Chapter 10

Purification of Immune Cell Populations from Freshly Isolated Murine Tumors and Organs by Consecutive Magnetic Cell Sorting and Multi-parameter Flow Cytometry-Based Sorting

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Abstract

It is well established that tumors evolve together with nonmalignant cells, such as fibroblasts, endothelial cells, and immune cells. These cells constantly entangle and interact with each other creating the tumor microenvironment. Immune cells can exert both tumor-promoting and tumor-protective functions. Detailed phenotypic and functional characterization of intra-tumoral immune cell subsets has become increasingly important in the field of cancer biology and cancer immunology. In this chapter, we describe a method for isolation of viable and pure immune cell subsets from freshly isolated murine solid tumors and organs. First, we describe a protocol for the generation of single-cell suspensions from tumors and organs using mechanical and enzymatic strategies. In addition, we describe how immune cell subsets can be purified by consecutive magnetic cell sorting and multi-parameter flow cytometry-based cell sorting.

Key words Tumor microenvironment, Immune cells, Myeloid cells, Magnetic cell sorting, Enrichment, Fluorescence-activated cell sorting (FACS), Solid tumor, Preclinical mouse model

1 Introduction

It is now well established that immune cells present in the tumor microenvironment play a critical role in tumor development and progression [1]. Most human and experimental tumors are abundantly infiltrated with various immune cell types. The presence of some of these immune cells, including regulatory T cells, macrophages, and myeloid-derived suppressor cells, is frequently associated with a poor prognosis [2–4], while other immune cells, including $CD8^+$ T cells and NK cells, frequently correlate with good prognosis [5, 6]. Experimental studies using transgenic mouse models for de novo tumorigenesis have been instrumental in identifying the functional significance of the various immune cell subsets in tumor development, progression, and therapy response [4, 7–11].

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In these transgenic tumor models, tumors develop spontaneously in their natural microenvironment and immune cells evolve together with tumor cells, thus closely recapitulating tumor development in the human setting [12]. For these reasons, careful phenotypic and functional characterization of the complexity of the inflammatory tumor microenvironment has been increasingly important.

There are various methods for the assessment of the type, the phenotype, and/or the activation status of immune cells in tumors and other organs. It is possible to either directly analyze freshly generated single-cell suspension by multi-parameter flow cytometry or isolate specific immune cell population by flow cytometry-based cell sorting for further culturing, protein, or RNA extraction analyses. Regardless of which of these techniques will be performed, single-cell suspensions need to be generated from tumor and organs. Here, we describe a protocol to mechanically and enzymatically process tumors and other organs to generate single-cell suspensions and to prepare the samples for flow cytometry analysis. Flow cytometry-based sorting is the ultimate method for the isolation of specific cell populations. However, depending on the number and percentage of the immune cells of interest, the actual sorting procedure might be time consuming. In order to accelerate the sorting procedure and to obtain a high purity of the desired cells, we have described a two-step

