Optimization and intra-assay validation of a multiparametric flow cytometric test for monitoring circulating TREGs

Iole Macchia*, Floriana Iacobone, and Francesca Urbani
Department of Oncology and Molecular Medicine, Istituto Superiore di Sanità (ISS), Rome, Italy
*Corresponding author: e-mail address: iole.macchia@iss.it

Contents

Abstract

Multiparametric flow cytometry (MFC) represents an essential tool for immune monitoring, and validation of MFC panels is a fundamental prerequisite in routine laboratory settings as well as for translational and clinical research purposes.

Regulatory T cells (TREGs) constitute a subset of CD4+ effector T cells that modulate the immune response in numerous settings, including autoimmune disease, allergy,

Methods in Cell Biology
ISSN 0091-679X
https://doi.org/10.1016/bs.mcb.2024.06.005

microbial infection, tumor immunity, transplantation, and more. These cells comprise a small fraction of total CD4+ T cells in human peripheral blood and mouse spleen. In oncology, TREG cells are highly relevant, as they are involved in the suppression of the anti-tumor response in many types of cancer, to the extent that the first immune checkpoint inhibitor approved for clinical use in humans was a monoclonal antibody directed against CTLA-4, a molecule functionally associated with TREGs. Due to all these factors, robust assays are mandatory to accurately determine TREG cell frequency and function.

Here, we describe the validation of an 8-color flow-cytometry protocol for TREG detection and analysis in a real-world laboratory scenario. The entire process includes the workflow plan and the standard operating procedure resembling each phase, from the panel design to the staining, acquisition, and analysis steps. Validation is planned to be performed in replicates on fresh whole blood samples derived from multiple healthy subjects. The analytical validity of the TREG cell assay is ensured by testing the intra-assay accuracy.

The detailed procedure for the entire process is accompanied by important troubleshooting suggestions and other useful tips.

1. Introduction

Multiparametric flow cytometry (MFC) is a powerful, sensitive, and quantitative analytical method that allows the identification of defined cell types within a mixed cell population, thanks to its unique ability to detect, at a single-cell level, multiple parameters simultaneously, by using several fluorochromes with different emission wavelength. MFC has been widely used to investigate the systemic immune profile of subjects, in translational and clinical studies.

Regulatory T cells (TREGs) are a subset of CD4+ T lymphocytes, with a role in regulating or suppressing other cells of the immune system; thus, they ensure the maintenance of immunological self-tolerance and immune homeostasis by preventing autoimmune responses against self-antigens (Levings, Allan, & Picc, 2006).

These cells can be produced within the normal thymus (natural TREGs) or outside it by differentiation of naïve T cells (adaptive TREGs). Natural TREGs (nTREGs) constitutively express the CD25 receptor (IL-2-receptor α-chain), required for their development and survival through IL-2 responsiveness (Niec & Waldmann, 2013), and the nuclear transcription factor Forkhead box P3 (FoxP3), which is necessary for their development and for maintaining their suppressive function; FoxP3 is also transiently expressed in activated non-suppressor T cells (Deng, Song, & Greene, 2019). Beyond FoxP3, the immunosuppressive functions of TREGs cells

are also influenced by other factors, including the Cytotoxic T lymphocyte antigen-4 (CTLA-4) and the glucocorticoid-induced TNF-receptor (GITR) (Wegrzyn, Kedzierska, & Obojski, 2023).

TREGs have been shown to suppress most immune cell populations, including lymphocytes, various types of macrophages, dendritic and B cells. In particular, they are able to suppress activation, proliferation, and cytokine production of $CD4^+$ T cells and $CD8^+$ T cells (Okeke & Uzonna, 2019).

Immuno-monitoring TREGs is crucial as these cells are involved in a wide range of diseases, including cancer, autoimmune diseases, infectious diseases, transplantation, and allergy (Sakaguchi et al., 2020). Since this lymphocyte subpopulation is altered in number and/or function in numerous pathologies, their frequency may be considered a promising immunological biomarker.

Increased levels of TREGs have been reported at tumor sites, draining lymph nodes as well as in the peripheral blood, in many types of human cancer, including melanoma, breast, colon, lung, prostate, liver, ovarian cancer as well as hematological malignancies (Plitas & Rudensky, 2020). TREGs are generally considered a poor prognostic factor since they may suppress the immune response against tumor antigens (Liyanage et al., 2002). In fact, high levels of circulating TREGs in melanoma patients have been correlated with poor overall survival and disease-free survival (Heldager et al., 2024).

For this reason, several studies, have attempted to reduce or deplete the number of these cells by immune checkpoint inhibitor (ICI) therapy, to enhance tumor immunity and to control tumor growth (Takeuchi & Nishikawa, 2016; Wolf et al., 2003). Indeed, some ICIs, such as Ipilimumab and Tremelimumab, are monoclonal therapeutic antibodies directed against CD152 (CTLA-4) molecules, characteristic receptors of these cells. Therefore, TREG levels in peripheral blood of melanoma patients at the time of diagnosis may be considered a potential biomarker not only for prognosis and survival, but even for prediction of response to ICIs.

To date, proposed unique cell markers have failed to fulfill the criteria of being exclusively expressed on these cells, since TREGs represent a heterogeneous cell population with distinct phenotypes and functional profiles (Miyara et al., 2009). Standardization of TREG staining by flow cytometry is challenging, notably because of the intracellular location of the FoxP3 transcriptional factor.

Up to now, rigorous analysis of multiparametric flow cytometry data, using various sample preparations and optimization steps for TREG cell

quantification, has highlighted certain surface and intracellular markers as essential for univocally identifying TREGs, which are often defined as CD3+CD4+CD25+FoxP3+CD127− cells (Biancotto, Dagur, Fuchs, Langweiler, & McCoy, 2012; Santegoets et al., 2015).

