Chapter 8

Analyzing the Tumor Microenvironment by Flow Cytometry

Yoon Kow Young, Alicia M. Bolt, Ryuhjin Ahn, and Koren K. Mann

Abstract

Flow cytometry is an essential tool for studying the tumor microenvironment. It allows us to quickly quantify and identify multiple cell types in a heterogeneous sample. A brief overview of flow cytometry instrumentation and the appropriate considerations and steps in building a good flow cytometry staining panel are discussed. In addition, a lymphoid tissue and solid tumor leukocyte infiltrate flow cytometry staining protocol and an example of flow cytometry data analysis are presented.

Key words Tumor microenvironment, Solid tumor, Immune cell infiltrate, Flow cytometry (FCM), Staining panel, Intracellular staining, Fluorescence-activated cell sorting (FACS), Multi-parameter, Isotype control, Spillover, Compensation, Fluorescence minus one (FMO)

1 Introduction to Flow Cytometry

1.1 General Information on Flow Cytometry

The tumor microenvironment consists of the basement membrane, extracellular matrix, immune cells, fibroblasts, and capillaries that form an intricate network at the primary tumor and metastatic niche to play an important role in tumor progression and metastasis. Flow cytometry is a quantitative tool that has been successfully used to characterize and isolate the heterogeneous components of the tumor microenvironment in clinical patient and cancer animal model samples. For example, using flow cytometry, researchers have identified increased levels of CD4+ CD25+ regulatory T-cells in the peripheral blood, tumor, and lymph nodes of breast and pancreatic cancer patients [1]. Using a mouse model of colorectal cancer, flow cytometry was used to determine increased levels of Gr1+ CD11b+ myeloid-derived suppressor cells in the tumor and spleen of animals, which played a role in enhanced angiogenesis and tumor growth [2]. In addition, researchers have used flow cytometry to characterize the infiltration of bone-marrow-derived myeloid and natural killer cells in the pre-metastatic niche in breast and melanoma cancer models [3]. In this chapter, we discuss flow cytometry

Josie Ursini-Siegel and Nicole Beauchemin (eds.), *The Tumor Microenvironment: Methods and Protocols*, Methods in Molecular Biology, vol. 1458, DOI 10.1007/978-1-4939-3801-8_8,

methodology and how to design and run a multi-parameter flow cytometry panel to characterize the tumor microenvironment at the site of primary tumor and metastatic niche.

Flow cytometry technology is an attractive tool for studying the tumor microenvironment, because it is a rapid and quantitative means to assess multiple markers (called parameters) in several cell populations from a cell suspension at single-cell resolution. Since its conception in the 1960s, there have been several major technological advances [4, 5]. Of these, the increase in the number of parameters that can be evaluated per sample has greatly advanced our understanding of cellular biology. Building and analyzing a multi-parameter flow cytometry staining panel can be challenging. However, smaller, more user-friendly instruments and software have made this technology more accessible.

Flow cytometers measure physical and fluorescent parameters of particles in suspension, which are termed "events." Examples of events could include cells, bacteria, organelles, or latex beads. Samples can be labeled with fluorescent probes or markers that include fluorescent dyes, fluorescent reporter genes, and fluorophores conjugated to antibodies or other proteins. Different fluorescent conjugates and dyes are commercially available and have excitation and emission characteristics that span the whole visible spectrum.

As mentioned, the power of flow cytometry rests on four main qualities. (1) *Data are acquired rapidly*. Analysis of thousands of events per second is routine. Due to the rapid speed at which data can be acquired, a large sample size can be analyzed, often in the order of millions of events. Having a large sample size effectively increases the statistical power of the data. (2) *Data are quantitative*. The fluorescence intensity observed is quantitative and therefore, the intensity for each probe compared to control can be directly related to the abundance of the parameter probed (protein content, DNA/RNA content, metabolites, etc.). (3) *Analyses can be multi-parametric*. Flow cytometry has the capability of simultaneously analyzing multiple fluorescent probes with each probe measuring a unique cellular parameter. Increasing the number of parameters increases the number of different populations and/or functions that can be analyzed simultaneously. Multiplexing multiple types of assays into one flow cytometry staining panel can increase the amount of data that can be acquired using a relatively small sample size. (4) *Every event is analyzed individually*. This allows us to simultaneously characterize multiple cell populations from a single sample. In addition, having a large number of events collected per sample makes it possible to focus on very rare cell populations by removing or "gating out" populations that are not of interest and "drill down to" or "gate into" a rare population of interests, even down to a single cell.

There are however several limitations in using flow cytometry. Flow cytometers can only analyze cells in suspension. Therefore, anatomical location of each cell within whole tissue is lost. Once

cultured cells are enzymatically treated (trypsinized) or organs mechanically dissociated, virtually all morphological parameters of the cell are altered. The cells retract their protrusions and extensions and become rounded. As we will see below, forward scatter (size) and side scatter (granularity) measurements are the only two physical parameters that are collected. Enzymes are often used to digest tissue into a single-cell suspension. These enzymes also have the potential of cleaving surface proteins of interest [6, 7]. In addition, when immunophenotyping, the antibody used to tag the cells can potentially activate them. This may alter the metabolism and expression of markers of interest. As is the case for immunofluorescent microscopy, cell fixation and permeabilization are required for intracellular immunophenotyping. This limitation impacts our ability to isolate live cells by fluorescence-activated cell sorting that differ in intracellular epitope properties.

