Chapter 19

Classification of the Immune Composition in the Tumor Infiltrate

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Abstract

Flow cytometry is one of the most suitable techniques for analyzing and classifying different cell suspensions derived from blood or others compartments. The characterization of all different cellular subtypes is made with different antibodies that detect surface or intracytoplasmic antigens. Here we describe the technique to thoroughly characterize immune cells from tumor infiltrates and proceed to isolation using single-cell sorting.

Key words Flow cytometry, FACS analysis, Immune system, Immune infiltrate, Tumor immunology, Single-cell sorting

1 Introduction

Fluorescence-activated cell sorting (FACS) is increasingly used worldwide in several areas of clinical and translational research [1, 2], for phenotypic characterization and functional activity by measuring cytokines, intracellular signaling and cell proliferation. It is a rapid, sensitive, quantitative, single-cell analysis technique that still may give impetus to new developments, such as the identification of circulating tumor cells (CTS) [3] or microvesicles [4, 5].

A flow cytometer is constituted of three main systems: fluidics, optics and electronics. The fluidics is the system used for particles transportation, in a single-cell suspension, through the hydrodynamic focusing in front of the laser beam, in the so-called interrogation point. Here cells are enlighten by the lasers and all the characteristics are sent to the optical system, where the fluorescence is divided by band pass filters to select each wavelength detected by photomultipliers (PMT). PMTs are the electronic system converting light signals in electronic signals, for further processing by the computer.

Flow cytometry is used for characterization of cells through scatter analysis and fluorescence.

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Scatter analysis describes the size (forward-scattered light or FSC) and internal complexity (side-scattered or SSC) according to the presence of granules and polylobate nuclei.

Fluorescence identifies the different types of white blood cells in lymphocyte, monocyte, macrophage, and granulocyte compartments. Inside the lymphocyte compartment there are many subpopulations, as T, B, NK, T-reg cell subsets that we cannot identify with scatter analysis. Consequently, we need surface markers to identify the specific cell subsets. These markers are called *Cluster Designation* (CD) and are identified by monoclonal antibodies that in flow cytometry are conjugated by fluorochromes [6].

Fluorochromes are particular molecules that, once irradiated by a laser source, emit fluorescence in a higher wavelength then the excitation one. Emission lights are recorded by the flow cytometer PMTs and data are showed as dots in a Cartesian plane called dot plot. The two axes represent the emission fluorescences and cells are represented as dots according to the amount of fluorescences expressed.

Since flow cytometry has always been used to detect different cell subpopulations in different blood diseases, we apply this method to detect immune subpopulations in tumor infiltrates. For this purpose, we process the surgical biopsies of different tumors in order to get a single-cell suspension. Subsequently we stained these samples with a large number of antibodies in order to obtain as much information as possible on cellular subpopulations. Identified cells can be easily isolated with a single-cell sorter: this instrument is suitable for recovering all the needed cells from the total suspension, making them available for further protein, RNA, and DNA analysis by next generation sequencing (NGS).

2 Materials

All antibodies and buffers should be stored at 4 °C and used within the expiration date. Renew and filter the buffers each week.

2.1 Samples Preparation Buffers

1. Washing buffer: phosphate buffered saline (PBS) without Ca^{2+}/Mg^{2+} with 0.5% BSA (*see* **Note 1**).
2. Digestion buffers: trypsin, Accutase, or collagenase type 1 (10 μg/mL) + DNAse (10 μg/mL). Prepare and use according to the type of the tissue (*see* **Note 2**).
3. Gradient buffer: Ficoll (*see* **Note 3**).
4. Blocking buffer: PBS 1–2% BSA or commercially available solutions blocking the Fragment Crystallizable (FcR) receptors (*see* **Note 4**).
5. Viability dyes: PI, 7-AAD, DRAQ5, DRAQ7, or DAPI.

