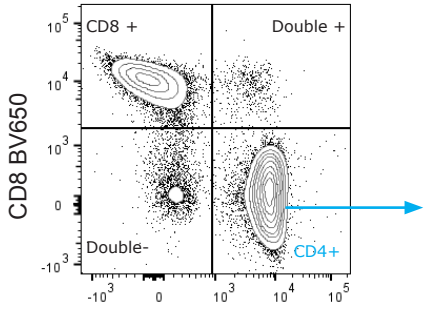

Fig. 8 Gated T cells identifying CD8 and CD4 populations.

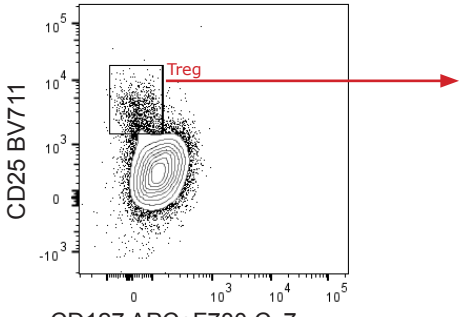

Fig. 9 Gated CD4+ cells identifying T reg cells.

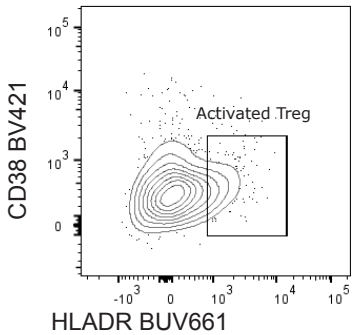

Fig. 10 Gated Treg cells identifying activated Treg cells.

T Helper Cell Subset Analysis

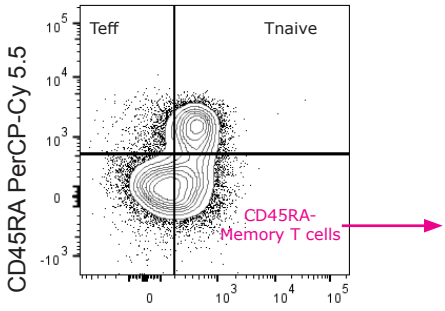

Fig. 11 Gated CD4+ T cells identifying CD45RA- memory T cells.

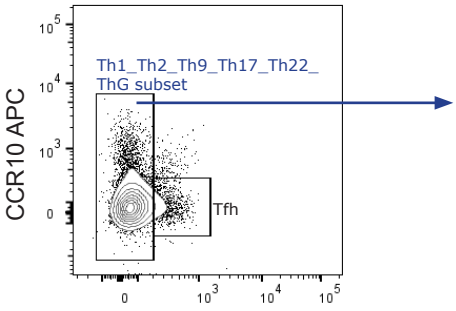

Fig. 12 Gated CD45RA- memory T cells identifying Tfh cells and a subset containing Th1, Th2, Th9, Th17, Th22, and ThG cells.

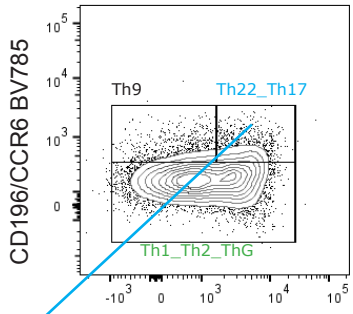

Fig. 13 Gated subset from Fig. 12 further analyzed to isolate Th9 cells and subsets containing Th22_Th17 and Th1_Th2_ThG cells.

	Count	% Total	% CD4
WBCs	71779	100.0%	----
CD4	16950	23.6%	100.00%
Memory	11300	15.7%	66.7%
Th2	5296	7.4%	31.2%
Th1	2438	3.4%	14.4%
Treg	1136	1.6%	6.7%
Th9	784	1.1%	4.6%
Tfh	949	1.3%	5.6%
Th17	649	0.9%	3.8%
Thg	546	0.8%	3.2%
Th22	177	0.2%	1.0%

Fig. 14 White blood cell quantification.

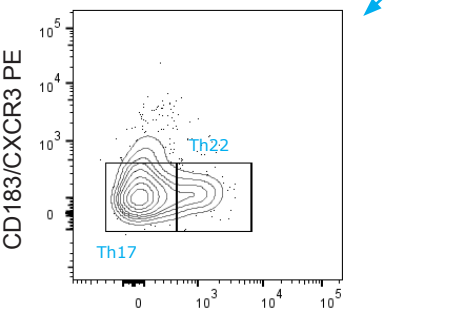

Fig. 15 Gated Th22_Th17 subset further analyzed to show distinct populations.

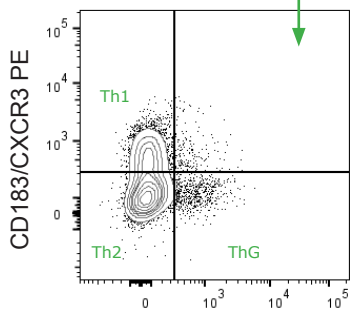

Fig. 16 Gated Th1_Th2_ThG subset further analyzed to show distinct populations.