Additional markers use for nTREGs are the above mentioned CTLA-4 and GITR, although it should be noted that these markers are also expressed by other T-cell types (e.g., activated T cells), so they are not in themselves unequivocally diagnostic. However, the role of these markers on other T cells is not clearly defined. The expression of intracellular CTLA-4 and FoxP3 is positively correlated with surface expression of CD25 and inversely correlated with expression of the CD127 marker (IL-7 receptor) (Liu et al., 2006). Higher levels of intracellular FoxP3 and CTLA-4 and lower levels of surface CD127 consistently distinguish CD25hi cells from CD25int/low and CD25− cells (Sakaguchi et al., 2020).

Other phenotypic markers (CD45RA and CD39) can define more precisely subsets of TREGs, including the CD45RA-CD39+ memory TREGs with ATPase-dependent suppressive activity (Borsellino et al., 2007; Miyara et al., 2009). TREGs can also produce soluble messengers, endowed with a suppressive function, including TGF-β, IL-10 and adenosine.

Overall, the validation of a panel for the immunomonitoring of TREGs is necessary to obtain reliable and comparable results (Pitoiset et al., 2018).

The purpose of this protocol is to optimize and validate a flow cytometric multiparametric assay for evaluating TREG frequency and functional status in human fresh peripheral blood, adaptable for use across different clinical and physiological scenarios. Here, we describe the validation process of an 8-color panel for identification and enumeration of human naïve/memory TREG subsets, focusing on the CD45RA-CD39+ memory suppressive subpopulation, utilizing markers (such as CD45, CD3, CD4, CD25, CD127, FoxP3, CD45RA, CD39), selected in the light of previous consensus reports (Santegoets et al., 2015). This panel can be also considered as a backbone to be supplemented with further drop-in markers. Specifically, this protocol will provide guidelines for intra-assay validation. Full inter-assay validation is not feasible in ex vivo fresh blood samples due to the possible time-dependent alterations in the distribution of certain immune cell subsets, including TREGs.

Based on our previous experience in harmonizing a MFC panel aimed at studying memory status of T cell subpopulations (Macchia et al., 2020), the protocol presented below is the outcome of a real-life validation conducted in our laboratory.

2. Materials

2.1 Common disposables

Below are listed the common disposable items routinely used in our laboratory (Note 1).

- 3 mL K2-EDTA blood collection tubes (e.g., #367838 Vacutainer tubes, BD, NJ, USA).
- 96 U-bottom well plates (e.g., #3879 Corning, NY, USA).
- 5 mL round bottom polystyrene test tubes (e.g., #352054 Falcon Corning, NY, USA) (Note 2).
- 1.5 mL safe lock vials (e.g., #022363204 Eppendorf, DE, EUR).
- Pipet tips (e.g., # F161630, # F161930, #F161670 D10/D200/D1000 Diamond tips Gilson, WI, USA).
- 25-, 10-, 5-, 2-mL sterile disposable pipettes (e.g., #357535, #357551, #357543, #357507, Falcon Corning, NY, USA).

2.2 Reagents

Below are listed the reagents routinely used in our laboratory (Note 3).

- Deionized water.
- Dulbecco-Phosphate Buffered Saline (DPBS), w/o Ca and Mg (e.g., #17-512F Lonza, CH, EU) (Note 4).
- Fetal bovine serum (FBS) (e.g., #35-079 Corning, NY, USA) (Notes 5 and 6).
- EDTA concentrate (e.g., #E7889 MilliporeSigma-Merck, MO, USA) (Note 7).
- Sodium azide concentrate (e.g., #71290 MilliporeSigma-Merck, MO, USA) (Note 8).
- Compensation beads (e.g., #B22804 VersaComp Antibody Capture Bead Kit, Beckman Coulter, CA, USA) (Note 9).
- Lysing solution 10× (e.g., #349202 BD Biosciences, CA, USA) (Note 10).
- Fluorescent-conjugated anti-human antibodies (Note 11) as per Table 1.
- FoxP3/Transcription Factor Staining Buffer Set (# 00-5523-00 e-Biosciences, MA, USA), which includes fixation/permeabilization 4× buffer, permeabilization buffer 10× and diluent (Notes 12 and 13).
- Fc Receptor Blocking Solution (e.g., # 422302 Human TruStain FcX™, Biolegend, CA, USA) (Note 14).

Table 1 MFC panel composition.

Cell localization	Antigen/ligand	Fluorochrome	Clone	µL/sample	ID#	Manufacturer	Nationality (US)
Surface	CD4	FITC	RPA-T4	10	555346	BD Pharmingen	CA
Intranuclear	FoxP3 (Note 49)	PE	PCH101	5	12-4776-42	e-Bioscience	MA
Surface	CD3	PE Cy5.5	SP34-2	10	552852	BD Pharmingen	CA
Surface	CD25	PE Cy7	M-A251	5	557741	BD Pharmingen	CA
Surface	CD127	APC	HIL-7R-M21	10	558598	BD Pharmingen	CA
Surface	CD39	APC CY7	A1	5	328226	Biolegend	CA
Surface	CD45RA	Pacific Blue	HI100	5	304123	Biolegend	CA
Surface	CD45	Krome Orange	J.33	20	B36294	Beckman Coulter	CA

2.3 Equipment

Below are listed the equipment routinely used in our laboratory (Note 15).