2.2 FACS Buffers

1. Staining buffer: PBS w/o Ca^{2+}/Mg^{2+}, 1% BSA, 1–2 mM EDTA (*see* **Note 5**).
2. Fixation buffer: 2–4% paraformaldehyde (PFA) solution (*see* **Note 6**).
3. Permeabilization buffers: 0.1% saponin PBS (*see* **Note 7**).

2.3 Antibodies and Beads

1. Purified monoclonal antibodies or fluorochrome-conjugated antibodies (Abs) according to the analysis needed (*see* **Note 8**) should be titrated before use by each laboratory.
2. Compensation beads to set the right compensation between fluorochromes in a multiparametric staining.
3. Calibration beads to check the daily performances of the instrumentation.

2.4 Tubes and Filters

1. 5 mL 12 × 75 mm FACS tubes.
2. 70–100 μm tube filters.

2.5 Sorting Buffers

1. Resuspension buffer: PBS, 1–2% BSA, 1–2 mM EDTA.
2. Recovering buffers: Complete medium (5–10%FBS, 25 mM HEPES in DMEM/RPMI).

3 Methods

All procedures have to be performed at room temperature except where differently indicated.

3.1 Samples Preparation

1. Partially digest the tumors or biopsies using the digestion buffer and make 3–5 different injections into the tissue with a syringe according to the size of tumor tissue, in order to smash easily the tissue afterward. Incubate for 30 min at 37 °C.
2. Take the sample and place it on a 100 μm nylon strain and use the syringe plunger to smash and get the single-cell suspension through the strainer. Block the digestion before proceeding with further steps with ice-cold complete medium and wash out the buffer (*see* **Note 9**).
3. Tissue homogenate can be stratified on Ficoll (density of 1077 g/mL) to separate the white blood cells (WBC) from parenchymal and dead cells or debris. Add 5 mL of Ficoll in a 15 mL Falcon tube and stratify 10 mL of tissue homogenate on it (*see* **Note 10**). Centrifuge at 400 × *g* for 30 min at room temperature (RT) with no brakes (*see* **Note 11**).
4. Leukocytes are separated by red blood cells (RBC), tissue cells, and debris. Leukocytes will form an opaque layer between the Ficoll and medium, all other cells will be in the pellet at the bottom of tube. Remove and discard the top part over the layer

to recover better the opaque interphase layer containing leukocytes. Take the interphase using a pipette and add to a fresh 50 mL Falcon tube containing PBS w/o Ca^{2+}/Mg^{2+}. Centrifuge and perform three washes with PBS to remove any trace of the Ficoll (*see* **Note 12**).

5. Resuspend the cell pellet in staining buffer and count the cells always before proceeding with staining. Divide the cells in the 12×75 mm tubes with minimum of 10^5 cells up to 1.5×10^6 cells maximum (*see* **Note 13**).

3.2 Staining Procedure

1. Take the amount of cells needed and proceed with FcR blockade at 4 °C for 10–15 min if your cells express high levels of Fc receptors.
2. Proceed directly with staining without washing. Abs are added in 100 μL of staining buffer for the pellet resuspension in the right concentration according to datasheet of the producer or according to the laboratory titrations [7].
3. Incubate for 15–20 min at room temperature (RT) protecting from light (*see* **Note 14**).
4. Resuspend the cells in wash buffer and centrifuge to discard the surnatant. Add 100–200 μL of resuspension buffer for the analysis (*see* **Note 15**).
5. If Abs are unconjugated, an indirect staining is needed. Primary Abs against the cell antigen is added with the same conditions as for a direct staining. After two washes in washing buffer, the cells are resuspended again in staining buffer for the binding with secondary conjugated Abs against primary Abs. Incubate for 10 min at RT in the dark and wash the cells. Resuspend as in **step 4**.
6. If the sample cannot be read in the same day of the preparation, proceed with the fixation with the Fixation buffer.
7. PI, 7-AAD, or DAPI (if UV or violet laser is provided) is added for dead cells discrimination just before analysis (*see* **Note 16**) and only if the cells are not fixed (*see* **Note 17**).