Myeloid Analysis

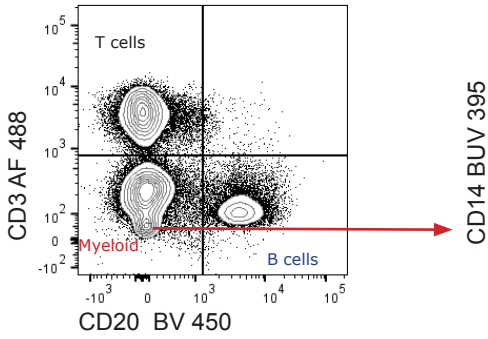

Fig. 17 Gated single, live WBCs with regions identifying T cells, B cells and Myeloid cells.

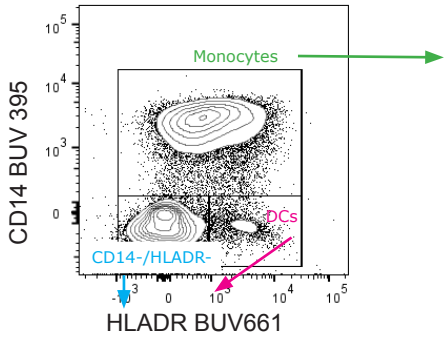

Fig. 18 Gated Myeloid cells gated to identify Monocytes, Dendritic cells and CD14-/HLADR- myeloid cells.

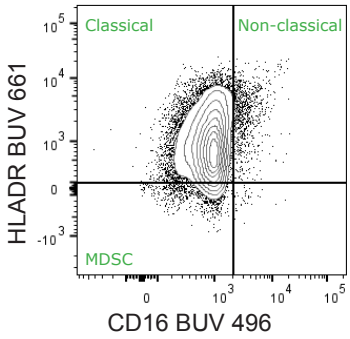

Fig. 19 Gated Monocytes identified as Classical, Non-classical and MDSC cells.

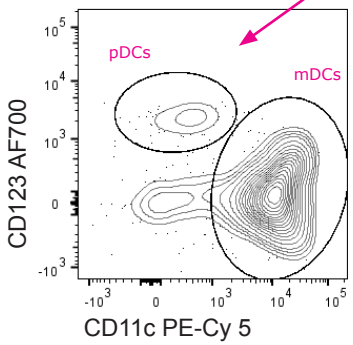

Fig. 20 Gated dendritic cells further analyzed to show pDCs and mDCs.

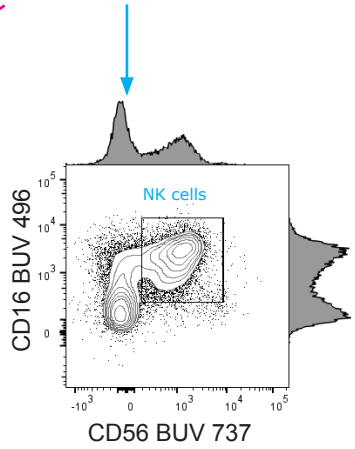

Fig. 21 Gated CD14-/HLADR- cells further analyzed to identify NK cells expressing CD16.

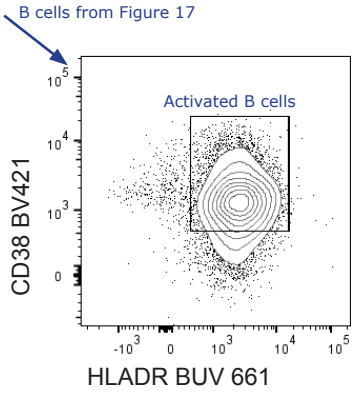

Fig. 22 Gated B cells further analyzed to show activated B cells.

Results

Human peripheral blood samples were stained, and data were acquired on the ZE5 Cell Analyzer. The resulting data were analyzed using FlowJo v10.2 Data Analysis Software. Figures 2–22 show the gating strategy used to identify the T cell subset populations of interest for the study. This information will be used to quickly and comprehensively immunophenotype patient samples from clinical trials of novel drugs aimed at enhancing the therapeutic benefit of allo-HSCT while minimizing GVHD.

References

1. H. T. Maecker, J. P. McCoy, R. Nussenblatt, Standardizing immunophenotyping for the Human Immunology Project. Nat Rev Immunol 12, 191–200 (2012).
2. G. Wingender, M. Kronenberg, OMIP-030: Characterization of human T cell subsets via surface markers. Cytometry A 87, 1067–1069 (2015).
3. Y. D. Mahnke, M. H. Beddall, M. Roederer, OMIP-015: Human regulatory and activated T-cells without intracellular staining. Cytometry A 83, 179–181 (2013).
4. J. Jiang, W. Guo, X. Liang, Phenotypes, accumulation, and functions of myeloid-derived suppressor cells and associated treatment strategies in cancer patients. Hum Immunol 75, 1128–1137 (2014).
5. G. Moncunill, H. Han, C. Dobano, M. J. McElrath, S. C. De Rosa, OMIP-024: Pan-leukocyte immunophenotypic characterization of PBMC subsets in human samples. Cytometry A 85, 995–998 (2014).