Highly Multiplexed Immune Cell Phenotyping with ChipCytometry™

Highlights

- ChipCytometry is a cyclic immunofluorescence imaging technique that can be used for higher multiplex analysis than flow cytometry and is compatible with both suspension cells and tissue samples.
- Here, 74 biomarkers were detected in a single PMBC sample using ChipCytometry.
- High plex immune analysis allows deep phenotyping, detection of rare cell types, and monitoring of cellular processes including activation markers and apoptosis indicators.

Introduction

Immune cell phenotyping is an important quantitative tool for immunology and immuno-oncology research, clinical trials, and therapeutic monitoring. Cell phenotyping classifies cells based on the patterns of surface markers they express and enables the identification and quantification of T cells, B cells, natural killer cells, dendritic cells, and some subsets within those categories[1]. The surface markers for cell identification are tagged with fluorescently labeled antibodies and detected via flow cytometry or immunofluorescence microscopy.

Deep cell phenotyping involves further classification based on additional differentiators between cell subtypes. Moving beyond basic cell phenotyping requires detection of many biomarkers on the same cells. Highly multiplexed analysis using flow cytometry or immunohistochemistry is technically challenging, as spectral space is limited and there is a low ceiling for the number of unique fluorescent signatures that can be detected at once.

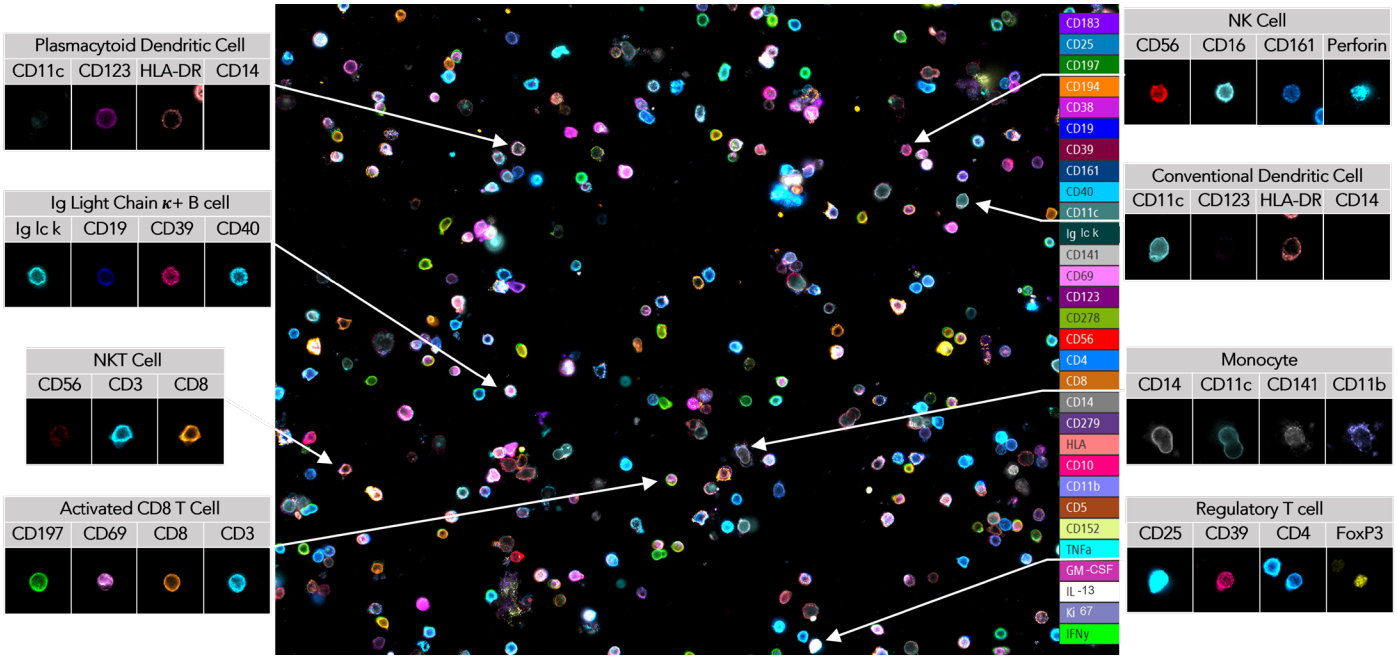

Figure 1. Image overlays demonstrate examples of cell phenotyping, with individual staining patterns of different cell types demonstrated in left and right insets. Select biomarkers are colored in overlay as indicated in legend.