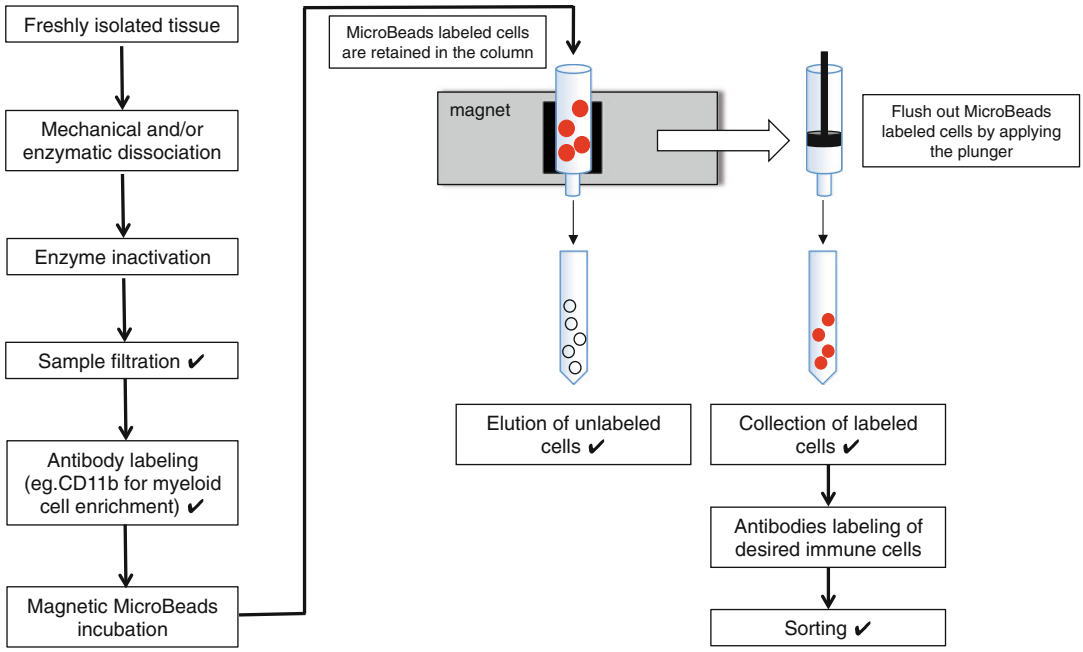

Fig. 1 Schematic workflow of the immune cell subset isolation by magnetic cell sorting and multi-parameter flow cytometry-based cell sorting. Ticks (✓) indicate steps to collect a small sample of cells that will be analyzed by flow cytometry at the end of the protocol

method for isolation of several myeloid cell subsets from tumors. Briefly, we first enrich the samples for myeloid immune cells by the use of magnetic cell sorting; then, we isolate immune cell subpopulations by flow cytometry-based sorting. A schematic overview of the protocol is depicted in Fig. 1. Although here we focus on the isolation of myeloid immune cells, researchers can adapt this protocol for the isolation of other type of cells, such as lymphocytes.

2 Materials

2.1 Generation of Single-Cell Suspensions from Freshly Isolated Solid Tumors and Organs

1. McIlwain Tissue Chopper (Ted Pella, Inc.) (*see* **Note 1**).
2. Enzyme digestion mix 1: 3 mg/mL Collagenase A in DMEM (serum free) (*see* **Notes 2** and **3**).
3. Enzyme digestion mix 2: 100 μg/mL Liberase in DMEM (serum free).
4. DMEM medium supplemented with 8% fetal bovine serum (FBS), 100 IU/mL penicillin, 100 μg/mL streptomycin (P/S), and 0.5 mM EDTA.
5. DMEM medium supplemented with 8% FBS, P/S.
6. Sorting buffer: IMDM, 2% FBS, 0.5% β-mercaptoethanol, 0.5 mM EDTA.
7. Erylysis buffer: 155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA in H_2O, pH 7.2–7.4.
8. 5000 U.I./mL Heparin sulfate.
9. Deoxyribonuclease I from bovine pancreas (DNase) dissolved to 10 μg/μL in 1× PBS.
10. FACS buffer: 0.5% BSA in 1× PBS.
11. Plunger from a 2 mL syringe.
12. Water bath or MACSmix™ tube rotator from Miltenyi Biotec.

Table 1
Example of an antibody panel for the purification of several myeloid cell populations by consecutive magnetic cell sorting and flow cytometry-based cell sorting

Antigen	Marker for	Fluorochromes/dye
CD11b	Myeloid cells	APC
F4/80	Macrophages	PE
Ly6G (1A8)	Neutrophils	FITC
Ly6C	Monocytes and neutrophils	eFluor450
	Live/dead	405 excitation

2.2 Purification of Immune Cell Subsets by Consecutive Magnetic Cell Sorting and Multi-parameter Fluorescence-Activated Cell Sorting (FACS)

1. AutoMACS™ Rinsing Solution supplemented with 0.5% BSA from Miltenyi Biotec (*see* **Note 4**).
2. Magnetic Micro Beads (Miltenyi Biotec) conjugated to an antibody with specificity for the fluorochrome of the antibody used in Subheading 3.3, **step 3** (*see* **Notes 4** and **5**).
3. QuadroMACS™ separator from Miltenyi Biotec (*see* **Notes 4** and **6**).
4. LS columns from Miltenyi Biotec (*see* **Notes 4** and **7**).