- Class A biological safety cabinet (e.g., #8511 Kottermann, DE, EU) (Note 16).
- Centrifuge (e.g., #5810R Eppendorf, DE, EU) (Note 17).
- Vortex (e.g., # VWRI444-1372 VWR, PA, USA).
- Micropipette Set (e.g., #F167370 Pipetman L Starter kit, Gilson, WI, USA).
- Flow Cytometer (e.g., Gallios Beckman Coulter, CA, USA) (Note 18).
- Pipettor (e.g., #075002 Pipet-Aid Drummond, PA, USA).

2.4 Software

Below are listed the software routinely used in our laboratory (Note 19).

- Flow cytometry acquisition and analysis software (e.g., Kaluza V1.3, Beckman Coulter, CA, USA).
- Analysis software (e.g., Microsoft Office 2021 Excel or SPSS statistical processor, IBM-SPSS V28, IBM Corporate New York, NY, USA) (Note 20).

3. Methods

3.1 Safety warning

This protocol involves the use of human blood derivatives. Operators should have undergone appropriate training and must adhere to laboratory safety guidelines for blood derivative products, including the proper adoption of personal protective equipment (PPE) and controlled environment precautions (CEP).

3.2 Preliminary procedures

1. The project workflow is planned, as illustrated in Fig. 1A.
2. Sample processing is set up as well (Fig. 1B), by in-house adapting manufacturer instructions of the eBioscience FoxP3/Transcription Factor Staining Buffer Set (Note 21).

3.3 Panel design

1. A 8-color MFC panel in liquid format is designed (Table 1 in Section 2.2), based on a previous consensus report (Santegoets et al., 2015), by selecting antibody clones of the following markers:

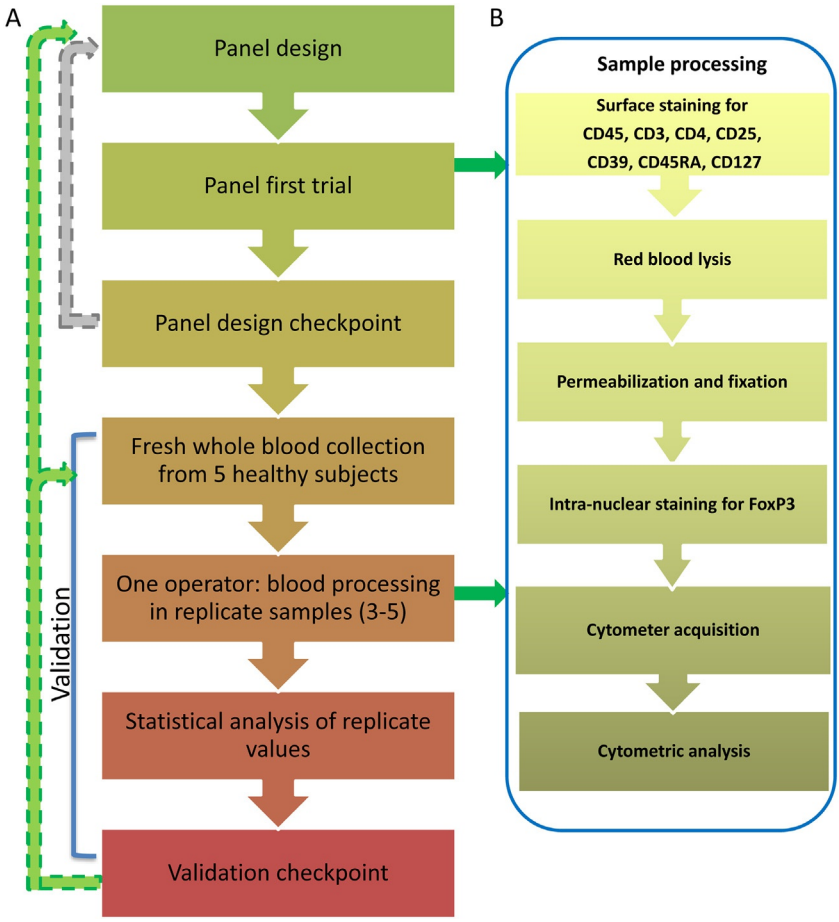

Fig. 1 Experimental workflow. (A) Flowchart of the validation of a flow cytometry panel for TREG monitoring in human fresh peripheral blood. Dotted arrows indicate potential re-design and re-validation steps. (B) Flow chart of sample staining, acquisition and analysis procedure.

- anti-CD45, -CD3, -CD4, -CD25, -CD127 and -FoxP3 conjugated antibodies, as minimally required markers.
- anti-CD45RA and -CD39, as additional markers.

2. Antibody-fluorochrome combinations are defined according to commonly recommended procedures for panel design (Flores-Montero et al., 2019) (Note 22).

3.4 Conjugated antibody titration

Each conjugated monoclonal antibody is titrated to define optimal working concentration. This procedure is strongly advised (Brando & Sommaruga, 1993), especially when constructing a multiparametric flow cytometry panel. The antibody titration procedure is not specifically addressed in this SOP, however, briefly, each antibody is tested at scalar dilutions in a twofold ratio starting from the dose recommended for the production batch (e.g., 10 µL, 5 µL, 2.5 µL, if manufacturer recommended dose is 10 µL). After acquisition, the optimal dose for each antibody is the one that shows the best resolution index (post-titration volumes are indicated in Table 1) (Notes 23 and 24).

3.5 Whole blood sample collection

1. Enrolled subjects sign written informed consent approved by the competent local ethics committee.
2. A 3 mL volume of whole blood is collected by venipuncture in K2-EDTA vacuum tubes from healthy volunteers (referred to as Subj#0, Subj#1 … #5) (Note 25). Subj#0 sample is dedicated to the first trial phase, while Subj#1 to #5 samples are dedicated to the validation phase.
3. Samples are left at room temperature until the beginning of the staining procedure, which is performed within 4 h from blood withdrawal.

