Multicolor immunophenotyping of human tumor cells using MACSQuant® Analyzer 16

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Introduction

Flow cytometric phenotyping is a powerful tool in a variety of applications, from basic research to diagnostics and clinical monitoring. Especially in the growing field of immunotherapy it is of utmost importance to understand steady-state immunity and the effects of intervening therapies on the immune system. In particular, approaches engaging T cells to attack tumors have shown to be efficacious in multiple cancers. Nevertheless, often only a subset of patients benefits from these therapeutic approaches. Immune monitoring with reliable assays is mandatory during follow up of patients and can provide useful information on the efficacy of therapy. This can be achieved e.g. by using more standardized reagents and procedures to reduce inter- and intra-assay variance. Especially in cases of limited sample availability, it is fundamental to increase the information derived from flow cytometric analysis. Here, we demonstrate standardization of flow cytometry assays by usage of a ready-to-use 8-color immunophenotyping cocktail developed for the analysis of whole blood samples. This cocktail consists of recombinantly engineered REAfinity™ Antibodies which display reduced background staining and ensure high lot-to-lot consistency. In order to address the increase of parameters applied during flow cytometric analysis, we demonstrate usage of a 12-color T cell panel, consisting of various activation and exhaustion markers and allowing an in depth characterization of tumor-infiltrating T lymphocytes using the MACSQuant® Analyzer 16.

Results

1 Flow cytometric immunophenotyping of dissociated tumor samples

Table 1

Specificity	Fluorochrome	AB clone	Purpose
Dead cells	7-AAD	n/a	Dump
CD3	PE	REA613	T cell lineage
CD4	VioBright™ 667	REA623	T_H
CD8	APC-Vio® 770	REA734	CTL
CD14	VioBlue®	REA599	Monocytes
CD16	VioBright 515	REA423	Granulocytes
CD19	PE-Vio 770	REA675	B cells
CD45	VioGreen™	REA747	Leukocytes
CD56	VioBright 515	REA196	NK cells

Resected colon cancer tissue was dissociated using the gentleMACS™ Dissociator and the Tumor Dissociation Kit, human. An aliquot of the dissociated tumor sample was stained with the 8-Color Immunophenotyping Kit, human, which is based on REAfinity™ Antibodies. This ready-to-use cocktail includes two new Vio® Dyes, VioBright™ 515 and VioBright™ 667, and it allows the addition of 7-AAD for dead cell exclusion (table 1). The stained cells were acquired on the MACSQuant® Analyzer 16 with the Express Mode Immunophenotyping_8_color_Kit_h.

Figure 1

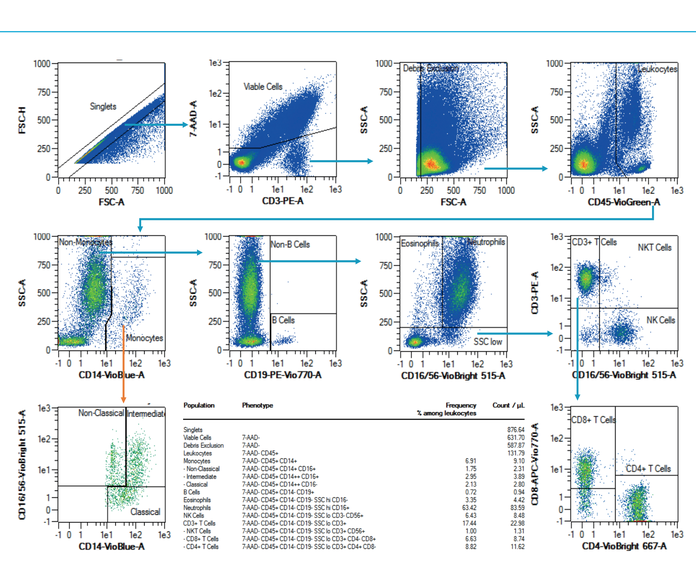


Figure 1 shows the gating strategy and layout of the automated Express Mode analysis: After gating on singlets, viable cells and FSC/SSC, all leukocytes were gated as CD45⁺ cells. Among leukocytes, Monocytes were discriminated based on their CD14 expression and further divided into classical, intermediate and non-classical monocytes via CD16. B cells were identified as CD19⁺. Among the non-B cell population, CD16 and SSC were used to distinguish neutrophils (CD16⁺SSC^{high}) and eosinophils (CD16-SSC^{high}) and a SSC^{low} population. Among these, T cells were further separated into CD8⁺ cytotoxic T cells (CTL) and CD4⁺ T helper (TH) cells. Lastly, NK cells were identified as CD56⁺ as well as a CD3⁺CD56⁺ population, which included NKT cells among others. Although developed for the analysis of whole blood samples, it could be shown that both cocktail and Express Mode can also be used for more demanding sample materials like dissociated tumors (melanoma, colon and ovarian cancer) and simplify their analysis. Furthermore, the example shown above emphasizes the need for high-parameter analysis, as only 3.6% of live cells were T cells.