1.2 Instrumentation

There are two main types of flow cytometers: flow cytometry analyzers and fluorescence-activated cell sorters (FACS). Both have the same analysis capability, but FACS has the additional ability to physically separate targeted populations into separate tubes for further downstream applications, such as in vitro culture or RNA, DNA, and protein isolation. Despite this added function, flow cytometers are based on the same components. There are three main components of a flow cytometer: fluidics, optics, and electronics (Fig. 1). The fluidics drives the instrument. In a large majority of instruments, pressurized sheath fluid hydrodynamically focuses the sample through the flow cells. At the interrogation point, fluidics and optics meet. High-power lasers are the excitation source of choice, since laser light is coherent and sample illumination time is extremely short. As a particle strikes the laser, light is scattered and excited fluorescent probes emit florescent light. Light-focusing and steering optics direct the scattered and fluorescent photons to detectors. Light detection optics, such as photodiodes and photomultiplier tubes (PMT), amplify and convert photon signals into electrical pulses. The electronics digitize each pulse in three integrations: pulse height, width, and area. Pulse height is the peak intensity of the pulse or the highest intensity measured. The width of the pulse is the time it takes for the event to pass through the laser or the time of flight. The area under the pulse is the sum of all heights. The area of the pulse is the preferred parameter to use for analysis, as it reflects the average intensity of each event.

The light deflection or scattering properties of each event allow for the detection of two physical parameters. Forward scatter (FSC) refers to the light deflected in front of or in line with the laser, which can tell us about the particle size. Larger events will scatter more light, increasing the FSC value. It is also important to note that other factors, such as refractive index and light absorption of the event, can also affect the FSC parameter readout by

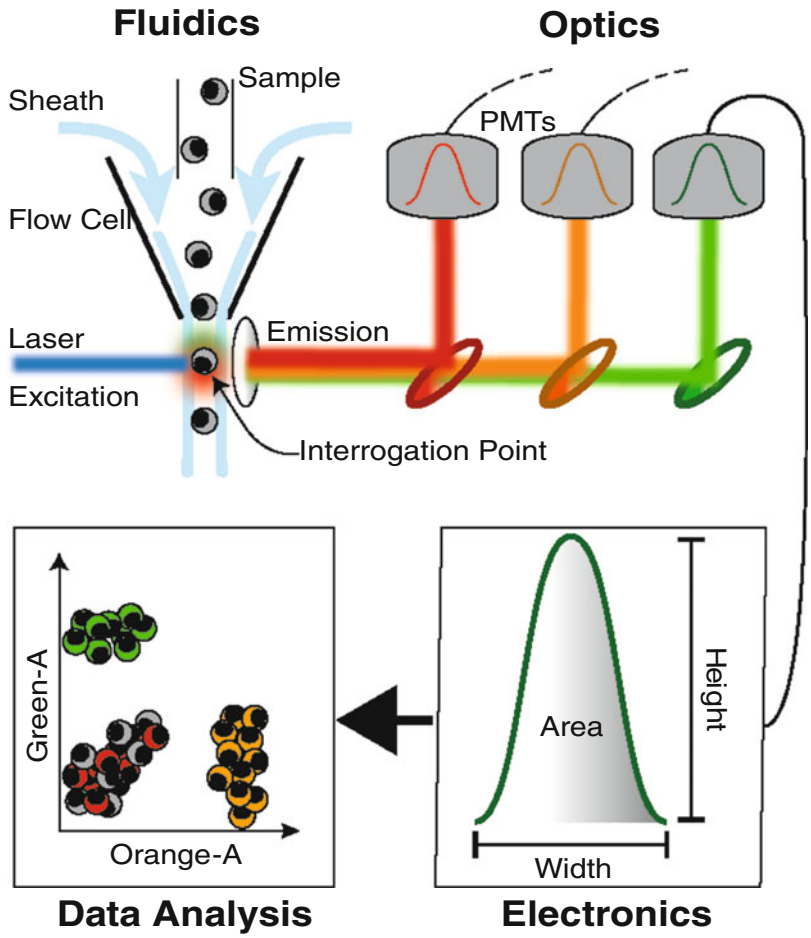

Fig. 1 Flow cytometry workflow. Flow cytometers are composed of three components: fluidics, optics, and electronics. Signals processed by the electronics are analyzed using flow cytometry software

about 2–5% [8]. The second physical parameter is side scatter (SSC), commonly referred to as granularity. Cells that have a rough outer membrane, granular vesicles, or an irregular internal structure scatter more light at right angles to the illumination source. The FSC and SSC parameters provide a rough estimate of the size and granularity of each cell, but also allow us to discriminate noise, debris, dead cells, and aggregates from the cells of interest.

