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OMIP-023: 10-Color, 13 Antibody Panel for In-depth Phenotyping of Human Peripheral Blood Leukocytes

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Purpose and Appropriate Sample Types

This panel was developed and optimized to determine the phenotype and activation of 15 different leukocyte subtypes in one run. Leukocytes are identified by expression of CD45 (leu-1) pan leukocyte antigen. Neutrophil (CD16), monocyte (CD14), T- (CD3), B-lymphocyte (CD19), and NK-cell (CD16 and CD56) markers are employed. Special gating strategy is used for subtyping of granulocytes (e.g., eosinophils, neutrophils, basophils). For further T-cell phenotyping, CD4/CD8 markers are used for differentiation of four T-cell subtypes, CD25/CD127 for regulatory T cell identification and CD3/CD16/CD56 for NKT-cells, additionally. Special gating strategies have been developed for B-cell, NK-cell, and monocyte subtyping. For detection and analysis of activation also further activation markers (HLA-DR, CD38, CD25, CD127, and CD69) are analyzed. This panel has been established for analysis of human RBC-lysed EDTA-treated whole blood samples and for cord blood (Table 1). Since the starting material is fresh EDTA-treated blood, dead cells are probably not an issue. Thus to save one channel for specific staining the vitality staining was not used in the panel.

Background

Determination of the phenotype of the major leukocyte subtypes and their state of activation is standard in clinical diagnosis and clinical research. But, up to date these antibody panels rely on multiple tubes with about five antibodies per tube to obtain the complete blood differential. Polychromatic flow cytometry, an all-in-one-tube measurement, shows clear advantage over multiple tube oligochromatic panels with reduced time for measurement and smaller sample volume needed. The latter is of special importance when only small sample volumes are available such as biomaterials taken from small children (1), liquor (2), or hypoplastic bone marrow (3). In addition, also the unequivocal identification of specific cell subtypes like Tregs or NKT-cells requires multiple phenotypic markers (4,5).

Therefore, our goal was to combine typical markers for leukocyte phenotyping used in routine commercial clinical assays such as Simulset (BD-Immunocytometry System, Heidelberg Germany) and develop a comprehensive panel for a ten-color three-laser flow cytometer. We combined the most relevant markers for the clear definition of major and minor subsets (backbone markers) and additional markers for further characterization such as states of activation.

To this end, we defined the leukocyte subsets that needed to be identified and the most commonly used lineage and activation markers. We tested a great variety of commercially available monoclonal antibodies and fluorochrome conjugates for their clear phenotypic identification of leukocyte subtypes. Optimal concentrations were tested by titration (Supporting Information Figs. 1A and 1B and 2A–2C). Also the optimal combination(s) for an unequivocal cell phenotyping and the clear detection of activation marker expression was tested (Supporting Information Table 4). We

Table 1. Summary table for the application of OMIP-023

Purpose	In-depth phenotyping of the main leukocyte subtypes in one run
Species	Human
Cell types	Fresh EDTA-treated peripheral blood, RBC-lysed and Fresh EDTA-treated Cord-blood
Cross reference	None

ended up with a ten-color 13-antibody panel that also relies on the multiple usage of one fluorescence channel for three different cell types (Table 2, Supporting Information Figs. 4A and 4B). Three surface antigens (CD8, CD14, and CD19) are measured in the FITC channel. They are expressed on: T-cells and NK-subsets (CD8), B-cells (CD19), monocytes, and dimly on granulocytes (CD14). Monocytes and granulocytes can therefore be clearly identified and separated from each other based on light scatter and co-expression of other lineage markers (details in Supporting Information). CD16 and CD56 are detected by PE-Cy7 label neutrophils (CD16), NK-, and NK-T cells (CD16/CD56) as well as monocyte subtypes (CD16). All four subsets are clearly differentiated from each other in a SSC-CD45-PB dot-plot.

