Vio® Dyes meet REAfinity™ Antibodies – antibody-fluorochrome conjugates for high-resolution multiparameter flow cytometry analysis

Cesar Evaristo, Marco Vahldieck, Susanne Krauthaeuser, Nicole Jansen, Niklas Wilske, Anne Richter, and Christian Dose
Miltenyi Biotec GmbH, Bergisch Gladbach, Germany

Introduction

Regulatory T (Treg) cells play a crucial role in the suppression of non-specific or excessive immune responses. Therefore, the detailed characterization of these cells and their function is of great interest. Treg cells generally express the surface markers CD4 and CD25, which are mainly used for initial identification purposes. Additional markers, including CD45RA, CD127, and in particular transcription factors such as FOXP3 and Helios, are used to distinguish subpopulations. Due to varying expression levels of these surface markers, the identification of cell subsets can be very challenging. Accordingly, the accurate analysis of Treg cell subsets and the determination of cell percentages by multicolor flow cytometry can be limited by the brightness of the fluorochromes used. To overcome these limitations, new fluorescent dyes and staining protocols have been created. Vio® 515 / VioBright™ 515 and Vio 667 / VioBright 667 are introduced as new flow cytometry dyes, preferentially conjugated to recombinantly engineered antibodies, i.e., REAfinity™ Antibodies. Rapid protocols were developed for staining hardly accessible intranuclear transcription factors. Based on comprehensive multicolor panels, these protocols allow cell surface and intracellular staining in less than an hour in total, using either PBMCs, whole blood, lysed blood or even Treg cells derived from any kind of pre-enrichment.

Methods

1 Development of a multicolor panel for Treg cell phenotyping

- Flow analysis was performed on the MACSQuant® Analyzer 10.
- Cells were stained with surface panels for 10 min at RT in the dark, washed, centrifuged, and resuspended in an appropriate buffer.
  - 1×10^6 cells from PBMCs or lysed blood
  - up to 100 µL whole blood
  - total fraction of enriched cells from MS Column
- For intracellular staining a rapid protocol was used, enabling processing of whole blood in less than 1 h and PBMCs, lysed blood, and pre-enriched cells in less than 45 min.
- Panels included new VioBright Dyes for surface staining and new Vio Dyes for intracellular staining (table 1).

Antibody-fluorochrome conjugates

Cell marker	Clone	Purpose	Fluorochrome	Detection channel	Order no.
CD3	REA613	T cell discrimination	VioGreen	V2	130-109-466
			PE-Vio 770	B4	130-109-463
CD4	REA623	Identification of T helper subset	VioGreen	V2	130-109-456
			VioBright FITC	B1	130-109-457
			VioBright 515		customized
			APC-Vio 770	R2	130-109-454
CD8	REA734	Identification of cytotoxic T helper subset	VioGreen	V2	130-110-684
CD25	REA570	Identification of Treg cells	VioBright 515	B1	customized
			PE	B2	130-109-020
			APC	R1	130-109-074
			VioBright 667	R1	customized
CD25*	4E3 (REA945**)	Identification of Treg cells	VioBright FITC	B1	130-104-323
			PE	B2	130-091-024
CD45	REA747	Identification of leukocytes	VioGreen	V2	130-110-637
			APC-Vio 770	R2	130-110-635
CD45RA	REA562	Identification of naive T cells	PE-Vio 615	B3	130-108-720
			APC-Vio 770	R2	130-108-717
CD45RO	REA611	Identification of memory T cells	PE-Vio 615	B3	130-109-434
CD127	REA614	Treg cell discrimination	PE	B2	130-109-435
			PE-Vio 770	B4	130-109-437
Helios	22F6	Identification of Treg cell subpopulations	VioBlue	V2	customized
			FITC	B1	130-104-000
Helios	REA829	Identification of Treg cell subpopulations	VioBlue	V2	customized
			FITC	B1	130-112-629
			PE	B2	130-112-630
Ki-67	REA183	Identification of Treg cell subpopulations	Vio 515	B1	130-108-830
			Vio 667	R1	130-111-761
FOXP3	REA944**	Identification of Treg cell subpopulations			customized
T-bet	REA102	Identification of Treg cell subpopulations	APC	R1	130-098-607

Table 1 * Clones REA570 and 4G10 are not suitable for labeling the enriched Treg cells. ** REA clones will be available later in 2017.

2 Gating strategy for phenotyping based on cell surface staining using an 8-color panel

Discrimination of Treg cell subsets was accomplished using the following gating strategy (fig. 1).