3.6 Panel compensation set-up

The compensation matrix is established by VersaComp bead staining for each single pre-titrated conjugated antibody, according to manufacturer instructions (Note 26). To generate a compensation matrix, automatic procedure is applied and manual adjustments are operated, if necessary.

3.7 Panel first trial

This phase is performed to set up the correct PMT voltages and to verify compensation matrix as well as to check the overall good outcome of the panel.

3.7.1 Sample collection

1. Blood is collected from 1 enrolled subject (Subj#0), as described in Section 3.5.

2. 200 μL of Subj#0 whole blood are pipetted into the following 5 mL round bottom polystyrene tubes:
   - 1 unstained tube.
   - 1 fully stained (8-color) tube.

3.7.2 Sample staining

Warning: Flow cytometry procedure must be performed in the dark to preserve fluorochrome incidental excitation.

3.7.2.1 Working solutions (Note 27)

Working solutions are prepared and kept at RT until usage. Appropriate volumes are calculated for each sample to be processed, as follows:

- Staining solution: DPBS supplemented with 2% FBS, 2 mM EDTA, 0.09% Sodium Azide (Note 28).
- Lysing solution 1×: concentrated solution is diluted 1:10 in deionized water (Note 29).
- Fixation/Permeabilization 1×: 4× Fixation/Permeabilization buffer is diluted 1:4 in Diluent (Note 30).
- Permeabilization Solution 1×: 10× Permeabilization Buffer is diluted 1:10 in distilled water (Note 31).

3.7.2.2 Surface staining

1. A mix of pre-titrated surface antibodies (Table 1 included in Section 2.2), diluted in staining solution in a final volume of 50 μL for each sample, is prepared (Notes 32 and 33).
2. 50 μL of the surface antibody mixture are added to the 8-color tube(s).
3. Cells are gently mixed and incubated 15′ at RT in the dark.
4. To lyse red blood cells, 3 mL of 1× BD Lysing Solution are directly added to each tube.
5. Cells are mixed by inversion 2–3 times and incubated 10′ at RT in the dark (Note 34).
6. Cells are centrifuged at 500×*g* for 5′ at RT.
7. Supernatants are discarded.
8. 4 mL of staining buffer are added to each tube.
9. Cells are centrifuged at 500×*g* for 5′ at RT.
10. Supernatants are discarded.
11. Pellets are resuspended in the residual volume of staining buffer.

3.7.2.3 Fixation, permeabilization and intranuclear staining

From this point onwards, it is possible to transfer the contents of the tubes into the wells of a 96-well U-bottom plate (Note 35). This operation

facilitates the management of a large number of samples. The volumes of the reagents relative to the plate procedure are indicated in parentheses.

1. 1 mL of freshly prepared fixation/permeabilization working solution 1× is added to each sample (250 μL) (see Section 3.7.2.1).
2. Cells are vortexed and incubated for 30′ at RT in the dark.
3. Cells are centrifuged at 800×*g* for 5′ at RT.
4. Supernatants are discarded (Note 36).
5. 2 mL of staining buffer are added to each sample (250 μL).
6. Cells are centrifuged at 800×*g* for 5′ at RT.
7. Supernatants are discarded (Note 37).
8. 1 mL of freshly prepared Permeabilization solution 1× (see Section 3.7.2.1) is added to each sample (250 μL).
9. Cells are centrifuged at 800×*g* for 5′ at RT.
10. Supernatants are discarded (Notes 38 and 39).
11. Cells are resuspended in the residual volume of Permeabilization Buffer 1×.
12. A dilution of freshly prepared anti-FoxP3 antibody is obtained in 1× permeabilization buffer (the appropriate pre-titrated volume of anti-FoxP3 is diluted in 50 μL of 1× permeabilization buffer for each sample) (Note 40).
13. 50 μL of diluted anti-FoxP3 are added to each sample and gently mixed.
14. Cells are incubated 30′ at RT in the dark.
15. 2 mL of staining buffer are added to each sample (250 μL).
16. Cells are centrifuged at 800×*g* for 5′ at RT.
17. Supernatants are discarded (Note 41).
18. Cells are resuspended in 300 μL of DPBS (Note 42).
19. Cells are left at 4 °C in the dark until acquisition (Note 43).

3.7.3 Sample acquisition

1. The unstained tube is run to set up morphological and fluorescent basal parameters.
2. The compensation matrix, previously calculated (see Section 3.6), is recalled. Manual adjustment can be performed, if necessary.
3. 250,000 total events (or 50,000 events in the Lymphocyte gate) are acquired for each 8-color-stained tube, with a flow rate <500 events/s for optimal resolution, recording each antibody fluorescence, FS-H, FS-A, SS-A and TIME channels (Note 44).