Table 2
Examples of antibody panels for the quantitative and phenotypic characterization of myeloid and lymphoid cell populations

Antigen	Marker for	Fluorochromes/dye
Lymphoid panel		
CD45	Immune cells	eFluor605NC
CD11b	Myeloid cells	APC-Cy7 eFluor780 (damp channel)
CD3	Lymphocytes	PE-Cy7
CD19	B cells	FITC
CD4	$CD4^+$ T cells	PE-Cy5
CD8	$CD8^+$ T cells	PerCP
CD49b	NK cells	APC
Granzyme B[a]	Lymphocyte activation	PE
IFNγ[a,b]	Lymphocyte activation	eFluor450
	Live/dead	Dye eFluor780
Antigen	Marker for	Fluorochromes and dye
Myeloid panel		
CD45	Immune cells	PerCP
CD11b	Myeloid cells	APC-Cy7 eFluor780
CD3	Lymphocytes	PE-Cy7
Ly6G (1A8)	Neutrophils	FITC
Ly6C	Monocytes and neutrophils	eFluor450
F4/80	Macrophages	PE
	Live/dead	Dye 405 excitation

[a]These are intracellular markers. For intracellular staining, we have good experience with BD Cytofix/Cytoperm Fixation/Permeabilization kit from BD Bioscience. Follow the manufacturer's instruction for the intracellular staining
[b]For IFNγ staining, before starting with Subheading 3.2, **step 3**, cells need to be stimulated with 50 ng/mL PMA, 1 μM ionomycin and GolgiPlug in IMDM, 8% FBS, and 0.5% β-mercaptoethanol for 3 h in the incubator at 37 °C

5. Antibodies: For the antibody combination used to isolate different myeloid cell populations, see Table 1 (*see* **Notes 8** and **9**).

2.3 Other Materials

1. 96-Well U-bottom plates.
2. 70 μm Filter cell strainers that fit on top of a 50 mL tube.
3. 15 and 50 mL Polypropylene tubes (*see* **Note 19**).
4. 5 mL Polypropylene tubes.
5. Antibodies: For the antibody combination used for quantitative and/or phenotypic characterization of immune cells, see Table 2 (*see* **Note 9**).
6. Flow cytometer (*see* **Note 10**).
7. Sorter (*see* **Note 11**).

3 Methods

Throughout the protocol, it is important to keep the samples on ice as much as possible, unless stated otherwise. Freshly harvested tissues should be collected in 1× PBS on ice and immediately processed. Single-cell suspensions are prepared as follows.

3.1 Tissue Digestion and Single-Cell Suspension

1. Mammary gland and mammary gland tumors: Chop the sample in a tissue chopper three times or until the tumor is dissociated (*see* **Note 1**). It is recommended to cut the tissues in smaller pieces with a scalpel before chopping to facilitate the procedure. Once chopped, incubate the sample in 10 mL enzyme digestion mix 1 with 25 μg/mL DNase in a 50 mL tube for 1 h at 37 °C in a shaking water bath (*see* **Notes 2**, **3**, and **12**).

   After digestion, stop the reaction by adding 20 mL DMEM, 8% FBS, P/S, and 0.5 mM EDTA and filter the sample through a 70 μm cell strainer in a 50 mL tube at 4 °C. Wash the cell strainer with 5 mL of DMEM, 8% FBS, P/S, and 0.5 mM EDTA to collect residual cells, centrifuge 300×*g* for 10 min at 4 °C, and discard supernatant. Immediately continue through Subheading 3.2 or 3.3.
2. Lungs: Chop the lungs three times in a tissue chopper (*see* **Note 1**) and incubate the sample in 2 mL digestion mix 2 in a 15 mL tube for 30 min at 37 °C in a shaking water bath (*see* **Note 12**). Stop the reaction by adding 5 mL DMEM, 8% FBS, and P/S and filter the sample through a 70 μm cell strainer in a 50 mL tube at 4 °C. Wash the cell strainer with 5 mL DMEM, 8% FBS, and P/S to collect residual cells, centrifuge at 300×*g* for 10 min at 4 °C, and discard supernatant. Immediately continue through Subheading 3.2 or 3.3.
3. Spleen and lymph nodes: Disperse the tissue through a 70 μm cell strainer (pre-wetted with 1× PBS) in a 50 mL tube by smashing it with a plunger while adding 1× PBS. Wash the cell

strainer with 5 mL 1× PBS in order to collect residual cells. Centrifuge at 300 × *g* for 10 min at 4 °C and discard supernatant. Resuspend the pellet of the spleen in 5 mL erylysis buffer for 5–10 min at RT in order to eliminate erythrocytes that might interfere with flow cytometry analyses. Filter the cells through a 70 μm cell strainer, centrifuge at 300 × *g* for 10 min, and discard supernatant. Immediately continue through Subheading 3.2 or 3.3.