Fluorescence emission is also measured at roughly right angles to the laser excitation source. Several optical components, such as lenses, fiber optics, dichroic mirrors, and band-pass filters, are used to steer and filter the emitted light, which is sent to the PMT. Each PMT is essentially collecting a discrete range of light wavelengths. The available excitation laser and the arrangement of the emission optics are highly customizable. The instrument layout of the optical components is referred to as the instrument optical configuration. It is important to note that the choice of probes must match

not only the assay, but also the instrument configuration. Improper fluorescence probe panel designs will negatively affect the sensitivity of the experiment; thus, it is recommended to consult with your flow cytometry core manager to determine your site's machine specifications, if applicable.

1.3 Panel Design

Immunophenotyping is a very common flow cytometry assay in which fluorophore-conjugated antibodies are used as probes to stain target cells with high avidity and affinity. This allows for rapid and easy phenotyping of each cell type in a heterogeneous sample according to the presence or absence of a combination of proteins. The epitope density of each event is also measured by assessing the intensity of the fluorescence, thus providing not only information on the presence or absence of an epitope, but also a quantitative measure of how many epitopes are present.

In the study of the tumor microenvironment, many researchers are interested in studying immune infiltrates, which can include, but are not limited to, lymphocytes (both T and B), macrophages, and myeloid derived-suppressor cells. These primary samples are heterogeneous, and thus it is important to be able to discriminate and identify different cell subpopulations within each sample. As mentioned, the probes must not only match the assay, but also the instrument configuration. Each fluorophore must be excitable by the available laser(s) and its emission must be detectable by one of the available emission filters.

With the right cocktail of probes, it is possible to affix each cell type with a unique set of fluorescent markers. Care must be taken to choose fluorochromes that are distinct. If two cellular proteins were stained with different probes conjugated to fluorophores with similar light excitation and emission spectra, the cytometer may not be able to differentiate them from one another. The emission profile of each fluorophore should be as spectrally separate as possible. Many manufacturers have spectra viewers to help choose the most compatible fluorophores when designing a new staining panel. However, in multi-parameter flow cytometry, the emission spectra of two distinct probes may overlap slightly and spill over into adjacent filters. Fluorescence spillover can be visually mitigated by compensation, but should be avoided or minimized as much as possible since it introduces error in the measurement [9, 10]. Spillover is seen as a spreading of the data or swelling of the negative population into affected parameters. Compensation helps to separate overlapping fluorescence so that respective populations can be more easily analyzed and gated. Adding compensation does not introduce measurement error into the data. It is the fluorescence spillover itself that causes data spread and decreases the sensitivity of the affected parameters [11]. Therefore, choosing two fluorophores that overlap increases background and decreases our ability to resolve dim double positives. The negative effect of

spillover is more apparent for very bright fluorescent signals and in turn, will decrease the sensitivity of fluorescence measured by adjacent detectors [10].

There are other important considerations when choosing fluorochromes. Fluorochromes differ in their intensity. Brilliant Violet™ 421 (BV421), phycoerythrin (PE), and allophycocyanin (APC) for example are amongst the brightest fluorophores currently available. Tagging the least abundant markers with these fluorochromes will increase our ability to visualize these events. In addition, considering the stability of the fluorophore is also important. APC and the GFP reporter proteins are large and more labile to formaldehyde and especially alcohol fixation. If a fixation step is required (intracellular staining), lower molecular weight fluorophores, such as Alexa Fluor 488, Cyanin 5 (Cy5), and Alexa Fluor 647, are more resistant to denaturation [12]. Tandem dyes consist of two dyes that are covalently coupled, so the light emission of the first excites the second by fluorescence resonance energy transfer (FRET) [13]. PE-Cy5 and APC-Cy7 dyes are common tandem dyes used in flow cytometry. For example, when PE (FRET donor) is excited by a laser, its emission can in turn excite Cy5 (FRET acceptor) and in turn emits a red shifted light (higher Stokes shift). This expands the range of fluorescence that can be emitted with a single excitation laser. However, tandem dyes are unstable and must be kept at 4 °C and protected from light [13]. The donor and acceptor can decouple causing the probes to fluoresce at the donor wavelength and the acceptor to aggregate, introducing false positive and artifacts. As in immunofluorescent microscopy, a secondary detection system, such as fluorescent-conjugated secondary antibody or avidin-biotin detection system, can be employed. Unconjugated primary antibodies can be easily labeled with a fluorescent secondary antibody, which has binding affinity to the primary antibody. Commercial kits are also available to directly conjugate primary antibody in-house. Biotinylated primary antibody coupled to a fluorescently coupled avidin secondary detection system is advantageous where there is low expression of epitopes. Since several fluorescent avidin can bind to each biotin, the signal is greatly amplified [14].

Primary samples, especially digested tissue, contain a lot of dead cells, cellular debris, and cells that are not of interest. It is sometimes very difficult to distinguish cells from dead cells and debris. Adding a live and dead discrimination marker will eliminate most of the debris and dead cells from the analysis. In addition, dead cells are highly autofluorescent and sticky and nonspecifically bind to dyes and antibodies, introducing false positives. Propidium iodide (PI) is often used because it is inexpensive and easy to use, but it cannot be used for fixed samples and its broad fluorescence emission is prohibitive. Several other live/dead dyes exist, including some that are amenable to fixation. In addition, introducing a marker of nucleated hematopoietic cells when studying immune infiltrates in a digested tumor sample is also wise (i.e.,

CD45). Teasing out these cells from all other events (debris, dead cells, and all other non-hematopoietic cells) simplifies the analysis of the target cells. These strategies can be adapted for any flow cytometry panel.