ChipCytometry is a cyclic multiplexed immunofluorescence technique that enables deep cell phenotyping via iterative rounds of staining with fluorescent antibodies, imaging with high-resolution microscopy, and photo-inactivation to remove fluorescent signal. High dynamic range images created by combining multiple exposures of every fluorophore allow for biomarker detection and quantification over six orders of magnitude. After data collection, images are analyzed with hierarchical gating to classify key immune cell types. The CellScape platform automates the ChipCytometry staining, imaging, and signal removal cycles and can analyze up to four cell suspension or tissue samples simultaneously.

Compared to flow cytometry, ChipCytometry provides several key benefits for immune cell phenotyping of cell suspensions: streamlined assay validation, small sample volume, and the ability to store and re-interrogate a sample later. The ChipCytometry workflow is compatible with any fluorescently labeled antibodies, including those traditionally used for flow cytometry or other applications. Here, ChipCytometry was used to detect, quantify, and analyze 74 clinically relevant biomarkers from a PBMC sample. This highly multiplexed dataset enabled deep immune profiling and cell phenotyping, including analysis of B cell subtypes, T cell subtypes, monocytes, dendritic cells, apoptosis indicators, and activation markers, all from a single sample and without assay optimization.

Methods

Blood from one healthy human donor was collected into a sodium citrate cell preparation tube, and PBMCs were isolated from the mononuclear layer[2]. Half of the sample was stimulated with PMA/Ionomycin[3]. Stimulated and unstimulated PBMCs were mixed in a 1:1 ratio and loaded into ChipCytometry microfluidic cell chips, following manufacturer's instructions (Canopy Biosciences). Prestaining for 5 markers was performed, and then the sample was fixed with fixation buffer before continuing with cyclic staining, imaging, and photo-inactivation using 93 fluorescently labeled antibodies. No optimization of staining order or antibody titration was performed, and antibody clones were selected based on their performance and suitability in single-plex assays only. After TL-based segmentation, fluorescence values for each marker and each cell were calculated. Bi-variant gating was then performed to quantify cells displaying each phenotype of interest.

Table 1. Gating strategy for basic immune phenotyping.

Cell Type	Biomarker Expression Pattern
T cells	CD45+ CD3+ CD14- CD56-
Dendritic cells	CD45+ CD3- CD56- CD14- HLADR+
B cells	CD45+ CD14- CD3- CD56- CD19+ CD20+
Monocytes	CD45+ CD14+
NK cells	CD45+ CD14- CD3- CD56+
NK T cells	CD45+ CD14- CD3+ CD56+
Plasma cells	CD45+ CD14- CD3- CD56- CD38+ CD138+

Results

High Plex Staining

Of the 93 fluorescent antibodies used in staining, 74 markers (80%) showed a specific signal after cycles of labeling, imaging, and photo-inactivation with no assay optimization. One undetected biomarker (active caspase-3) was expected to be negative in a typical PBMC population. Five additional biomarkers showed a positive but non-specific signal.

Cell Phenotyping

Using the highly multiplexed dataset, immune cells were classified based on biomarker expression profiles. Figure 1 is an image overlay demonstrating the microscopy output of highly multiplexed ChipCytometry, with select examples of identified cell types highlighted. Figures 2-6 illustrate the quantitative outputs from deep immune cell phenotyping.

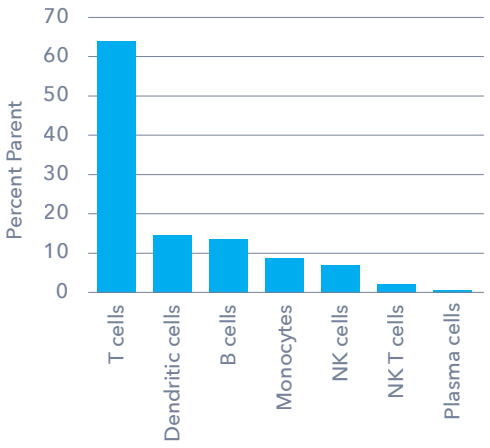

Figure 2. Analysis of main immune cell populations. Parent reference was all CD45+ immune cells.