4. Blood: Collect 1 mL of blood in 50 μL heparin sulfate. Add 5 mL erylysis buffer and incubate for 5–10 min at RT in a 15 mL tube. Centrifuge at 300 × *g* for 10 min at 4 °C and pour off the supernatant. Add again 5 mL erylysis buffer for 5–10 min at RT, centrifuge at 300 × *g* for 10 min at 4 °C, and pour off the supernatant. Immediately continue through Subheading 3.2.

Once the single-cell suspensions have been prepared, the samples can either be directly analyzed by flow cytometry in order to quantitatively and phenotypically characterize cell populations (*see* Subheading 3.2) or prepared for cell sorting (*see* Subheading 3.3).

3.2 Quantitative and/or Phenotypic Characterization of Immune Cells by Multi-parameter Flow Cytometry

1. Resuspend single cells in 5–10 mL FACS buffer (for a small tumor and for one lymph node we recommend 5 mL; for large tumors, spleen, and lung, we recommend 10 mL) and count the cells. Plate ≈ 2×10^6 cells per antibody combination in a 96-well plate U-bottom shape.
2. Centrifuge the plate in a cold plate—centrifuge at 300 × *g* for 2 min and flick out supernatant.
3. Add 50 μL of antibody mix (*see* **Note 9**), gently mix by pipetting, and incubate the cells for 30 min at 4 °C in the dark. In Table 2, an example of an antibody combination for myeloid and lymphoid cells is shown.
4. Wash the wells twice with 150 μL of FACS buffer, centrifuge the plate at 300 × *g* for 2 min, and flick out supernatant. Resuspend in 50–100 μL of FACS buffer and analyze the sample with a flow cytometer (*see* **Notes 9** and **10**).

Although immune cell subsets can be directly sorted by flow cytometry from the prepared single-cell suspensions, it is our experience that a better yield and purity can be obtained by a two-step process involving pre-enrichment for total myeloid immune cells by magnetic cell sorting, followed by purification of the desired immune cell subset(s) by multi-parameter flow cytometry-based cell sorting. Throughout the protocol, we advise to collect small samples of cells for analysis by flow cytometry at the end of the procedure to check the enrichment by the column and the purity of the sorted cells.

3.3 Immune Subset Purification by Consecutive Magnetic Cell Sorting (See Note 4) and Multi-parameter Flow Cytometry-Based Cell Sorting

1. Resuspend single cells in 5–10 mL of sorting buffer (for a small tumor and for one lymph node we recommend 5 mL; for large tumors, spleen, and lung, we recommend 10 mL) and count cells.
2. Centrifuge cells at 300×*g* for 10 min at 4 °C and discard supernatant.
3. To enrich the sample for myeloid cells, we use the marker CD11b (*see* **Note 8**). Resuspend the sample in sorting buffer containing the fluorochrome-conjugated CD11b antibody (e.g.: CD11b-APC) and incubate for 30 min at 4 °C in the dark. Use 50 µL of antibody mix per 10^7 cells (*see* **Note 13**). We advise to collect a small sample (≈50,000 cells) and store it in the 96-well plate at 4 °C. This sample will be used later for flow cytometry analyses and will be referred to as the "labeled" fraction.
4. Wash cells with 1–2 mL of sorting buffer per 10^7 cells (*see* **Note 13**). Centrifuge the cells at 300×*g* for 10 min at 4 °C and discard supernatant.
5. Resuspend cell pellet in 80 µL of sorting buffer per 10^7 cells (*see* **Note 13**). Add 20 µL per 10^7 cells of magnetic Micro Beads conjugated with an antibody against the CD11b-fluorochrome used in Subheading 3.3, **step 3** (e.g.: anti-APC Micro Beads) (*see* **Notes 5**, **13**, and **14**). Incubate for 20 min on ice in the dark.
6. Wash cells by adding 1–2 µL of sorting buffer per 10^7 cells (*see* **Note 13**). Centrifuge the cells at 300×*g* for 10 min at 4 °C and discard supernatant.
7. Resuspend up to 10^8 cells in 500 µL of AutoMACS™ Rinsing Solution and filter the cell suspension through a 70 µm cell strainer on a 50 µL tube to eliminate clumps that might obstruct the column (*see* **Note 15**). Wash the cell strainer with 100 µL of AutoMACS™ Rinsing Solution to collect residual cells.
8. Depending on the number of labeled cells, choose the appropriate column, and place it in the magnetic field of a MACS separator (*see* **Notes 6** and **7**). Prepare empty 15 mL tubes underneath the column in order to collect the flow-through.
9. After rinsing the column with 3 mL of AutoMACS™ Rinsing Solution, apply cell suspension once the flow-through has stopped. Let the column empty by gravity.
10. Wash column three times with 3 mL of AutoMACS™ Rinsing Solution and collect the flow-through that contains the CD11b– cells. Add new buffer only when column reservoir is empty.
11. Remove the column from the magnetic field and place it into a new 15 mL tube. Pipette 6 mL of AutoMACS™ Rinsing Solution into the column and immediately flush out the CD11b+ cells by firmly pressing the plunger into the column (*see* **Note 16**).