2 Validation of a 12-color T cell panel

Table 2

Specificity	Fluorochrome	AB clone	Purpose
Dead cells	7-AAD	n/a	Dump
CD3	Brilliant Violet 650™	OKT3	Lineage
CD4	Brilliant Violet 605™	RPA-T4	T_H
CD8	Brilliant Violet 570™	RPA-T8	CTL
CD25	Brilliant Violet 421™	BV96	T_{regs}
CD39	APC-Vio® 770	REA739	Differentiation
CD69	VioGreen™	REA824	Activation / Differentiation
CD103	PE-Vio 615	REA803	Homing receptor / Differentiation
CD137	APC	REA765	Activation
CD223	PE	REA351	Checkpoint inhibitor
CD279	VioBright™ 515	REA1165	Checkpoint inhibitor
CD366	PE-Vio 770	REA635	Checkpoint inhibitor

In order to validate our in-depth 12-color T cell panel (table 2) and to show that different expression levels of the included markers can indeed be detected, isolated T cells were taken into culture and stimulated via CD3 and CD28 (T cell TransAct™). A sample was taken after 24, 48 and 72h. Cells were then labelled with antibodies against the different specificities mentioned above and afterwards acquired on the MACSQuant® Analyzer 16.

Figure 2A

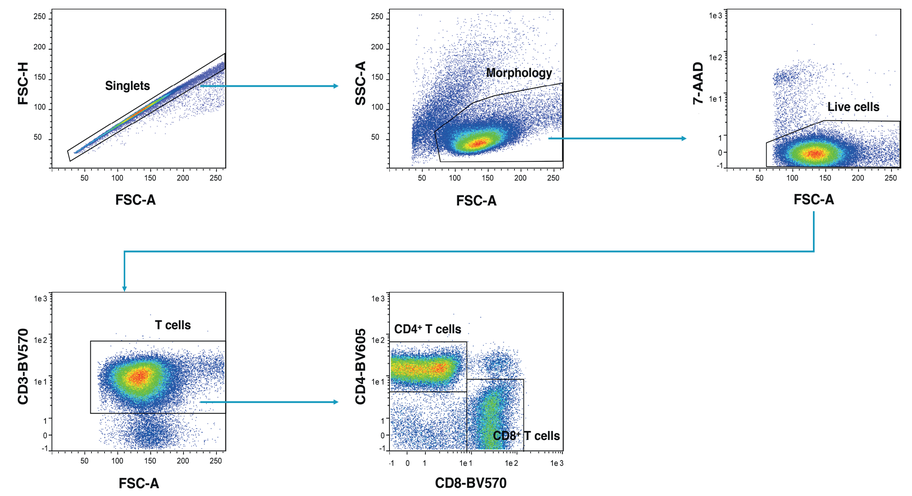
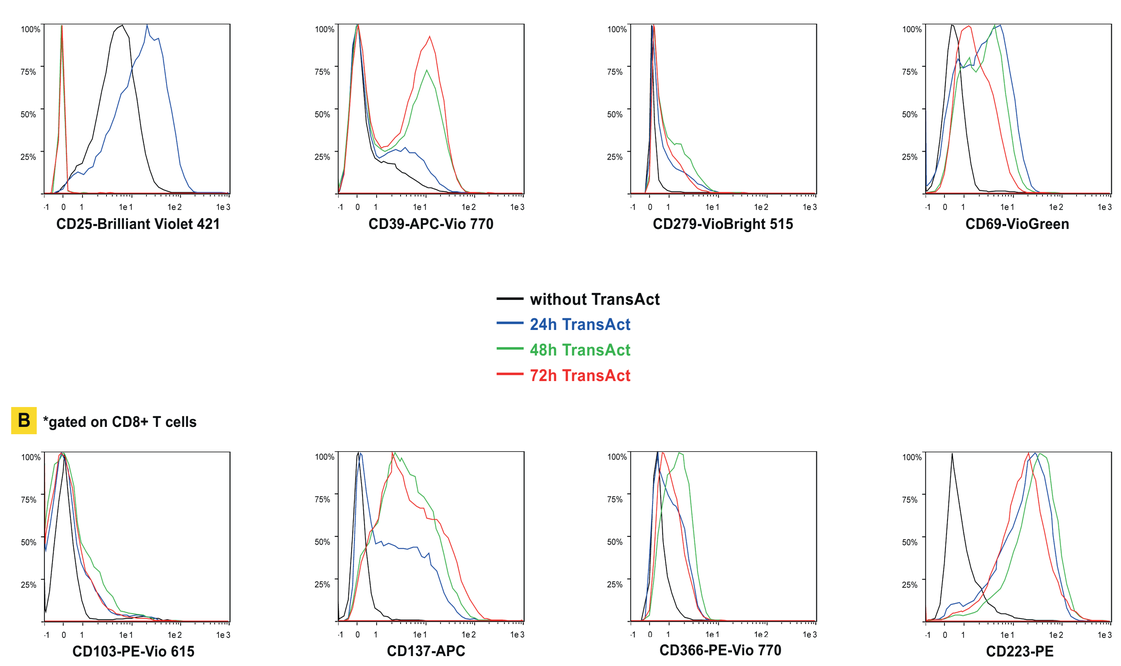