3.7.4 MFC analysis

1. Gating strategy (Fig. 2A) is performed by Kaluza software. For each 8-color sample, the following plots are drawn:

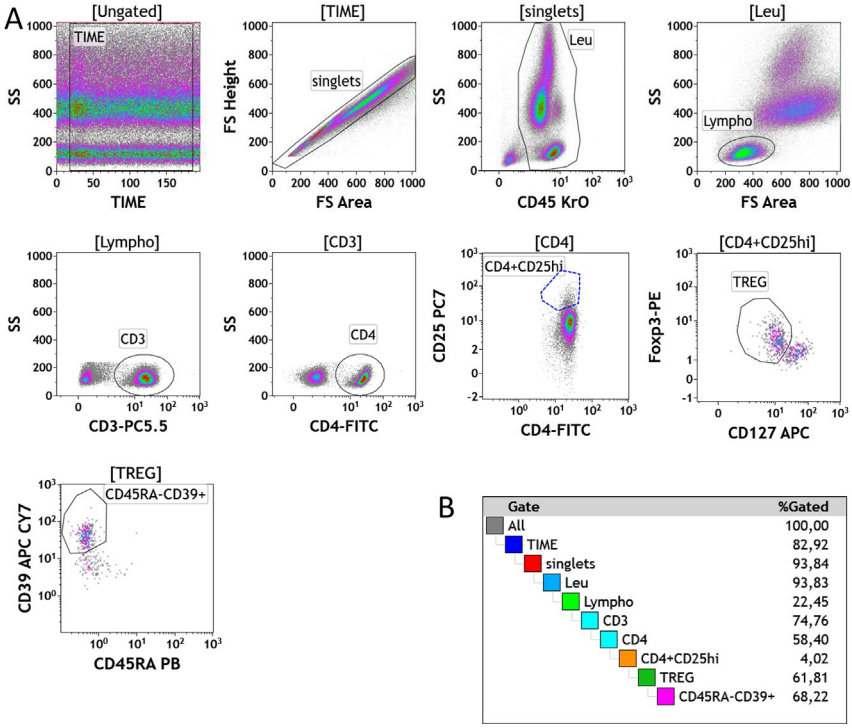

Fig. 2 Gating strategy and gate statistics for TREGs. (A) Representative dot plots from a healthy subject whole blood sample. TREGs are defined as CD3+CD4+CD25hiCD127-Foxp3+. Suppressive memory TREGs are defined as CD45RA-CD39+. (B) Representative gate statistics for whole blood samples.

- A TIME vs SS-A plot to select a stable acquisition time lapse by a gate (TIME).
- Within TIME: a FS-H vs FS-A plot to select SINGLET events.
- Within the singlet event gate: a CD45 vs FS-A plot to select LEUKOCYTES.
- Within the leukocyte gate: a FS-A vs SS-A plot to identify the LYMPHOCYTE population.
- Within the lymphocyte gate: a CD3 vs SS-A plot to identify CD3+ lymphocytes.
- Within the CD3+lymphocyte gate: a CD4 vs SS-A plot to identify CD4+ lymphocytes.
- Within the CD4+lymphocyte gate: a CD25+/CD4+ plot to identify CD4+CD25high events.

- Within the CD4+CD25high event gate: a CD127 vs FoxP3 plot, to identify CD127-FoxP3+ events, also known as *TREGs*.
- Within the TREG gate: a CD39/CD45RA plot, to identify CD45RA-CD39+ events, also described as *memory TREGs with ATPase-dependent suppressive activity*.

2. For each .fcs file, gate statistics are generated by Kaluza software (Fig. 2B).

3.7.5 First trial checkpoint

First trial is meant to assess the feasibility of compensation, fluorochrome compatibility, overall performance of the analysis, and to define the gating strategy design, as well. If the panel composition did not meet the requirements for proper acquisition and analysis (lack of compensation or other issues), the entire process is considered for repetition until a satisfactory result is achieved (Fig. 1A gray dotted arrow). The subsequent panel validation is launched when a satisfactory checkpoint result is achieved.

3.8 Panel validation

The entire procedure (blood collection, staining, acquisition) is conducted within the same day for each subject under study.

3.8.1 Sample collection

1. Blood is collected from 5 enrolled subjects (Subj#1 to #5), as described in Section 3.5.
2. 200 μL of each blood sample are aliquoted in three different 5 mL U-bottom polystyrene tubes (a, b, c) (Note 45).

3.8.2 Sample staining

Sample staining is performed as described in Section 3.7.2.

3.8.3 Sample acquisition

1. On the cytometer acquisition software, the PMT set up and compensation matrix derived from the first trial session are recalled. If needed, compensation matrix is manually adjusted before acquisition.
2. 250,000 total events (or 50,000 events in the lymphocyte gate) are acquired for each 8-color-stained tube, with a flow rate <500 events/s for optimal resolution, recording each antibody fluorescence, FS-H, FS-A, SS-A and TIME channels (Note 46).

3.8.4 MFC analysis

1. Each .fcs file is analyzed according to the gating strategy described in Section 3.7.4 and in Fig. 2A.
2. For each .fcs file, gate statistics is generated by Kaluza software (Fig. 2B).

3.8.5 Statistical analysis

1. Each .fcs file gate statistics are exported from Kaluza software into an Excel file or into a statistical processor database (Note 47).
2. To explore results, distribution of each parameter of interest by each subject analyzed is graphed (Fig. 3A).
3. Outliers are appropriately eliminated.
4. To estimate intra-assay precision, Coefficient of Variation (CV = SD/Mean) for each parameter of interest, related to each subject, is calculated. The median CV for each parameter and for each subject, is calculated as well (Fig. 3B). CV values below 0.20 are generally considered as optimal (Note 48).
5. A heatmap of calculated CVs is drawn for a quick overview of the results (Fig. 3B).
6. Observations are provided based on the obtained results, highlighting any parameters of interest that may be more prone to excessive variability.

3.8.6 Validation checkpoint

The opportunity to review the panel composition, staining method, gating strategy or final analysis should be considered in case one or more parameters of interest are found to be insufficiently accurate. The entire procedure should be repeated until satisfactory results are achieved (Fig. 1A, green dotted arrow).

4. Concluding remarks

The protocol described herein offers an easy and integrated workflow to validate a panel designed to assess frequency and activation status of circulating TREGs on a small volume of whole blood samples. The described panel may be adapted to the characterization of PBMC or tissue infiltrating TREGs and can also be further expanded by adding supplementary markers capable of qualifying the maturation and exhaustion state of TREGs cells even more finely (Manuszak, Brainard, Thrash, Hodi, & Severgnini, 2020).