All flow cytometers are not alike and differ in their optical configuration. Thus, fluorescence measurements obtained from one instrument to another might differ greatly. It is recommended to perform a titration assay of all the fluorescent markers, even when using published assays. The background fluorescence and the positive signal should fit the linear reading range of the PMT. Several protocols have been published on how to titrate fluorescent markers properly [15, 16]. At an optimal titer, the signal-to-noise ratio is at maximum and the separation between the positive and negative populations will be at its greatest (making it easier to identify your positive population). A sample that is stained above or below the optimal titer will have lower signal-to-noise ratio. When the sample is stained with increasing number of reagents, the negative cells will start nonspecifically binding these markers, increasing background fluorescence and decreasing the separation between negative and positive fluorescence values. On the other hand, as we decrease staining reagent, the negative signal stays low but the positive signal will decrease until it merges with the negative.

1.4 Flow Cytometry Controls

A flow cytometry experiment is only as good as its controls. Three main groups of controls should be run during each assay: instrument controls, sensitivity controls, and biological controls [17]. Instrument controls include the negative or unstained controls and compensation controls. Sensitivity controls are the gating controls, which help to identify the positive cell populations. Biological controls are also gating controls with the added advantage of being biologically relevant. These three controls are an integral part of every flow cytometry experiment, stained the same day and acquired at the same time as the experimental samples. Here is a brief overview of each of these controls.

1.4.1 Instrument Controls

Unstained Control

Any unstained particle that is excited by a laser emits a baseline amount of fluorescence, autofluorescence. Cells typically show higher autofluorescence in the green spectrum. The unstained control allows us to adjust the PMT voltage gain for each detector, with the goal of adjusting the PMT gain to place the autofluorescence above the noise of the instrument. Some labs prefer to adjust the autofluorescence to three standard deviations above noise and others will set the population at a fixed intensity (above noise) for each parameter being read. Positive signal will clear the autofluorescence threshold and still be within the reading range (dynamic range) of the detector. If it is too bright, the PMT gain can be readjusted lower, putting the positive population in range. If possible, it would be wise to decrease the intensity of the positive signal for the next experiment by decreasing the titration of fluorescent label.

Compensation Control

The second set of necessary instrument controls are compensation controls. In conventional digital cytometers, compensation is an algorithm that is used to correct fluorescence spillover. For example, a flow cytometry experiment is set up to analyze the level of GFP transfection and PE antibody-labeled cells. The cytometer is equipped with a blue laser to excite both fluorophores and green and orange emission filters to collect GFP and PE fluorescence, respectively. While most of the light emitted from GFP-labeled cells is in the green spectrum, GFP-positive cells will also emit orange fluorescence in lesser intensity. The GFP green emission filter will collect most of the GFP green spectrum. However, portions of the light emitted by GFP cells will also spill over into the orange emission filter. All GFP cells will also be PE positive. With a GFP compensation control sample tube, the fluorescence spillover into PE can be properly compensated.

In order to calculate the correct fluorescence spillover compensation value, each compensation control sample tube must meet the three golden rules [18]. First, each compensation tube must contain a single positive signal, stained with the same fluorochrome as the experimental panel. Second, the autofluorescence of the negative and positive population must be identical. Third, the positive portion of the control must be as bright or brighter than the experimental sample.

The sample used for the compensation controls can be different from the test sample as long as the golden rules are met. For example, splenocytes can be a source of T-cells to prepare compensation sample tubes instead of precious tumor T-cell infiltrates. In addition, for low-expressing epitopes or rare cell types, compensation beads stained with the test-conjugated antibody are an excellent substitute. Compensation beads are engineered to capture the test antibody. It is important that the positive signal is as least as bright as the test sample, because the compensation value is more accurately calculated with a brighter fluorescence signal. A dimly stained compensation control will most often result in an underestimation of the compensation value (under-compensation). It is strongly recommended to use software automated compensation tools, because performing compensation manually will often result in overcompensation.

1.4.2 Sensitivity Controls

Sensitivity controls are negative controls. They are guides to determine the boundary between autofluorescence and positive specific antibody-binding fluorescence. There are three sensitivity controls: isotype control, fluorescence minus one (FMO) control, and antibody competition control [17].

Isotype Control

An isotype control antibody is an antibody of the same isotype as the test antibody, but has no affinity for antigen being tested. Thus, this antibody has the same constant region, but not the same variable region. In addition, if the primary test antibody is conjugated to a

fluorochrome, the isotype control is also conjugated with the same fluorochrome. Traditionally, isotype controls have been used as absolute gating controls. However, there are strong arguments that refute the validity of using this type of control as a gating control [9, 17, 19, 20]. Isotype controls can be useful in certain situations. For example, cultured primary cells may show increased nonspecific binding and an isotype control can help evaluate the increased level of background staining. When performing an intracellular stain with a conjugated primary antibody, it is difficult to wash off nonspecific antibody trapped in the cell. The isotype control can show how effective the washing steps were. Some immune cell types have high levels of Fc receptors, which can bind the staining antibody. When staining these cells, the Fc receptors need to be blocked adequately. In this case, an increase in isotype control staining will show incomplete Fc receptor blocking. Once a complete Fc receptor blocking protocol is determined, the isotype control can be omitted. However, as mentioned, the increase in background shown using isotype controls is inaccurate and only qualitative.