- CD45 was used for leukocyte identification.
- Gating of leukocytes via FSC-A vs. SSC-A.
- Doublets were excluded with a singlet gate in FSC-H vs. FSC-A.
- Exclusion of dead cells using DAPI-negative cells.
- T cells were identified by CD3 and CD4 staining, followed by discrimination of Treg cells based on CD25 and CD127.
- Further Treg cell subsets were analyzed by CD45RA and CD45RO staining.
- Cell percentages, mean fluorescence intensities (MFI), and stain indices (SI) were compared for B1 and R1 VioBright Dyes (fig. 2)

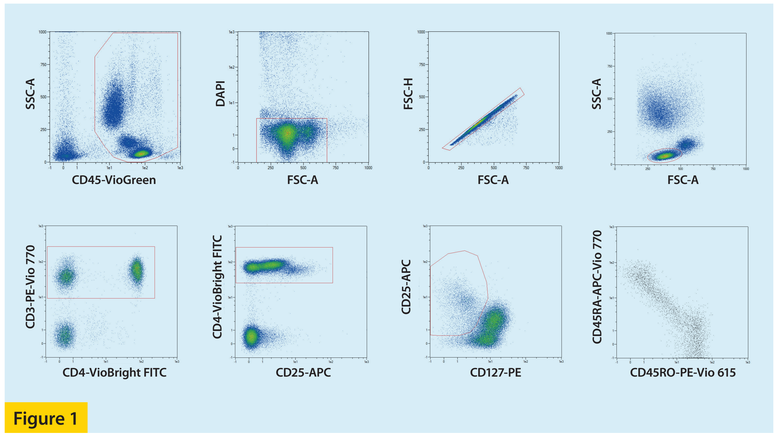
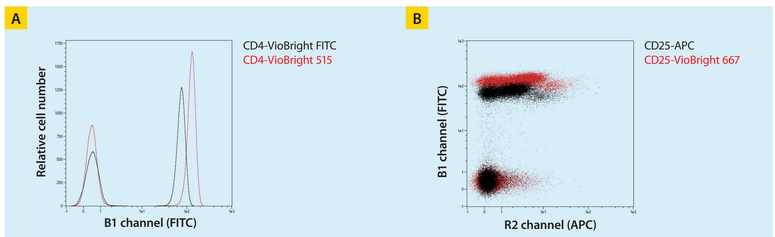


Figure 1

Results

1 Eight-color panel including VioBright™ 515 and VioBright 667 for Treg cell phenotyping

Whole blood was lysed and washed after surface staining with the 8-color panel. CD4-VioBright™ FITC vs. CD4-VioBright 515 (fig. 2A) and CD25-APC vs. CD25-VioBright 667 (fig. 2B) were compared in terms of cell percentages and MFI / SI (fig. 2C). The multicolor panel was verified for the analysis of Treg cells derived from whole blood, lysed blood, PBMCs, and pre-enriched cells (not shown).



C

Marker	Dye	Percentage of target cells	MFI	SI
CD4	VioBright FITC	58.2*	76	107
	VioBright 515	58.8*	129 (+70%)	222 (+207%)
CD25 (REA570)	APC	10.1**	7.64	n.d.
	VioBright 667	10.4**	13.11 (+72%)	n.d.

* $CD4^{++}$ among $CD3^+$ cells **$CD25^+$ among $CD4^{++}CD127^-$ cells

Figure 2

2 Comparison of intranuclear staining using Anti-Helios clone 22F6 vs. the corresponding REA clone

A rapid intranuclear staining protocol was used to label PBMCs. The panel included CD3-VioGreen™, CD4-APC-Vio 770, CD25 (REA570)-PE, CD127-PE-Vio 770, and CD45RA-PE-Vio 615.
Anti-Helios (22F6)-VioBlue® was compared to Anti-Helios (REA829)-VioBlue in the absence or presence of FcR Blocking Reagent in terms of background staining, MFI, and cell percentages (fig. 3). The multicolor panel was verified for the analysis of Treg cells derived from whole blood, lysed blood, PBMCs, and pre-enriched cells (not shown).

