Introduction
With the advent of flow cytometry into the clinical arena as a diagnostic tool, high-dimensional multicolor analysis has become increasingly recognized as invaluable for the diagnosis and classification of leukemia. In the past, clinicians were restricted by the number of channels available to construct multicolor panels of leukocyte markers. As a result, defining populations of normal and aberrant blood cells often required splitting valuable samples into multiple tubes to achieve the needed resolution.

The ZE5™ Cell Analyzer, when configured with five lasers and 27 channels of fluorescence detection, allows all the markers necessary to classify chronic/acute lymphoproliferative leukemia disorders (CLL/ALL) to be mixed into a single tube. This panel combines 19 surface membrane markers of both cluster of differentiation and Ig species with TCR expression to successfully dissect aberrant leukocyte populations present in a patient with chronic B-cell lymphocytic leukemia (B-CLL).

Materials and Methods
Antibodies
CD3 BUV496, CD4 BUV661, CD19 BUV737, TCRgd BUV395, CD8 BV711, CD45 VioGreen, CD56 BV785, Kappa Light Chain BV421, CD7-BV650, HLADR VioBlue, CD117 BV605, CD33 FITC, CD34 PE-Vio770, CD64 PE-CF594, CD11b PE, CD14 PE-Cy5, CD20 APC-Vio770, CD38 APC-R700, Lambda Light Chain APC

Blocking Buffer
PBS + 50 µg/ml mouse, rat, rabbit IgG + 1% BSA + 1% glucose + 20 mM EDTA + 0.1% azide

Washing Buffer
PBS + 1% BSA + 1% glucose + 20 mM EDTA + 0.1% azide

Beads
AbC Total Antibody Compensation Bead Kit (Thermo Fisher Scientific)

Media
RPMI 1640 or DMEM + 10% FBS

Cell preparation
Normal PBMCs and B-CLL patient PBMCs were thawed and stained using standard procedures. Thawed samples were placed into 5 ml of prewarmed cell media in 50 ml polypropylene centrifuge tubes. Cells were counted and assessed for viability using the TC20™ Automated Cell Counter.
A 19-Color Multiparameter White Blood Cell Panel Designed for the Immunophenotyping of Normal and Malignant Leukocytes by Flow Cytometry

(a prepared cocktail containing all 19 antibodies. Antibody concentrations used were based on manufacturer recommendations. Samples were incubated on ice, protected from light, for 45 min to an hour. Cells were washed twice and resuspended into 1 ml of ice cold wash buffer. They were stored on ice and protected from light until acquisition on the ZE5 Cell Analyzer. Gating was carried out using FlowJo Software (Tree Star, Inc.). Stained compensation beads were used as single-stained controls (data not shown).

Table 1. Nineteen-Antibody White Blood Cell Panel

<table>
<thead>
<tr>
<th>Marker</th>
<th>Cell Distribution</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>Mature T-cells and thymocytes</td>
<td>T-cell activation signaling and regulation of TCR expression</td>
</tr>
<tr>
<td>CD4</td>
<td>T-helper cells, regulatory T-cells, monocytes, and macrophages</td>
<td>T-cell activation, thymic differentiation, and receptor for HIV</td>
</tr>
<tr>
<td>CD19</td>
<td>B-cells (but not plasma cells) and follicular dendritic cells</td>
<td>Regulator of B-cell development, activation, and differentiation</td>
</tr>
<tr>
<td>TCRgd</td>
<td>T subset</td>
<td>Antigen recognition</td>
</tr>
<tr>
<td>CD8</td>
<td>Thymocyte subsets and cytotoxic T-cells</td>
<td>Coreceptor for MHC class I molecules</td>
</tr>
<tr>
<td>CD45</td>
<td>Hematopoietic cells (not erythrocytes and platelets)</td>
<td>Critical for B- and T-cell receptor–mediated activation. Also required for thymic selection</td>
</tr>
<tr>
<td>CD56</td>
<td>NK, T subset, neurons, some large granular lymphocyte leukemias, myeloid leukemias</td>
<td>Adhesion</td>
</tr>
<tr>
<td>Kappa light chain</td>
<td>Immunoglobulin light chain</td>
<td>Antibodies are produced by B lymphocytes, each expressing only one class of light chain. Once set, light chain class remains fixed for the life of the B lymphocyte. In a healthy individual, the total kappa to lambda ratio is roughly 3:1</td>
</tr>
<tr>
<td>CD7</td>
<td>Thymocytes, T-cells, natural killer cells, and pluripotent hematopoietic stem cells</td>
<td>T-cell costimulation. Interacts with SECTM1</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>MHC class II cell surface receptor</td>
<td>Presentation of peptides to CD4+ T lymphocytes</td>
</tr>
<tr>
<td>CD117</td>
<td>Hematopoietic stem cells and progenitors</td>
<td>Receptor for stem cell factor or c-kit ligand</td>
</tr>
<tr>
<td>CD33</td>
<td>Monocytes, granulocytes, mast cells, and myeloid progenitors</td>
<td>Lectin activity and adhesion. A receptor that inhibits the proliferation of normal and leukemic myeloid cells</td>
</tr>
<tr>
<td>CD34</td>
<td>Hematopoietic stem cells and progenitors and capillary endothelial cells</td>
<td>Cell adhesion (via L-selectin). Possible role in early hematopoiesis through mediation of the attachment of stem cells to the bone marrow extracellular matrix or directly to stromal cells</td>
</tr>
<tr>
<td>CD64</td>
<td>IgG Fc receptor monocytes and macrophages</td>
<td>Binds to the Fc region of IgG with high affinity</td>
</tr>
<tr>
<td>CD11b</td>
<td>Granulocytes, monocytes, natural killer cells, T- and B-cells, and dendritic cells</td>
<td>Integrin alpha-M</td>
</tr>
<tr>
<td>CD14</td>
<td>Monocytes, macrophages (myelomonocytic cells), Langerhans cells, and granulocytes</td>
<td>Receptor of complex of LPS and LBP</td>
</tr>
<tr>
<td>CD20</td>
<td>B-lymphocyte surface antigen B1 and T- and B-cell subsets</td>
<td>B-cell activation and proliferation</td>
</tr>
<tr>
<td>CD38</td>
<td>Variable expression levels on most hematopoietic and some nonhematopoietic cells. High levels on plasma cells, early T- and B-cells, activated T-cells and germinal center B-cells</td>
<td>Regulator of cell activation, proliferation, and adhesion</td>
</tr>
<tr>
<td>Lambda light chain</td>
<td>B-cells</td>
<td>Antibodies are produced by B lymphocytes, each expressing only one class of light chain. Once set, light chain class remains fixed for the life of the B lymphocyte. In a healthy individual, the total kappa to lambda ratio is roughly 3:1</td>
</tr>
</tbody>
</table>