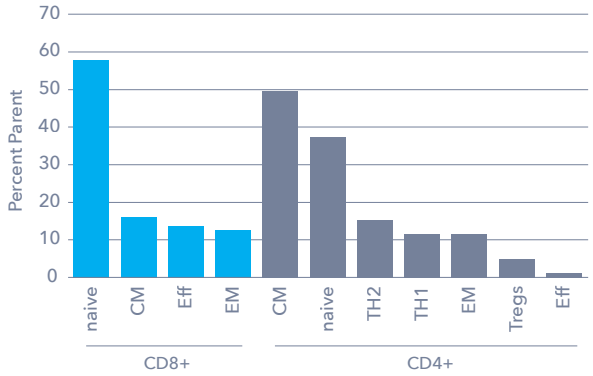

Figure 3. Phenotyping of CD4 and CD8 T cells. Parent reference for CD8+ T cell subpopulations was CD8+ cytotoxic T cells (cyan), and parent reference for CD4+ T cell subpopulations was CD4+ T helper cells (gray).

Of the isolated PBMCs, 83% were CD45+ immune cells. The largest proportion of the analyzed immune cells were CD3+ T cells, at 64% (Figure 2). A majority of CD8+ T cells were naive T cells at 60%, whereas CD4+ T cells mainly comprised of central memory CD4 at 50% (Figure 3). Use of such a highly multiplexed assay enabled a more detailed analysis of T and B cell markers to perform profound phenotyping. Exhaustion and expression of immune checkpoint receptors like CD274/CD279 (PD-L1/PD1) as well as transcription factors were analyzed in the T and B cell populations (Figures 4 and 5).

In addition to analyses of T and B cells, monocytes and dendritic cells were examined in detail by the analysis of activation markers (CD86), interleukins, and surface markers like CD11c and CD123 (Figure 6).

Biochemical Process Monitoring

The highly multiplexed ChipCytometry staining workflow also enabled analysis of biochemical processes in the PBMC sample. The absence of active Caspase-3 staining supports that there were no apoptotic cells present. 10% of the leukocytes were positive for the proliferation marker Ki-67 and 8% showed expression of the mitosis marker p-Histone H3 (Ser10), indicating that a proportion of the analyzed immune cells is proliferating or shows mitotic activity (Figure 7).

The immune cell response to chemical stimulation was measured by activation markers like cytokines IFNγ and TNFα or surface markers like CD69. The PBMC sample, which was a 1:1 mix of non-activated and activated cells, showed significant induction of several lymphocyte activation markers (Figure 7).

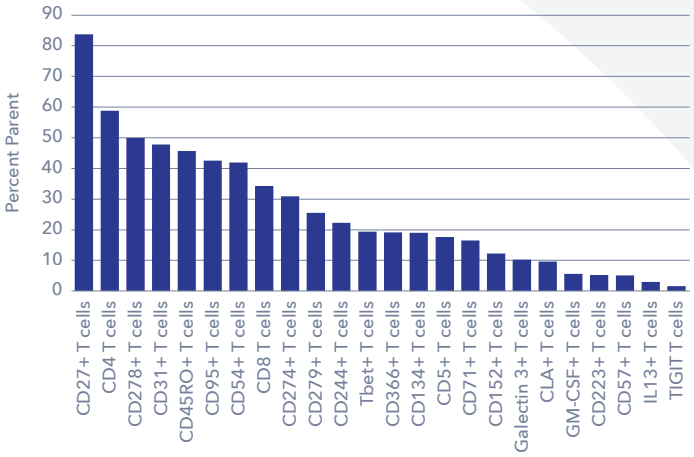

Figure 4. Detailed analysis of T cell subpopulations. Parent reference was all CD3+ T cells.