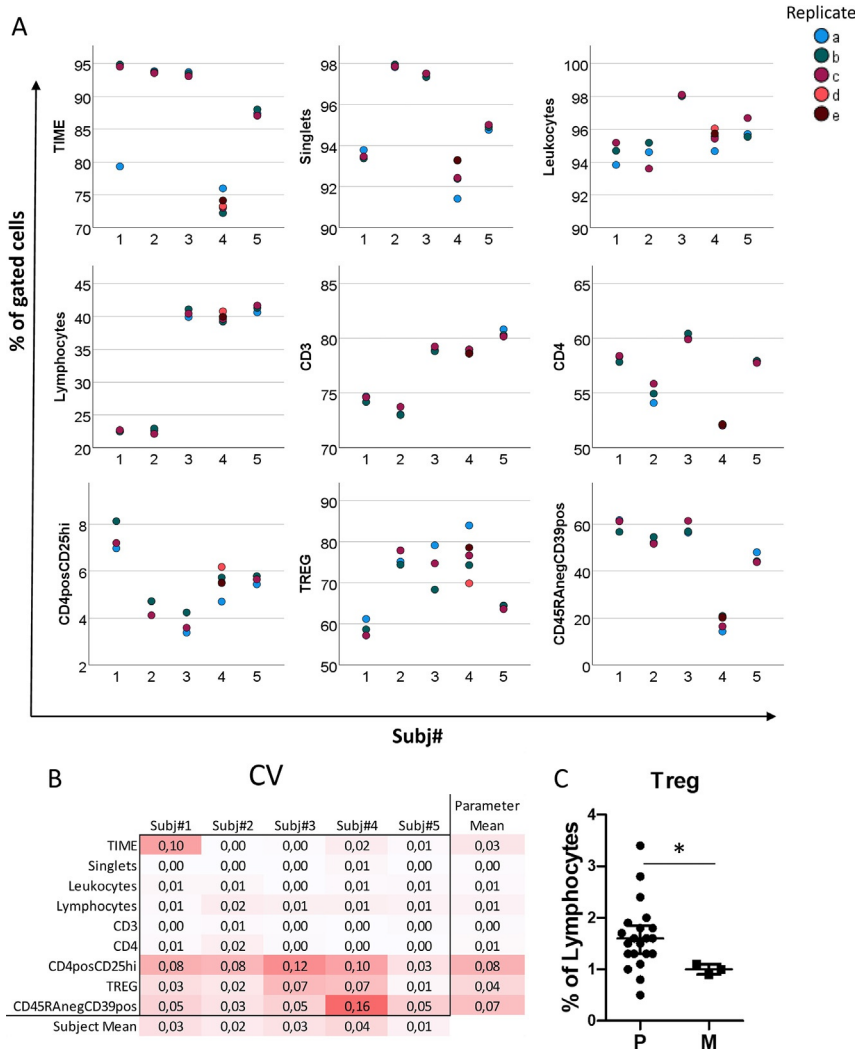

Fig. 3 Parameter distribution, CV heatmap and an applicative example. (A) Raw frequency distribution of subpopulation of interest. (B) Heatmap of CV values calculated for each parameter of interest on replicate results. (C) An applicative example: TREG frequency was assessed in primitive (P, $n = 21$) and metastatic (M, $n = 3$) breast cancer patients. *P calculated by U-Mann Whitney non-parametric test (unpublished data).

The described panel has passed intra-assay validation in our laboratory, and we have been applying it in several clinical and translational studies, such as in the context of breast cancer patients, where we found that primitive cancer patients had statistically significant higher levels of TREG cells if compared to metastatic patients (Fig. 3C, unpublished data).

A subsequent step could involve verifying its inter-laboratory reliability through a harmonization project, making it applicable in the context of immunological monitoring within multicenter studies (Macchia et al., 2020).

5. Notes

1. Catalog number and provider are indicated as a reference, but equivalent disposable items with equivalent characteristics may be used.
2. Check compatibility with your own flow cytometer.
3. Reagents with equivalent characteristics may be used, except for the indicated rat anti-human FoxP3 and the FoxP3/Transcription Factor Staining Buffer Set, which are strongly recommended.
4. Not classified under the Hazardous Products Current Regulations.
5. Heat-inactivated for 30′ at 56 °C.
6. This substance is classified as not hazardous according to the current regulation.
7. H332—Acute toxicity, Inhalation. H373—Specific target organ toxicity—repeated exposure, Respiratory Tract.
8. H300 + H310 + H330—Fatal if swallowed, in contact with skin or if inhaled. H373—May cause damage to organs (Brain) through prolonged or repeated exposure if swallowed. H410—Very toxic to aquatic life with long lasting effects.
9. Not classified as hazardous according to current regulation.
10. H302 + H312 + H332—Harmful if swallowed, in contact with skin or if inhaled. H314—Causes severe skin burns and eye damage. H317—May cause an allergic skin reaction. H335—May cause respiratory irritation. H341—Suspected of causing genetic defects. H350—May cause cancer. H370—Causes damage to organs. H373—May cause damage to organs through prolonged or repeated exposure. H402—Harmful to aquatic life.
11. The fluorescent-conjugated antibodies contain no substances which at their given concentration, are considered to be hazardous to health.
12. This reagent is strongly recommended since, in our laboratory, it has been compared with several other commercial kits, yielding the best results for the intra-nuclear staining with eBioscence anti-human FoxP3.
13. H317—May cause an allergic skin reaction. H341—Suspected of causing genetic defects. H350—May cause cancer. H370—Causes damage to organs.
14. This item is not a hazardous substance and does not contain hazardous ingredients or substances according to current regulation.