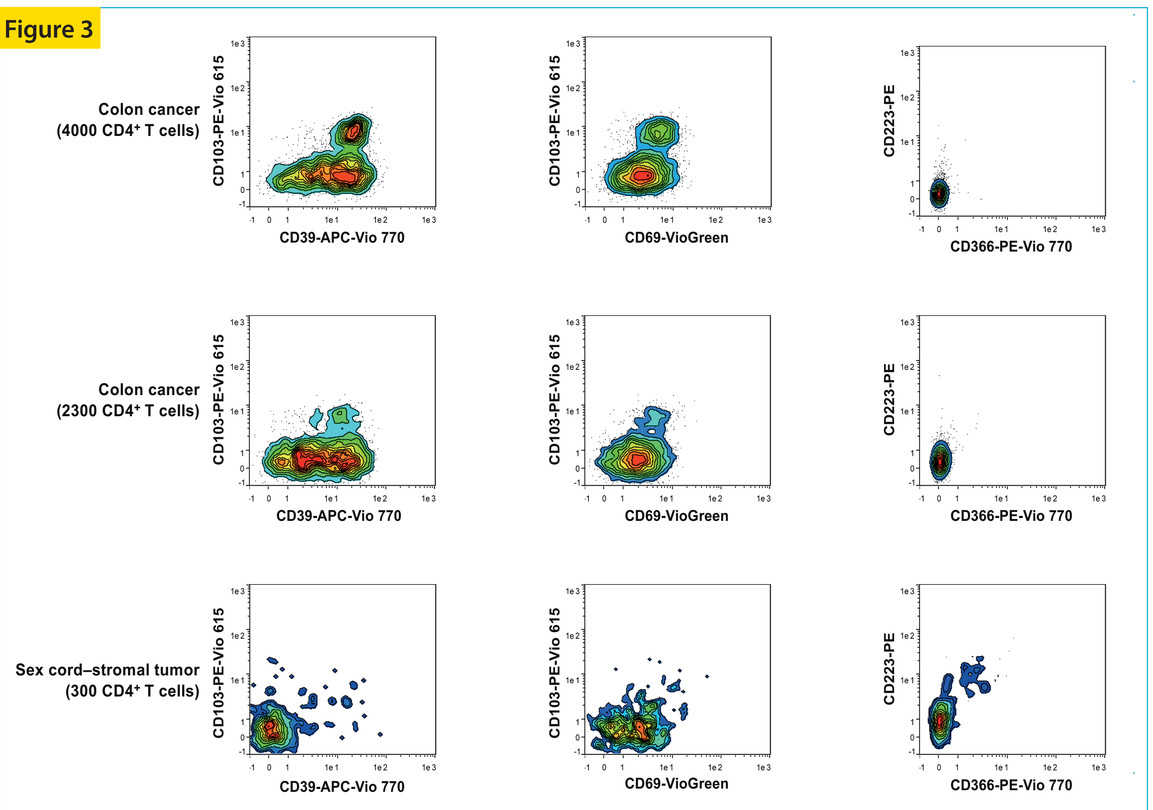
Figure 2B



After gating on singlets, FSC/SSC and living cells, T cells were separated into CD8⁺ cytotoxic T cells (CTL) and CD4⁺ T helper (T_H) cells (fig. 2A). The expression kinetics of the eight stained activation or differentiation markers were analyzed via histogram overlays of all time points tested. Four markers are shown for each T cell subpopulation (figure 2B).

3 Analysis of T cells in dissociated tumor samples

Figure 3



Three different dissociated tumor samples were stained with the 12-color T cell panel and acquired on the MACSQuant® Analyzer 16. As an example, three marker combinations are displayed for CD4⁺ T cells along with their total number in the analyzed sample.

Conclusion

- The 8-Color Immunophenotyping Kit, human and its associated Express Mode can simplify and standardize the analysis of dissociated tumor samples
- We developed a 12-color panel for the in-depth analysis of T cells from dissociated tumor
- Due to the limited sample material and very low numbers of T cells in the dissociated tumor, high-parameter analysis is needed. This demand can be met with our MACSQuant® Analyzer 16 and the exemplary panel shown here.

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