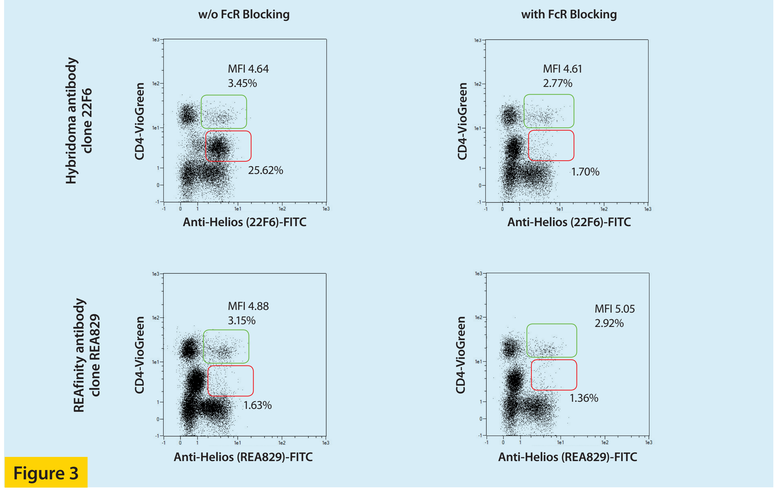


Figure 3

3 Intranuclear staining protocols for cells from different starting materials

Three rapid protocols were established that enable intranuclear staining of PBMCs in 40 min (fig. 5A) and whole blood in 50 min (fig. 5B), as well as on-column staining in 45 min (fig. 5C). Figure 4 shows a comparison of the processing times for the rapid protocols from Miltenyi Biotec vs. four intranuclear staining protocols from other providers. Different panels were designed that comprised basic markers such as CD3, CD4, CD25 (REA570) / CD25 (4E3), and CD127 (REA614) by default. Clone 4E3 was used for staining enriched $CD25^+$ cells, thus avoiding epitope hindrance caused by the CD25 antibodies used for enrichment. Intranuclear markers included Helios, FOXP3, and/or Ki-67 (fig. 5).

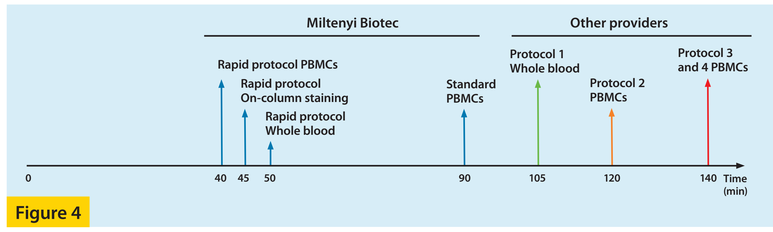


Figure 4

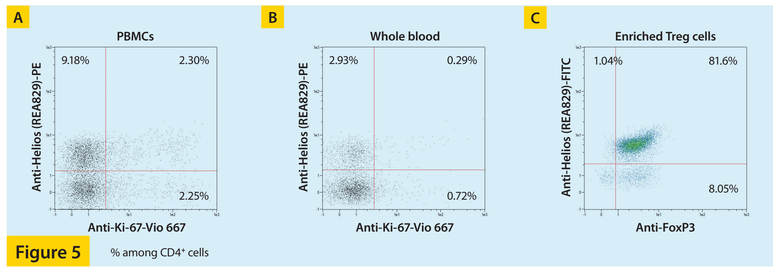


Figure 5 % among $CD4^+$ cells

4 Simultaneous detection of three transcription factors in $CD4^+$ and $CD8^+$ cells

The rapid intranuclear staining protocol for PBMCs was used to detect Helios, Ki-67, and T-bet simultaneously in $CD4^+$ and $CD8^+$ cells. The antibody panel included CD4-APC-Vio 770, CD8-VioGreen, CD25 (REA570)-PE, CD127-PE-Vio 770, CD45RA-PE-Vio 615, Anti-Helios (REA829), Anti-Ki-67, and Anti-T-bet (fig. 6).

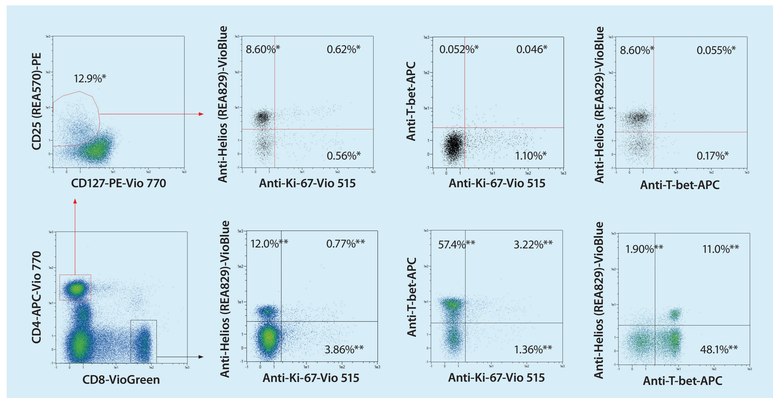


Figure 6 * % among $CD4^+$ cells ** % among $CD8^+$ cells

Conclusion

- Introduction of four new fluorescent dyes, Vio 515 / VioBright 515 and Vio 667 / VioBright 667, characterized by high fluorescence intensity.
- Development of new eight-color panels, which include VioBright 515 and VioBright 667 for surface staining, and Vio 515 and Vio 667 for intracellular staining, enabling high-resolution analysis of Treg cells in PBMCs, lysed blood, and whole blood.
- Introduction of REAfinity Antibodies conjugated to the brightest Miltenyi Biotec dyes, such as the new VioBright Dyes, and also PE-Vio 615 and PE-Vio 770, avoiding non-specific binding to monocytes.
- Introduction of rapid staining protocols for detection of transcription factors such as FOXP3, Helios, Ki-67, and T-bet, either using PBMCs, lysed or whole blood, or pre-enriched $CD25^+$ cells.
- Using the rapid FOXP3 staining protocol, processing time is up to twice as fast in comparison to other protocols.