15. Equipment with equivalent characteristics may be used.
16. To be turned on at least 15 min prior to use to allow for airflow stabilization.
17. To be used at 500–800×*g*.
18. At least 2 lasers: 488 and 630/640 nm. It must undergo an internal quality control of alignment (required), sensitivity and linearity (highly recommended).
19. Software with equivalent characteristics may be used.
20. Statistical processor is optional, since equivalent analysis and graphs can be obtained by Excel software.
21. https://www.thermofisher.com/it/en/home/references/protocols/cell-and-tissue-analysis/protocols/staining-intracellular-antigens-flow-cytometry.html
22. These procedures ensure proper matching between the markers under examination and the fluorophores conjugated to them, preventing bad staining artifacts such as excessive spillover or fluorochrome spread.
23. Antibody titration can indeed be conducted on the biological matrix of interest following relevant protocols. However, low-expressed markers might yield faint results. Therefore, it is advisable to utilize commercial ad hoc beads to mitigate this issue.
24. Resolution index = (xi − xo)/(square root of SDi squared + SDo squared), where "i" represents positive events and "o" represents negative events, "x" is the mean, and "SD" is the standard deviation.
25. Whole blood samples are taken by a specialized medical or nurse, as per current regulations.
26. Panel compensation can be conducted either on the biological matrix of interest or on VersaComp beads (https://www.beckmancoulter.com/wsrportal/techdocs?docname=B25652AA.pdf), or similar. However, low-expressed markers might yield faint results when detected on biological samples. Therefore, it is advisable to utilize commercial ad hoc beads to mitigate this issue.
27. An excess volume of each working solution should be calculated to avoid potential pipetting errors, when processing multiple samples.
28. Staining solution can be stored at +4 °C for 1 month.
29. Dilute the 10× concentrate 1:10 with room temperature (20–25 °C), deionized water. The prepared solution is stable for 1 month when stored in a glass or high-density polyethylene (HDPE) container at room temperature.
30. It is advisable to use a freshly prepared dilution.

31. As in the previous note.
32. Appropriate volume for each antibody to be diluted is established within the pre-titration phase described in Section 3.4.
33. If multiple samples are processed, it is recommended to prepare the mixture with a margin of excess to avoid issues related to potential pipetting errors.
34. Observe if the color of the liquid becomes transparent red, confirming that lysis of the red blood cells has occurred.
35. Avoid using adjacent wells to prevent cross-contamination.
36. In the case of using 96 U-bottom plates, supernatants can be discarded by inverting the plate, ensuring that the cellular pellet remains adherent to the plastic.
37. As in the previous note.
38. As in the previous note.
39. Please note that, after fixation/permeabilization, the cell pellet will become small and faint, thus possibly less visible.
40. See Notes 33 and 34.
41. See Note 37.
42. The staining of FoxP3 is sensitive to the fixation method. Cell fixation with paraformaldehyde is not recommended for FoxP3 detection (Timperi, Barnaba, & Piconese, 2017).
43. Acquisition must be performed within 2 h from staining since a delay may result in altered expression of some parameters.
44. During acquisition preview, gates are adjusted within the FSC-H vs SSC-H plot, doublets are excluded by creating a combination of same-channel bivariate plots utilizing Area vs Height (i.e., FSC-A vs FSC-H). If necessary and your software allows it, adjust the biexponential scale. Adjust each detector so that all populations are clearly defined, and the events of interest are not pushed up against axes.
45. Subj#4 samples is tested in 5 replicates (a, b, c, d, e) in our real-life project (Fig. 3A).
46. An elevated number of events is necessary to assure statistical significance also for rare cell populations (Cossarizza et al., 2021).
47. See Note 21.
48. As per example in Fig. 3B, in our real-life validation study, all parameters of interest passed accuracy testing.
49. There are several commercial anti-human FoxP3 Abs that may give slightly different results. In our opinion, the PCH101 clone (eBioscience) gives the most reliable results in combination with CD127 (Law et al., 2010).

Acknowledgments

We would like to thank the enrolled subjects who have voluntarily donated blood, the Blood Bank of the Umberto I Polyclinic, the cytometry facility at Istituto Superiore di Sanità (FAST), as well as Mrs. Fabiola Diamanti, and Mr. Teodoro Squatriti for their technical support. We also thank Valentina La Sorsa for her valuable help in revising the article.

Competing interests

Each author declares no conflict of interest that could significantly influence the objectivity or integrity of the results presented in this scientific paper.

References

Biancotto, A., Dagur, P. K., Fuchs, J. C., Langweiler, M., & McCoy, J. P. (2012). OMIP-004: In-depth characterization of human T regulatory cells. *Cytometry. Part A: The Journal of the International Society for Analytical Cytology*, *81*(1), 15–16. https://doi.org/10.1002/cyto.a.21158.

Borsellino, G., Kleinewietfeld, M., Di Mitri, D., Sternjak, A., Diamantini, A., Giometto, R., et al. (2007). Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. *Blood*, *110*(4), 1225–1232. https://doi.org/10.1182/blood-2006-12-064527.

Brando, B., & Sommaruga, E. (1993). Nationwide quality control trial on lymphocyte immunophenotyping and flow cytometer performance in Italy. *Cytometry*, *14*(3), 294–306. https://doi.org/10.1002/cyto.990140310.

Cossarizza, A., Chang, H. D., Radbruch, A., Abrignani, S., Addo, R., Akdis, M., et al. (2021). Guidelines for the use of flow cytometry and cell sorting in immunological studies (third edition). *European Journal of Immunology*, *51*(12), 2708–3145. https://doi.org/10.1002/eji.202170126.

Deng, G., Song, X., & Greene, M. I. (2019). FoxP3 in T reg cell biology: A molecular and structural perspective. *Clinical and Experimental Immunology*, *199*, 255–262. https://doi.org/10.1111/cei.13357.