We performed detailed analysis of fluorochromes and antibodies from different providers in order to compare and optimize reagent combinations for the characteristics of our instrument for successful 10-color flow cytometry (FCM). The simultaneous analysis of several different parameters (typically 12–15: two related to light scatter properties of the cells and 10–13 to immunophenotype and activation) contributes to increasing both the specificity and sensitivity of the test. Systematic review of issues related to sampling, preparation, instrument settings, spillover and compensation, specific and unspecific labeling and other reagent performance, and general principles of panel construction was performed. Our data clearly indicate the feasibility of the new OMIP and encourage further evaluating it as an alternative to standard methods. We show in this study that all main players of lymphocytes can be precisely measured using this multiparameter FCM approach. This assay is rapid, and requires a minimum amount of blood. Moreover, this assay is precise with minimal contamination between populations. Most importantly, the 10-color panel is an important tool to study the interactions between different immune cell populations during diseases, and to better understand the role that these cells play in disease diagnosis, prediction, or progression (6,7).

Ten-colors open a broad possibility to stain many lineage and activation markers at the same time in one sample, but the list of important markers is very long. CD45, CD14 staining is necessary for the differentiation of leukocytes (lymphocytes, monocytes, neutrophils, basophils, eosinophils) in combination with the removal of the not leukocyte related debris. CD3, CD4, CD8, CD56, and CD19 are needed for identification of T-, NK-, and B-cells. 10 colors could not cover all the interesting markers. The scale of physiologically important activation and adhesion related markers is very broad: CD25, CD69, HLA-DR, CD54, CD127, CD11a, CD11b, CD18 and this is a non-comprehensive list. Question arises how we can obtain the most detailed information using 10 fluorochromes. One idea is to combine more parameters on the same color analyzing it by the same detector. In this OMIP, we show the use of three parameters on one color. We have selected the CD14, CD19, or CD8 antigens because in principle either none or only one of them is present on the same cell. CD14 is expressed on monocytes (and at low level on neutrophils) but not on lymphocytes. By contrast, CD19 and CD8 are exclusively on B-, or T-, and NK cells. By using the side scatter signal or in combination with CD14 or CD45

Table 2. Summary table for the antibodies of the panel OMIP-023

SPECIFICITY MAB	AB CLONE	FLUOROCHROME	PURPOSE	CELL TYPE
CD45	J.33	PB	Pan-leukocyte antigen	All leukocytes
CD3	SP34-2	V500	T-cells	T-cells
CD8	B9.11	FITC	Cytotoxic T-cells	T-cells
CD4	SK3	APC-H7	T-helper cells	T-cells
CD25	B1.49.9	ECD	IL-2 Receptor α	Treg
CD127	R 34.34	APC-Ax700	IL-7 Receptor α	Treg
CD19	J3–119	FITC	B-cells	B-cells
CD38	LS198.4.3	PE-Cy5.5	Activated T and B-cells	Plasmacells
HLA-DR	Immu-357	APC	MHC-II	B-cells, monocytes
CD16	3G8	PE-Cy7	Fcγ Rec III	Monocytes, NK, neutrophils
CD56	N901(NKH-1)	PE-Cy7	N-Cam	NK, NKT
CD14	RMO52	FITC	LPS co-receptor	Monocytes
CD69	TP1.55.3	PE	Early activation	Neutrophils

Ab, antibody; APC, allophycocyanine; Ax, Alexa; Cy, cyanine; ECD, energy-coupled dye; FITC, fluorescein isothiocyanate; H7, Highlight 750; MAB, monoclonal antibody; NK, natural killer cells; PB, Pacific blue; PE, phycoerythrin; Treg, regulatory T-cells; V500, Horizon V500.