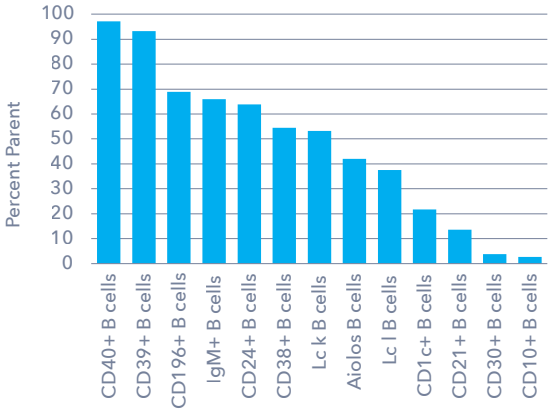

Figure 5. Profound analysis of B cell subpopulations. Parent reference was all B cells.

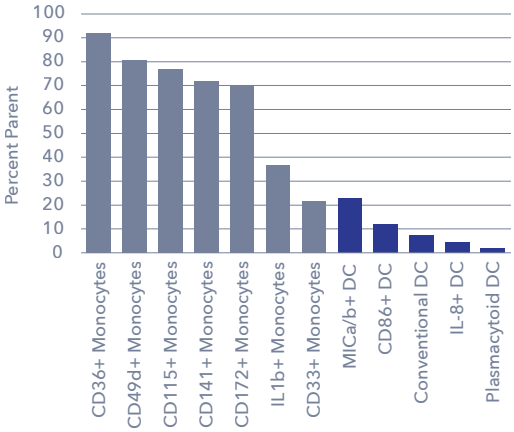

Figure 6. In depth analysis of monocytes and dendritic cells (DC). Parent references were all monocytes (gray) or all DC (indigo).

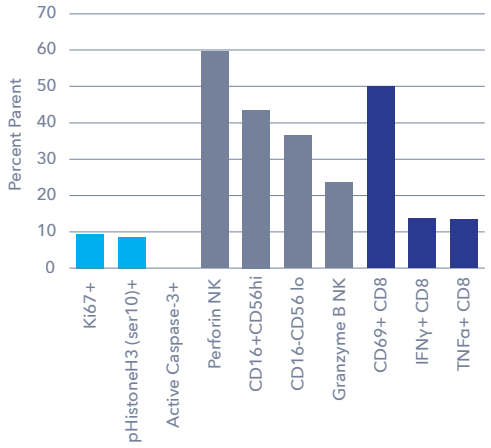

Figure 7. Analysis of markers involved in proliferation, regulation, apoptosis, and activation. Parent reference for proliferation and apoptosis markers was all CD45+ immune cells (cyan). Parent reference for NK cell subpopulations was all NK cells (gray). Parent reference for CD8+ cells was all CD8+ cytotoxic T cells (indigo).

Summary

Using ChipCytometry for cyclic immunofluorescence imaging of a PBMC sample, 74 unique biomarkers were detected, which is far more than can be detected by standard flow cytometry or other multiplex immunofluorescence methods. A successful specific staining rate of 80% without any optimization demonstrated the robust nature of ChipCytometry using commercially available antibodies. Further, this high level of multiplex is not the upper limit that can be achieved with ChipCytometry. Optimization of high plex assays should allow additional markers to be detected on a single sample. Parameters to further increase plex include adjusting staining order so that sensitive epitopes are detected first, optimizing antibody titers, or increasing staining times.

The high level of multiplex demonstrated here enabled deep phenotyping and cell subtyping, detection of rare cells, and monitoring of cellular processes including apoptosis indicators and activation markers. Deep phenotyping supports researchers and clinicians in understanding biology with extraordinary depth.

References

1. Pitoiset, F., Cassard, L., El Soufi, K., Boselli, L., Grivel, J., Roux, A., Klatzmann, D.,Chaput, N., & Rosenzwajg, M. (2018). Deep phenotyping of immune cell populations by optimized and standardized flow cytometry analyses. Cytometry part A, 93(8), 793-802.
2. Puleo, A., Carroll, C., Maecker, H., & Gupta, R. (2017). Isolation of PBMCs Using Vacutainer® Cellular Preparation Tubes (CPTTM). Bio-protocol, 7(2), e2103-e2103.
3. Ai, W., Li, H., Song, N., Li, L., & Chen, H. (2013). Optimal method to stimulate cytokine production and its use in immunotoxicity assessment. International journal of environmental research and public health, 10(9), 3834-3842.

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