Fluorescence Minus One Control

Background fluorescence is affected in large multi-parameter staining assays, because increasing the number of fluorescent markers increases the inevitable fluorescence spillover. As mentioned above, fluorescence spillover increases spread of the data, increases background, and decreases sensitivity of the parameter(s) affected by spillover. This is visualized as a swelling of the background fluorescence. FMO is a very good gating guide, because it helps to determine the threshold between autofluorescence and positivity [17, 19]. An FMO control sample is stained with all the fluorescent markers within a given panel with the exception of one. Some laboratories spike in the isotype control matching the omitted test antibody marker. However, as mentioned above, this is not recommended, because isotype controls do not accurately delineate the background fluorescence boundary. With proper panel design, the amount of spillover can be minimized. FMOs account for the combined spillover effect of all other markers onto the parameter for which the stain has been omitted. They are especially important in samples where there is not a clear delimitation between background and positive fluorescence. Even when the fluorescence spillover compensation is miscalculated, FMOs can help gate for the real positive events. It is strongly recommended to do all FMOs for the first few flow cytometry assays. The use of FMOs can then be re-evaluated or even omitted for the parameters that can be easily gated. Please keep in mind that FMOs are not perfect, as we will see below.

Antibody Competition Control

To access the specificity of the antibody, the sample can be stained with a fluorescently labeled antibody with the addition of increasing amounts of a competing unlabeled antibody. If the antibody is specific, there will be a decrease of positive events with increasing unlabeled antibody. This control is useful when the specificity of the test antibody is in doubt or you are validating a new antibody.

1.4.3 Biological Controls

A biological control sample lacks the target protein or has a baseline amount of this protein of interest. This control sample is stained with the full staining panel. It allows identification and gating of positive events with certainty. It takes into account all the spillover effects just like in the FMO controls, but it is biologically relevant to the assay, for example, if we were to study the level of pSTAT1 in cancer cells. In this assay, the FMO control would not do justice because all cells express some pSTAT1. The FMO would show that the cells have no pSTAT1, which is improbable. A fully stained, unstimulated cancer cell sample will serve as a much more accurate baseline biological control. Cancer cells stimulated with IFNγ, which would increase pSTAT1, can also serve as a positive biological control. An increase or decrease in fluorescent intensity compared to baseline in pSTAT1 can then be accessed when treated.

2 Materials

2.1 Sample Preparation

1. 50 mL Conical tubes.
2. 1.5 mL Eppendorf tubes.
3. Centrifuge.
4. RPMI or DMEM Media.
5. FBS.
6. 1× Phosphate-buffered saline (PBS).
7. 1.5 or 2 mL Rubber pestles.
8. EDTA/PBS.
9. Chemical dissociates: DNase I (final concentration at 0.3 mg/mL) and collagenase B (final concentration at 2.4 mg/mL).
10. 70 μm Nylon cell strainers.
11. 37 °C Plate shaker.
12. Hemocytometer or automated cell counter.

2.2 Staining

External/Internal Staining

1. Centrifuge.
2. Cold 1× PBS.
3. Cold FACS buffer: 500 ml 1× PBS, 5% sodium azide, 5% FBS.
4. 1.5 mL Eppendorf tubes.
5. 5 mL Polystyrene round-bottom "FACS" tubes.
6. 96-Well V-bottom, clear, polystyrene microplates.
7. Live/Dead Marker.
8. Live/Dead Fixable Aqua Dead Cell Stain Kit, for 405 nm excitation.

9. Live/Dead Fixable Near-IR Dead Cell Stain Kit, for 633 or 635 nm excitation.
10. Fc block: Purified Rat Anti-Mouse CD16/CD32 (Mouse BD Fc Block).
11. Fluorescently-conjugated primary antibodies.
12. Vortex.
13. Fixation/permeabilization buffer (*see* **Note 1**).
14. Normal rat or mouse serum.

3 Methods

3.1 Sample Preparation

This protocol will focus on characterizing the immune cell infiltrates in primary tumors, although other cell types could certainly be incorporated into a staining panel. Analysis can also be performed in lymphoid tissues where it serves as a good control of the immune cell infiltrates in the primary tumor. This type of analysis also helps to distinguish systemic versus tumor-specific activation of immune cells.

The sample preparation will obviously depend upon the type of tissue. In general, solid tumors will need to be enzymatically digested in order to acquire a single-cell suspension. Other organs, particularly lymphoid tissues, can be dissociated through mechanical means.