3.3 FASC Analysis

1. The tumor infiltrate analysis consists of detecting many subpopulations in the white blood cell compartment. A multiparametric staining will be necessary to identify all the different subpopulations. In this case, a wide range of fluorochromes can be used for a variety of Abs. The compatible fluorescences and the most widely adopted are: FITC, PE, PE-Cy5, PrCP-Cy5.5, PE-Cy7, APC, APC-Cy7, BV421, BV510 [8].
2. Single color controls are required to set up the compensation matrix. It is good practice to use the BD CompBeads or polystyrene microparticles coupled to an antibody specific for the

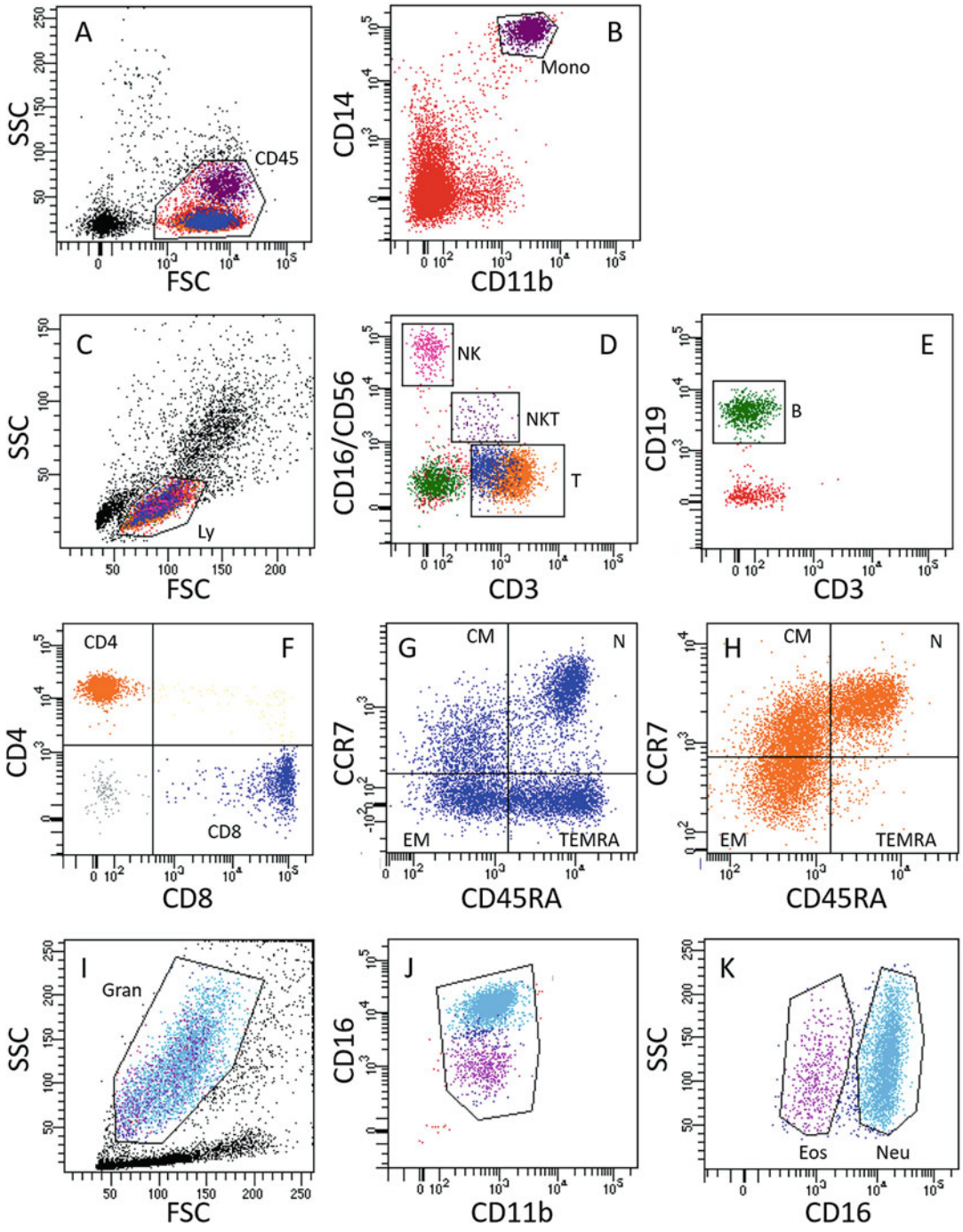

Fig. 1 Representative flow-cytometric panel of human blood cell sample analysis. (**a**) CD45+ gate on leukocytes. (**b**) Monocytes isolation identified as $CD11b^{+}CD14^{+}$ cells. (**c**) Morphological gate on the lymphocytes discriminating the cellular debris according to FSC and SSC. (**d**) Identification of T cells ($CD3^{+}$), NK ($CD16^{+}CD56^{+}CD3^{-}$), and NKT ($CD3^{+}CD16^{dim}$) subsets. (**e**) Gated on $CD3^{-}CD16^{-}CD56^{-}$ it is possible to identify B cells ($CD19^{+}$). (**f**) $CD4^{+}$ (Th) and $CD8^{+}$ (CTL) expression gated on $CD3^{+}$ cells. Identification of naïve

Kappa light chain of mouse, rat, or rat/hamster Ig to perform the single staining controls according to the manufacturer's protocol [7] (*see* **Note 18**).

3. Panel Design Strategy: The different Abs against antigens for a tumor infiltrate analysis are many, according to the analysis the researchers are interested in. The most studied are CD45, CD3, CD8, CD4, CD11b, CD11c, CD14, CD19, CD20, CD62L, CD27, CD28, CD68, CD86, CD138, CD206, MHCII, etc. in order to detect all different subpopulations of T, B, macrophages, monocytes, etc. (*see* **Note 19**).