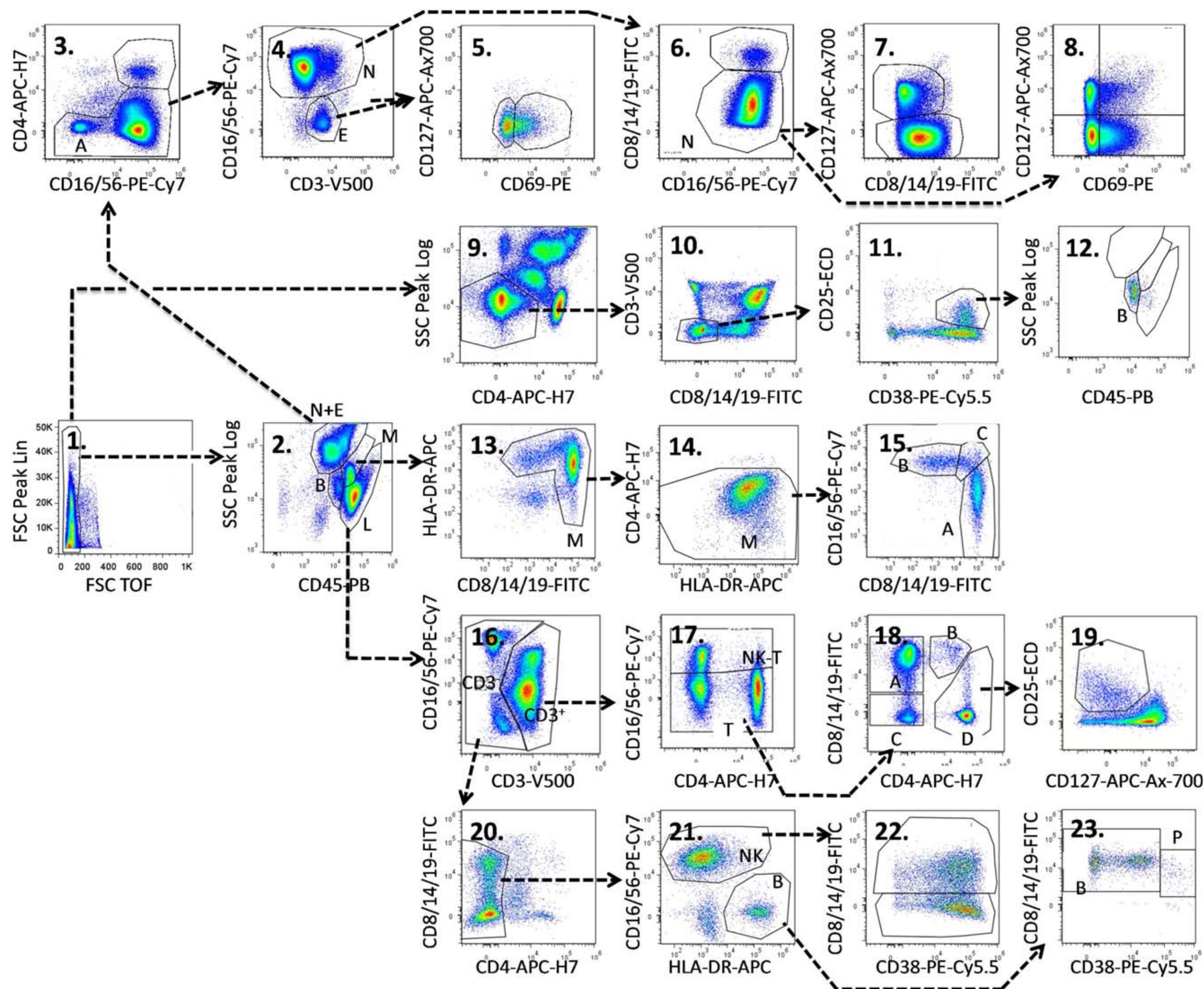

Figure 1. Gating strategy for analyzing the 13 antibody 10-color panel. Erythrocytes of fresh EDTA-treated whole blood samples were lysed (RBC-lysed whole blood). Leukocytes were stained with 13 antibodies and analyzed by 10-color flow cytometry. In the first step, air-bubbles, non-single events (1) and CD45$^-$ events (remaining erythrocytes, thrombocytes, and debris) were excluded (2) from the analysis. Neutrophilic and eosinophilic granulocytes (N+E), basophils (B), monocytes (M), and lymphocytes (L) were differentiated in a SSC-CD45-PB dot-plot (2). Granulocytes were further sub-differentiated into neutrophils (N) and eosinophils (E): CD4$^+$ events were excluded (3), CD16$^-$ E (4) were further differentiated in a CD69$^-$ and CD69$^+$ subset (5). CD8$^+$ events were excluded from the neutrophilic granulocyte gate (6), CD127$^-$ and CD127$^+$ subsets (7) as well as CD69$^-$ and CD69$^+$ subsets were differentiated (8), additionally. Basophils were gated in a CD4-SSC dot-plot (9): gated on SSC^{lo}, CD4$^-$ events (9); CD8/CD3 double negative events (10); were identified as CD38$^+$,CD25$^+$ events (11) and backgated on CD45-SSC (12). From the monocyte (M) gate (2) lymphocytes were excluded in a CD8/19-HLA-DR dot-plot (13), as well as CD4^{hi} events (14). M were finally sub-differentiated in three subpopulations based on their CD16 and CD14 expression (15) with classical/typical monocytes (**A**: CD14^{++}, CD16$^-$), non-classical/atypical monocytes (**B**: CD14$^+$, CD16^{++}) and intermediate monocytes (**C**: CD14^{++}, CD16$^+$). Lymphocytes (L, 2) were differentiated in CD3$^+$ (T-cells and CD16/56$^+$ NKT cells) and CD3$^-$ (B-cells and CD16/56$^+$ NK cells) events (16). The CD3+ events were differentiated in a CD16/56$^+$ NK-T and CD16/56$^-$ T-cell subpopulation (17). T-cells were further subdivided in a CD8$^+$ subset (cytotoxic T-cells, 18A), a CD4$^+$ subset (T-helper cells, 18D) with CD25$^+$/CD127$^-$ Tregs (19), CD4, and CD8 double positive events (18B) as well as double negative events (18C). CD4+ events were excluded from the CD3$^-$ population of plot 16 (20). NK and B-cells were differentiated based on their HLA-DR and CD16/56 expression (21). Two NK subsets were further sub-differentiated by CD8 expression: a NK CD8$^+$ subset and a NK CD8$^-$ subset (22). B-cells were subdivided in plasma cells (CD19$^-$, CD38$^+$) and CD19$^+$ B-cells (23).