Results

PBMCs from a healthy normal donor were stained using a 19-color panel and run on the ZE5 Cell Analyzer. Results demonstrated that all of the common markers were easily identifiable with no need for FMO controls.

PBMCs from a chronic lymphocytic leukemia patient were then stained with the same 19-color panel. Though the majority of cell populations remained unchanged in this panel, they could be useful for identification of other leukemia subtypes in additional samples. The panel easily identified the patient sample as a chronic B-cell leukemia based on:

1. The expression of CD19 along with an aberrant expression of surface kappa light chain and lambda light chain, indicating a monoclonal B-cell population (100% expression of only one type light chain).
2. Lowered expression of CD38 on CD20-expressing cells.
**Normal White Blood Cell Immunophenotype**

**General Subset Identification**

Fig. 1. Normal peripheral blood subset analysis shows typical distribution of all common markers. These panels show NK-cell, B-cell, and T-cell sets, along with T-cell subsets of T-helper (Th) and cytotoxic T (Tctl) cells.

**B-Cell Subset Identification**

Fig. 2. Normal peripheral blood subset analysis of B-cell subset. These panels show the distribution of surface membrane kappa and lambda light chains, CD20 with CD38 co-expression, and CD19 with CD34 co-expression.

**Myeloid-Cell Subset Identification**

Fig. 3. Normal peripheral blood subset analysis of myeloid-cell subset. These panels show the expected distribution of CD14, CD11b, CD64, and CD33 antibodies.

**Diseased White Blood Cell Immunophenotype**

Fig. 4. Aberrant expression of CD19 and surface membrane Ig light chain lambda and reduced expression of CD38 on CD20-positive B-cells are indicative of chronic B-cell lymphocytic leukemia.

Overlay of total population of normal PBMCs with B-CLL sample, showing almost complete dominance of lymphocyte population based on CD45 and side scatter.

Same total population now overlaid with CD3 and CD19, showing nearly complete dominance of CD19-positive B-cell population in the B-CLL sample.

Overlay of B-cell gated populations from normal PBMCs and B-CLL sample. Normal PBMC B-cells will display a ratio of surface membrane Ig light chain of kappa to lambda. The B-CLL sample shows a single chain expression, indicating a monoclonal population of B-cells.

Overlay of B-cell gated populations from normal PBMCs and B-CLL sample. Normal PBMC B-cells will have a relatively high expression of CD20 and CD38. The same gated population from a B-CLL patient shows a diminished CD38 expression.
Diseased White Blood Cell Immunophenotype

**Normal Blood**

**B-Cells** 11.0%

**T-Cells** 70.3%

**CD19 BUV737**

**CD20 APC-Vio770**

**CD3 BUV496**

**CD20 APC-Vio770**

**B-Cells** 89.6%

**CD34 PE-Vio770**

**Lambda LC APC**

**Kappa LC BV421**

**CD19 BUV737**

**CD38 APC-R700**

**CD34 PE-Vio770**

**Kappa light chain BV421**

**Fig. 5.** Diseased peripheral blood subset analysis comparing the profile of normal blood to that of a patient with chronic B-cell lymphocytic leukemia. Note the loss of CD38 expression along with an increase in overall B-cell population in the disease state.

**Conclusions**

- Large-panel multiparameter design and analysis is straightforward using the ZE5 Cell Analyzer’s 27 fluorescent detection channels
- A 19-color panel, designed from existing literature, could allow classification of CLL/ALL disorders utilizing a single tube with the combined antibodies on the ZE5 Cell Analyzer

Visit bio-rad.com/ZE5 for more information.

Data were originally presented as Poster #B86 at the 2017 32nd Congress of the International Society for Advancement of Cytometry (CYTO) meeting (Boston, MA).

AbC is a trademark of Thermo Fisher Scientific. Cy is a trademark of GE Healthcare. FlowJo is a trademark of FlowJo, LLC. Vio, VioBlue, and VioGreen are trademarks of Miltenyi Biotec GmbH.

For Research Use Only. Not for use in diagnostic procedures.