3.1.1 Solid tumor

1. Dissect primary tumor and cut into small pieces in ice-cold 1× PBS.
2. Chemically dissociate tumor samples by incubating tumor in a 50 mL conical tube with 10 mL media supplemented with 5% FBS and 2.4 mg/mL collagenase B (*see* **Note 2**).
3. Incubate for 1.5–2 h at 37 °C on a shaker.
4. Monitor frequently and only allow reaction to continue until all the tissue is dissociated.
5. After incubation, put in 20 mL of PBS. Pipet up and down four times to dissociate tissue.
6. Centrifuge samples for 7 min at 300 × *g* at room temperature.
7. Remove supernatant, add 6 mL media containing 0.3 mg/mL DNase I, and dissociate pellet (*see* **Note 2**).
8. Incubate for 15–30 min at 37 °C on a shaker.
9. Centrifuge samples for 7 min at 300 × *g* at 4 °C.
10. Remove supernatant and resuspend in 10 mL of 1 mM EDTA/PBS. Pipet up and down to dissociate tissue.
11. Pass dissociated cells through a 70 μm filter, and rinse filter with PBS.

12. Count cells using hemocytometer or automated cell counter.

(Protocol optimized in Dr. Josie Ursini-Siegel's Laboratory, McGill University.)

3.1.2 Spleen or Other Lymphoid Tissues

1. Dissect spleen and dissociate tissue using a rubber pestle in a 1.5 mL tube with 1× PBS.
2. Pass dissociated cells through a 70 μm filter, and rinse filter with 1× PBS.
3. Count cells.

3.2 Staining Protocol

Once a single-cell suspension is obtained, cells can be stained for multiple parameters. In general, staining to detect external or cell surface antigen is performed first, followed by fixation/permeabilization steps, and staining for intracellular antigens.

3.2.1 External Staining

1. Resuspend $2–10 \times 10^6$ cells (depending on cell populations that you want to analyze and the cell type you are measuring; *see* **Note 3**) per sample in 25 μL PBS and place into staining tubes/plate (*see* **Note 4**).
2. Add 25 μL of pre-titrated Live/Dead stain mix to each sample.
3. Incubate for 30 min on ice in the dark.
4. Add 100 μL PBS per sample; spin at 350 × *g*, 4 °C, for 5 min; and decant (*see* **Note 5**).
5. Add 25 μL Fc block mix to each sample.

   Purified Rat Anti-Mouse CD16/CD32 (Mouse BD Fc Block): 2 μL per sample diluted in 23 μL FACS buffer.
6. Incubate for 30 min on ice in the dark.
7. Add 25 μL of antibody mix, already titrated antibody concentrations (*see* **Note 6**).
8. Incubate for 30 min on ice in the dark.
9. Add 100 μL FACS buffer per well; spin at 350 × *g*, 4°C, for 5 min; and decant.
10. For samples stained with only fluorescently conjugated primary antibodies against cell surface markers, add 100 μL of FACS buffer to each well and transfer to FACS tubes containing ~300 μL FACS buffer. These are ready for analysis. For samples that require a fluorescently conjugated secondary antibody, follow **steps 7–10** again.

3.2.2 Internal Staining

11. For samples that require staining of internal cell markers, stain all external markers first by following **steps 1–10**, and then continue on with **steps 12–20**.

12. Add 100 μL FACS buffer to each sample and transfer to a FACS tube containing 900 μL fixation/permeabilization buffer and vortex.
13. Incubate at 4 °C for 30 min in the dark.
14. Add 2 mL FACS buffer, spin at 300 × *g* for 5 min, and decant.
15. Resuspend in 100 μL FACS buffer.
16. Add 2 μL normal mouse/rat serum.
17. Incubate for 15 min at room temperature.
18. Add titrated internal antibodies for 30 min at room temp in the dark.
19. Add 2 mL FACS buffer, spin at 300 × *g* for 5 min, and decant.
20. Resuspend in 400 μL FACS buffer. These samples are ready for analysis.

3.3 Sample Analysis and Gating Strategies

For analysis of immune cell infiltrates within the tumor tissue, it is important to isolate the immune cells from the tumor cells at the beginning of the analysis. A helpful tip is to use a marker of nucleated hematopoietic cells to pull out the immune cell populations from the tumor cells. The leukocyte-specific marker CD45 was chosen for this analysis. Aggregates were gated out using FSC-A vs. FSC-H and SSC-A vs. SSC-H and live cells were selected. T- and B-cells were quantified using CD3 and B220, respectively. CD3– and B220– cells were then further subdivided using myeloid cell markers. The granulocytic MDSC population is CD11b+ and Gr1+. The other portion of the CD11b+ population that is Gr1– can be further subdivided by F4/80 staining. Gr1– CD11b+ F4/80– cells are monocytes and Gr1– CD11b+ F4/80+ cells are macrophages (Fig. 2). Separate FMO controls should be performed on the tumor cells to know where to place positive cell population gates for each cell type analyzed.