it is possible to distinguish monocytes and lymphocytes. For differentiation of CD19 and CD8 cells and NK cells CD3 and CD16/56 markers were used.

The final panel has a very low false-positive rate, a very low limit of detection, high sensitivity, and reproducibility. One of the main goals was to develop cost-effective staining conditions, e.g. especially needed for large cohort studies. Although a concentration of 0.1 µl antibody allows for many CD markers clear discrimination of positive and negative cell populations, 2 µl antibody per 100 µl blood sample were used

to minimize pipetting errors. Increasing the incubation time to 2 h allows the usage of such low concentrations (see Supporting Information Figs. 1 and 5). Using lower concentrations of lineage specific antibodies has an additional advantage, because it results in reduced spillover into the channels where activation markers with low level of expression are measured.

The developed panel is displayed in Figure 1. The gating strategy is based on the principles of: removal of noise signals from air bubbles and disturbing debris, discrimination of doublets (FSC-TOF), and discrimination of $CD45^{-}$ erythrocytes. $CD45^{+}$ cells (all leukocytes) are further differentiated into neutrophilic and eosinophilic granulocytes (SSC^{hi}), monocytes (SSC^{med}), basophilic granulocytes (SSC^{med}), and lymphocytes (SSC^{lo}) with slightly decreased FSC. Apart from the assessment of relative and absolute counts of leukocyte subtypes, information is provided on the amount of antigen expressed per cells and whether the distribution pattern of antigen expression is homogeneous or heterogeneous in a defined subpopulation. Thus, quantitative analysis of antigen expression is essential and is very useful for the identification of different subtypes, for the differentiation of activated leukocytes and the correlation of antigen expression profiles in health and disease.

Similarity to Published OMIPs

This panel can be used for in-depth phenotyping of peripheral blood samples as well as cord blood in one tube. The panel is in a manner similar to OMIP-001, 002, 004, 007, 010, 012, 015, and 018 (3–5,8–12).

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