4. Before starting with the analysis proceed always and every day with the check performance and tracking (CS&T) (*see* **Note 20**).
5. Design the correct gates, discriminating between positive and negative cells and accordingly perform the sample recording. Gating strategy is the fundamental part of flow cytometry analysis in order to take into consideration the right subpopulation to be studied and sorted. The best way to determine the fluorescence gating strategy is to use fluorescence minus one (FMO) controls [9] (*see* **Note 21**).
6. Representative results: all different leukocytes subpopulations can be analyzed. Since the analysis is to be done on tumor infiltrate, it is better to select all the leukocytes with CD45 as first (Fig. 1a). Then, a morphological gate is necessary to identify the lymphocytes (Fig. 1c, d) detected as T cells ($CD3^+$) divided in the two major subsets T helper ($CD4^+$) and cytotoxic T lymphocytes ($CD8^+$) (Fig. 1f). Both $CD4^+$ and $CD8^+$ cells could be further divided on the basis of CD45RA and CCR7 (or in alternative CD62L) staining into maturational subsets, defined as naïve $CCR7^+CD45RA^+$, central memory $CCR7^+CD45RA^-$, effector memory $CCR7^-CD45RA^-$, and terminally differentiated memory cells $CCR7^-CD45RA^+$ (Fig. 1g, h). T-reg cells gated on $CD4^+$ as $CD127^-CD25^{hi}FoxP3^+$ [10] is very important in the tumor infiltrate analysis because T-regs represent one of the immunosuppressive subpopulations that drive the tumor escape mechanisms. B cells (Fig. 1e) can be identified as $CD19^+CD20^+$, with the subpopulation of B-regs [11] identified as $IgD^-IgM^-CD1d^{hi}CD24^{hi}CD38^{hi}$. Naïve ($CD27^-$) and memory ($CD27^+$) B cells can be also analyzed. Plasma cells