4 Notes

1. The fixation/permeabilization buffer is often recommended by the supplier, which is optimized for their antibody and should be used when possible. If no buffer is supplied, the fixation/permeabilization buffer will need to be optimized for each antibody [21]. In our experience, we prefer the Foxp3 Fix/Perm Staining Buffer from eBioscience for this step.
2. Some chemicals used to dissociate tumor tissue have been reported to alter cell surface marker expression on immune cells [6, 7]. Make sure to validate your specific protocol to determine how the chemical dissociation process affects surface marker expression on the cell types you are planning on analyzing in your panel before performing your experiments.
3. You should also consider how many cells you should stain per sample. In the context of analyzing immune cell components,

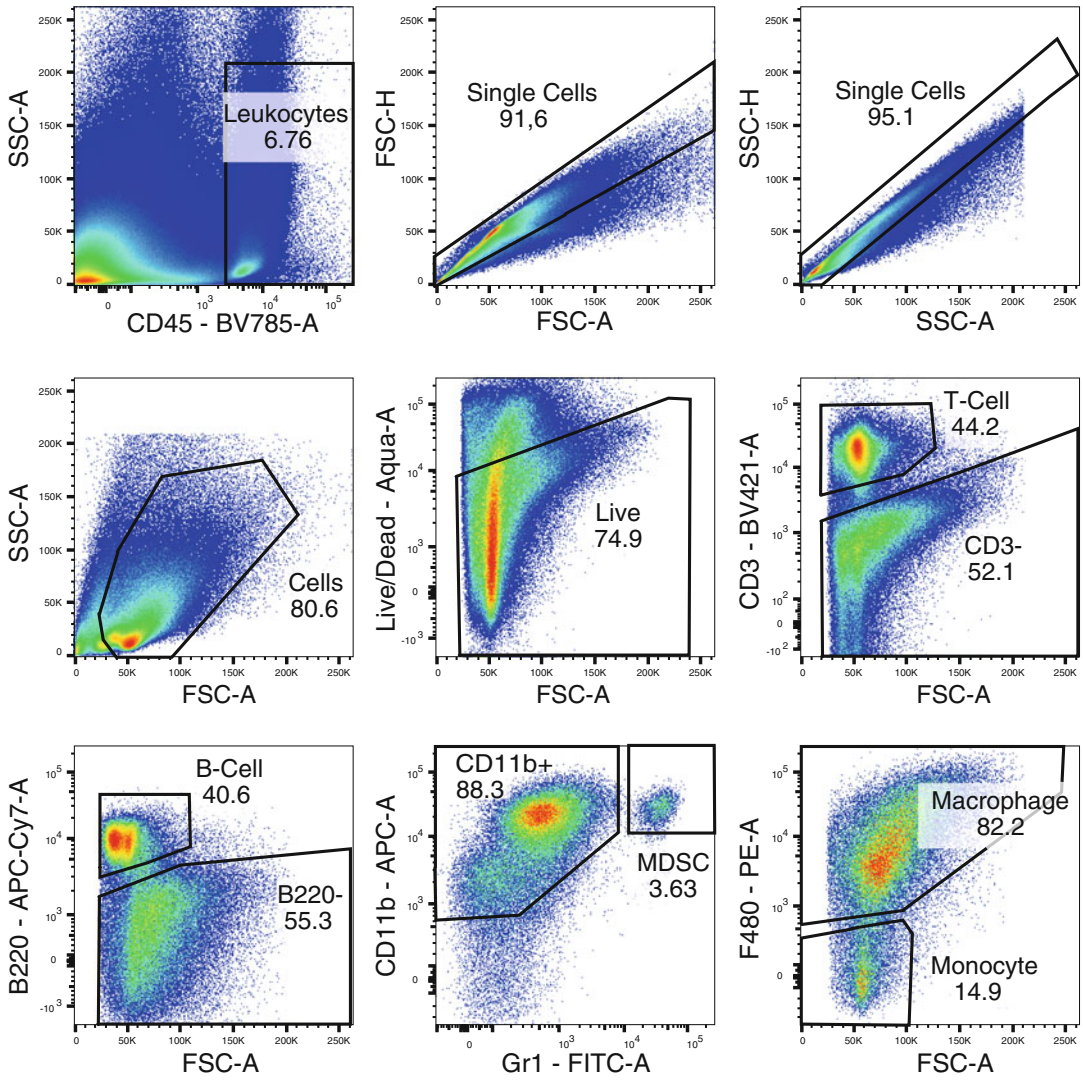

Fig. 2 Example of gating schematics to characterize the immune cell infiltrates in a primary tumor sample. A primary mouse mammary tumor was dissociated and cells were stained with fluorescently conjugated antibodies specific for immune cell subpopulations. The following markers were used in this analysis: Fixable Live/Dead Aqua, CD45 BV785, CD3 BV421, B220 APC-Cy7, CD11b APC, Gr1 FITC, and F4/80 PE

cell types from lymphoid tissues, such as spleen or thymus in which almost all the cells are lymphoid in origin, require a fewer number of cells to analyze than a more heterogeneous tissue type, such as a tumor where the number of immune cells in the total cell population is lower.

4. Samples can either be stained in tubes (FACS tubes) or 96-well microplates (with V-bottom) depending on sample volume. Staining in microplates is recommended for larger experiments to facilitate washing.
5. It is important to optimize centrifuge speed conditions in order to reduce the amount of sample lost in the wash steps.