Flores-Montero, J., Kalina, T., Corral-Mateos, A., Sanoja-Flores, L., Pérez-Andrés, M., Martin-Ayuso, M., et al. (2019). Fluorochrome choices for multi-color flow cytometry. *Journal of Immunological Methods*. https://doi.org/10.1016/j.jim.2019.06.009.

Heldager, N., Bjerregaard, H., Persson, G., Bojesen, S., Vauvert, T., & Hviid, F. (2024). Current research in immunology an increase in regulatory T cells in peripheral blood correlates with an adverse prognosis for malignant melanoma patients—A study of T cells and natural killer cells. *Current Research in Immunology*, *5*(September 2023), 100074. https://doi.org/10.1016/j.crimmu.2023.100074.

Law, J. P., Hirschkorn, D. F., Owen, R. E., Biswas, H. H., Philip, J., & Lanteri, M. C. (2010). The importance of Foxp3 antibody and fixation/permeabilization buffer combinations in identifying CD4+ CD25+ Foxp3+ regulatory T cells. *Cytometry A*, *75*(12), 1040–1050. https://doi.org/10.1002/cyto.a.20815.

Levings, M. K., Allan, S., & Picc, A. (2006). Functional dynamics of naturally occurring regulatory T cells in health and autoimmunity. *Advances in Immunology*, *92*(2006), 119–155. https://doi.org/10.1016/S0065-2776(06)92003-3.

Liu, W., Putnam, A. L., Xu-yu, Z., Szot, G. L., Lee, M. R., Zhu, S., et al. (2006). CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4 + T reg cells. *The Journal of Experimental Medicine*, *203*(7), 1701–1711. https://doi.org/10.1084/jem.20060772.

Liyanage, U. K., Moore, T. T., Joo, H.-G., Tanaka, Y., Herrmann, V., Doherty, G., et al. (2002). Prevalence of regulatory T cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. *The Journal of Immunology*. https://doi.org/10.4049/jimmunol.169.5.2756.

Macchia, I., La Sorsa, V., Ruspantini, I., Sanchez, M., Tirelli, V., Carollo, M., et al. (2020). Multicentre harmonisation of a six-colour flow cytometry panel for Naïve/memory T cell Immunomonitoring. *Journal of Immunology Research*, *2020*, 1–15. https://doi.org/10.1155/2020/1938704.

Manuszak, C., Brainard, M., Thrash, E., Hodi, F. S., & Severgnini, M. (2020). Standardized 11-color flow cytometry panel for the functional phenotyping of human T regulatory cells. *Journal of Biological Methods*, 7(2), 1–6. https://doi.org/10.14440/jbm.2020.325.

Miyara, M., Yoshioka, Y., Kitoh, A., Shima, T., Wing, K., Niwa, A., et al. (2009). Functional delineation and differentiation dynamics of human CD4 + T cells expressing the FoxP3 transcription factor. *Immunity*, *30*, 899–911. https://doi.org/10.1016/j.immuni.2009.03.019.

Niec, R. E., & Waldmann, H. (2013). The plasticity and stability of regulatory T cells. *Nature Reviews Immunology*, *13*(6), 461–467. https://doi.org/10.1038/nri3464.

Okeke, E. B., & Uzonna, J. E. (2019). The pivotal role of regulatory T cells in the regulation of innate immune cells. *Frontiers in Immunology*, *10*(April), 1–12. https://doi.org/10.3389/fimmu.2019.00680.

Pitoiset, F., Barbié, M., Monneret, G., Braudeau, C., Pochard, P., Pellegrin, I., et al. (2018). A standardized flow cytometry procedure for the monitoring of regulatory T cells in clinical trials. *Cytometry Part B - Clinical Cytometry*, *94*(5), 621–626. https://doi.org/10.1002/cyto.b.21622.

Plitas, G., & Rudensky, A. Y. (2020). Regulatory T cells in cancer. *Annual Review of Cancer Biology*. https://doi.org/10.1146/annurev-cancerbio-030419-033428.

Sakaguchi, S., Mikami, N., Wing, J. B., Tanaka, A., Ichiyama, K., & Ohkura, N. (2020). Regulatory T cells and human disease. *Annual Review of Immunology*, *38*, 541–566. https://doi.org/10.1146/annurev-immunol-042718-041717.

Santegoets, S. J. A. M., Dijkgraaf, E. M., Battaglia, A., Beckhove, P., Britten, C. M., Gallimore, A., et al. (2015). Monitoring regulatory T cells in clinical samples: Consensus on an essential marker set and gating strategy for regulatory T cell analysis by flow cytometry. *Cancer Immunology, Immunotherapy*, *64*(10), 1271–1286. https://doi.org/10.1007/s00262-015-1729-x.

Takeuchi, Y., & Nishikawa, H. (2016). Roles of regulatory T cells in cancer immunity. *International Immunology*, *28*(8), 401–409. https://doi.org/10.1093/intimm/dxw025.

Timperi, E., Barnaba, V., & Piconese, S. (2017). Phenotypic and functional analysis of the suppressive function of human regulatory T cells. *Methods in Molecular Biology*, *1514*, 139–151. https://doi.org/10.1007/978-1-4939-6548-9_12/FIGURES/2.

Wegrzyn, A. S., Kedzierska, A. E., & Obojski, A. (2023). Identification and classification of distinct surface markers of T regulatory cells. *Frontiers in Immunology*, *13*(January), 1–12. https://doi.org/10.3389/fimmu.2022.1055805.

Wolf, A. M., Wolf, D., Steurer, M., Gastl, G., Gunsilius, E., & Grubeck-Loebenstein, B. (2003). Increase of regulatory T cells in the peripheral blood of cancer patients. *Clinical Cancer Research*.