Fig. 1 (continued) ($CCR7^+CD45RA^+$), central memory (CM, $CCR7^+CD45RA^-$), effector memory (EM, $CCR7^-CD45RA^-$), and terminally differentiated effector RA+ ($CCR7^-CD45RA^+$) subpopulations gated on $CD4^+$ (**g**) and $CD8^+$ (**h**) cells. (**i**) Morphological gate on granulocytes identified as higher SSC in comparison to lymphocytes and monocytes. (**j**) Subsequent gate on myeloid cells ($CD11b^+$) and identification of neutrophils ($CD16^{high}$) and eosinophils ($CD16^{dim}$) (**k**)

(PC) can be identified as $CD19^+CD20^-CD138^+$. NK cells as $CD16^+CD56^+$ gated on $CD3^-CD19^-$ cells, but NKT as $CD3^+CD16^+CD56^+$ are easily identified (Fig. 1d). Monocytes can be identified as $CD14^+CD11b^+$ (Fig. 1b) and eventually also as $CD4^{dim}$ gated on higher SSC width respect to lymphocytes. Macrophages are selected as $CD11b^+CD68^+$ and divided in proinflammatory M1 ($MHCII^+$) and tumor associated macrophages M2 ($CD206^+$) [12]. For myeloid derived suppressor cells (MDSC) there is still no complete accordance but they can be identified as $CD14^{+/-}CD33^+$MHC-II^- [13]. Dendritic cells are selected as $SSC^{hi}CD14^-CD11b^+CD11c^+$MHC-$II^+CD86^+CD83^+$ [10]. Gated on granulocyte scatter (SSC^{hi}) it is possible to identify eosinophils as $CD66b^+CD16^-$, neutrophils as $CD66b^+CD16^+$, and basophils as $CD66b^+CD16^+CD294^+$ [9] (Fig. 1).

3.4 Single-Cell Sorting

FACS is a highly sophisticated technique for purifying cell populations at the highest degree of purity, reaching 95–100% of the sorted population [7].

Therefore, this technique shows its best results in experiments where high purity is an essential requirement (e.g., microarray analysis) [14]. In the last years the development of ultrahigh-speed sorters has further extended the possibilities of application of flow sorting in clinical settings. The FACS potential clinical applications may now include purification of blood stem cells from human blood for therapeutic purposes [15], applications in cancer therapy [1], amniocentesis replacement [7], and sorting human sperm [16].

Single-cell sorting has a great impact on cellular development analysis [17]. Lymphocyte development of Ig or TCR gene rearrangement can be amplified by PCR and the genetic basis of the immune response characterized [18].

With the development of the clone sorting system, it becomes possible to identify the phenotype of the single cell that self-renewed or gave rise to differentiated progeny.

1. Once the populations have been determined, select them with a designated gate and recover only the specific cells into collection tubes. Up to four gated populations can be sorted at once. For plate sorting, only one population at a time can be taken.
2. Select the appropriate nozzle depending on the cell type to be sorted. For sterile sort, sterilize the instrument. If a sterile sort is performed, the cells can be cultured.
3. Run the experimental sample tube at 4 °C, turn on deflection plates, and sort the sample.
4. Set the selection and sorting mask on the single-cell sorting, in order to discard all doublets and to take just one cell in one well

of 96-or 384-well plate containing a lysis buffer to lyse the cells and at the same time preserve the RNA/DNA content.