6. All antibody concentrations within a given panel should be titrated for each cell type analyzed, taking into account the total number of cells you plan to stain per sample in your experimental design.
7. New technologies, such as imaging cytometry and mass cytometry, are bridging the gap between different technological fields and allow for more data parameters to be acquired at a single-cell resolution. Imaging cytometry allows for simultaneous visualization of the cells [22]. Mass cytometry, flow cytometry in tandem with mass spectroscopy, has brought multi-parameter analysis to a whole new level [23]. In mass cytometry, rare earth metals are used instead of fluorochromes, which eliminate fluorescence spillover, a major hurdle when combining several fluorescent probes in one staining panel. It is challenging to build panels exceeding 12 fluorescent parameters, but with mass cytometry, simultaneous detection of 30 or more parameters is possible. Since the instrumentation cost and/or availability of these young technologies are still limited, we focused the previous protocol on using classical flow cytometers to analyze components in the tumor microenvironment.

Acknowledgements

We acknowledge grant support from the Canadian Institutes of Health Research (MOP115000 to K.K.M.) and Jewish General Hospital Foundation's continued support of the LDI Flow Cytometry Facility. R.A. is supported by a fellowship from the Fonds de Recherche du Québec-Santé.

References

1. Liyanage UK, Moore TT, Joo HG, 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. J Immunol 169:2756–2761
2. Yang L, DeBusk LM, Fukuda K, Fingleton B, Green-Jarvis B, Shyr Y et al (2004) Expansion of myeloid immune suppressor Gr+ CD11b+ cells in tumor-bearing host directly promotes tumor angiogenesis. Cancer Cell 6:409–421
3. Sceneay J, Chow MT, Chen A, Halse HM, Wong CS, Andrews DM et al (2012) Primary tumor hypoxia recruits CD11b+/Ly6Cmed/Ly6G+ immune suppressor cells and compromises NK cell cytotoxicity in the premetastatic niche. Cancer Res 72:3906–3911
4. Hulett HR, Bonner WA, Barrett J, Herzenberg LA (1969) Cell sorting: automated separation of mammalian cells as a function of intracellular fluorescence. Science 166:747–749
5. Kamentsky LA, Melamed MR, Derman H (1965) Spectrophotometer: new instrument for ultrarapid cell analysis. Science 150:630–631
6. Ford AL, Foulcher E, Goodsall AL, Sedgwick JD (1996) Tissue digestion with dispase substantially reduces lymphocyte and macrophage cell-surface antigen expression. J Immunol Methods 194:71–75
7. Autengruber A, Gereke M, Hansen G, Hennig C, Bruder D (2012) Impact of enzymatic tissue disintegration on the level of surface molecule expression and immune cell function. Eur J Microbiol Immunol 2:112–120

8. Shapiro HM (2005) Practical flow cytometry, 4th edn. Wiley, New York, p 736
9. Hulspas R, O'Gorman MR, Wood BL, Gratama JW, Sutherland DR (2009) Considerations for the control of background fluorescence in clinical flow cytometry. Cytometry B Clin Cytom 76:355–364
10. Maecker HT, Frey T, Nomura LE, Trotter J (2004) Selecting fluorochrome conjugates for maximum sensitivity. Cytometry A 62:169–173
11. Roederer M (2001) Spectral compensation for flow cytometry: visualization artifacts, limitations, and caveats. Cytometry 45:194–205
12. Schmid I, Dagarag MD, Hausner MA, Matud JL, Just T, Effros RB, Jamieson BD (2002) Simultaneous flow cytometric analysis of two cell surface markers, telomere length, and DNA content. Cytometry 49:96–105
13. Hulspas R, Dombkowski D, Preffer F, Douglas D, Kildew-Shah B, Gilbert J (2009) Flow cytometry and the stability of phycoerythrin-tandem dye conjugates. Cytometry A 75:966–972
14. Kaplan D, Smith D (2000) Enzymatic amplification staining for flow cytometric analysis of cell surface molecules. Cytometry 40:81–85
15. Hulspas R (2001) Titration of fluorochrome-conjugated antibodies for labeling cell surface markers on live cells. Curr Protoc Cytom Chapter 6:Unit 6.29
16. Stewart CC, Stewart SJ (2003) Titering antibodies. Wiley, New York, pp 4.1.1–4.1.13
17. Maecker HT, Trotter J (2006) Flow cytometry controls, instrument setup, and the determination of positivity. Cytometry A 69:1037–1042
18. Roederer M (2002) Compensation in flow cytometry. Curr Protoc Cytom Chapter 1:Unit 1.14
19. Herzenberg LA, Tung J, Moore WA, Herzenberg LA, Parks DR (2006) Interpreting flow cytometry data: a guide for the perplexed. Nat Immunol 7:681–685
20. O'Gorman Maurice RG, Thomas J (1999) Isotype controls—time to let go? Cytometry 38:78–80
21. Holmes K, Lantz LM, Fowlkes BJ, Schmid I, Giorgi JV (2001) Preparation of cells and reagents for flow cytometry. Curr Protoc Immunol Chapter 5:Unit 5.3
22. McGrath KE, Bushnell TP, Palis J (2008) Multispectral imaging of hematopoietic cells: where flow meets morphology. J Immunol Methods 336:91–97
23. Giesen C, Wang HA, Schapiro D, Zivanovic N, Jacobs A, Hattendorf B et al (2014) Highly multiplexed imaging of tumor tissues with subcellular resolution by mass cytometry. Nat Methods 11:417–422