5. Store the plate at −80°C for further analysis of gene expression and RNAseq or single cell NGS.

4 Notes

1. The use of Ca^{2+}/Mg^{2+} buffers may increase the formation of cellular aggregates or doublets that will be discarded in the analysis, with the consequence of a reduction in number of analyzed cells.
2. Many organs from mouse or human tissue biopsies need to be digested before staining to get a single-cell suspension. All different tissues need to be tested for the best enzymatic digestion in order not to interfere with the epitope expression. Moreover, the enzymatic treatment can interfere on cell viability. For this reason, it is best to add a viability marker (PI, 7-AAD, DAPI, etc.) during the analysis.
3. As a gradient separator, Ficoll is appropriate to isolate the viable cells and remove dead cells, fragments and debris.
4. Stain without FcR blockade may incur to unspecific binding especially if staining involved B cells, monocytes, and macrophages. In this case, FcR blocking is always required. Alternatively, there are many new Abs that are mutated in the Fc sequence: these Abs do not bind in an unspecific way. In this case, this step can be avoided.
5. EDTA buffers help to maintain the single-cell suspension avoiding the cluster and doublets formation. Check always which is the best concentration of EDTA for the cells, normally 1 to 2 mM of EDTA is ok.
6. The fixatives use depends on the type of intracellular staining needed. For staining of intracytoplasmic antigenic proteins, cytokines, and chemokines, 2–4% PFA buffer is the most widely used. This buffer is used also at the end of staining to fix and keep the cells in the fridge before the analysis. Other buffers may contain ethanol 70% for staining of intranuclear transcription factors. Alternatively, commercially available Fix&Perm Buffers from different suppliers are used.
7. Fix&Perm buffer is suitable for intracytoplasmic staining. In this case, cells should be kept at 4 °C for longer and particularly difficult staining. Fixed cells cannot be recovered from cell sorting for cellular culture.
8. Monoclonal Abs are to be used in preference to the polyclonal Abs due to high unspecific binding.

9. Homogenization of tissues should always be performed on ice, working on a petri dish to cut and smash the tissue.
10. Pay attention not to mix the sample with the Ficoll, otherwise it will prevent the white blood cell band formation.
11. Brakes during start and stop of centrifugation may interfere with the layer formation of WBC; deactivate this option from the centrifuge. Set the centrifuge at RT and not at 4 °C because low temperatures interfere with the WBC layer formation.
12. Ficoll is a glucose-based solution and may interfere with cell viability if not removed correctly after the stratification. Proceed always with at least three washes.
13. If the amount of cells is low, it is also possible to seed the cells into a 96-well plate and proceed with staining directly there. Add the mixture of Abs, wash the cells adding 150–200 μL of washing buffer, and centrifuge the plate with the plate adaptor. Discard the supernatant quickly flipping the plate into the sink.
14. Perform staining always in the dark to prevent fluorochromes from being destroyed by direct light, above all if tandem dyes are used.
15. If the cells are prone to clump, make sure to have a single-cell suspension without aggregates before analysis, check on the microscope and if any proceed with a further filtration step with a 70 μm strainer.
16. PI and 7-AAD are detected on PrCP channel but may interfere with the PE. Make sure to set all the compensations with the other channels in the correct way.
17. If fixation is performed, it will not be possible to use discriminator of viable staining because these particular dyes are able to pass the membrane of injured cells and bind the DNA. If cells are already fixed the membrane could not be intact anymore. Pay attention to avoid this step if cells are fixed.
18. Compensation is necessary to remove the spectrum overlap of one fluorescence between two detectors. CompBeads (BD) allow to make an automatic compensation by calculating an algebraic matrix for all the fluorophores used in the experiment. In a multiparametric experiment, it is a good practice, and it is very helpful for the researcher as it reduces the setup times of the experiment.
19. For best results, the panel design should consider the fluorochromes brightness. Bright fluorochromes should be conjugated to antibodies detecting low expressed markers. On the contrary, markers that are well expressed and provide a good separation between negative and positive cell populations must be used with less brilliant fluorochromes.

20. CS&T is a daily check made by the operator in order to see if the laser power has the same performance during the time. The CS&T beads (BD) are required to run the software in an automatic way. This performance check is always required in order to work always in the same condition during the experiment progress in different days.
21. In an FMO (fluorescence minus one) control tube, all reagents used in the experiment, are included except one. Create as much FMO tubes as the number of fluorochromes used. For example, if staining is made of three Abs conjugated with FITC, PE, and APC, the FMO tubes will be FMO FITC (only with PE and APC fluorescence), FMO PE (only with FITC and APC), and FMO APC (only with FITC and PE). The FMO helps to discriminate between dimly stained and broad negative populations. The FMO tubes are useful to determine where to place the positivity markers in a plot.

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