12. Count both CD11b+ and CD11b– cell fractions. We advise to collect a small sample (≈50,000 cells) to put in the 96-well plate at 4 °C. These samples will be used later for flow cytometry analyses and will be referred to as "CD11b–" and "CD11b+" fractions.
13. Spin down CD11b+ cells at 300×*g* for 10 min at 4 °C and discard supernatant.
14. Resuspend the CD11b+ cell fraction in the AutoMACS™ Rinsing Solution containing antibodies specific for the immune cell populations of interest. An example can be found in Table 1. Calculate 50 µL antibody mix per 10^7 cells (*see* **Notes 9** and **13**). If the number of cells is low, use at least 5 µL of antibody mix. Incubate cells for 30 min on ice in the dark.
15. Wash cells by adding 1–2 mL of AutoMACS™ Rinsing Solution per 10^7 cells (*see* **Note 13**). Centrifuge the cells at 300×*g* for 10 min at 4 °C and discard supernatant. Resuspend cells in AutoMACS™ Rinsing Solution at a concentration of 20×10^6 cells per mL (*see* **Notes 17** and **18**), add live/dead marker if necessary, and take it to the sorter (*see* **Note 11**). Collect sorted cells in 5 ml DMEM and 8% FBS in polypropylene tubes (*see* **Note 19**).
16. An example of a gating strategy to sort macrophages, neutrophils, and monocytes with markers described in Table 1 is shown in Fig. 2. After gating out duplets and gating on live cells, plot CD11b and F4/80. After gating on the CD11b+ F4/80– population, plot Ly6G and Ly6C. CD11b+ F4/80+ cells are macrophages; Ly6G+ Ly6C+ cells are neutrophils; Ly6G– Ly6C+ cells are monocytes.
17. We advise to collect a small sample of each sorted cell type (≈50,000 cells) and put it in the 96-well plate at 4 °C. These samples will be used for flow cytometry analysis.
18. Analyze the samples collected in Subheading 3.3, **steps 3**, **12**, and **17** by flow cytometry. These samples will give an estimation of the CD11b enrichment by the column and the sorting purity. *See* Fig. 3 for an example.

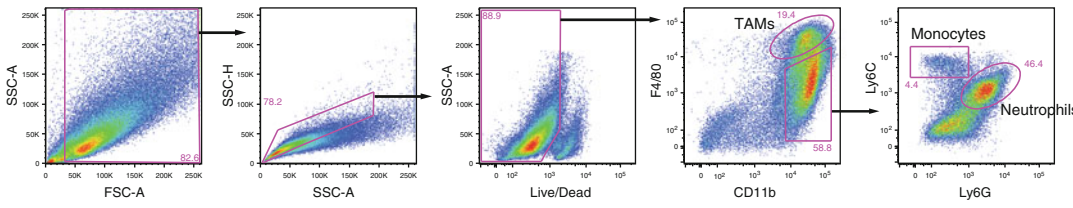

Fig. 2 Dot plots of a murine mammary gland tumor illustrating the flow cytometry gating strategy. After gating out duplets and dead cells, the macrophage population is gated as CD11b+ F4/80+. In the CD11b+ F4/80– fraction, neutrophils are Ly6G+ Ly6C+ and monocytes are Ly6C+ Ly6G–

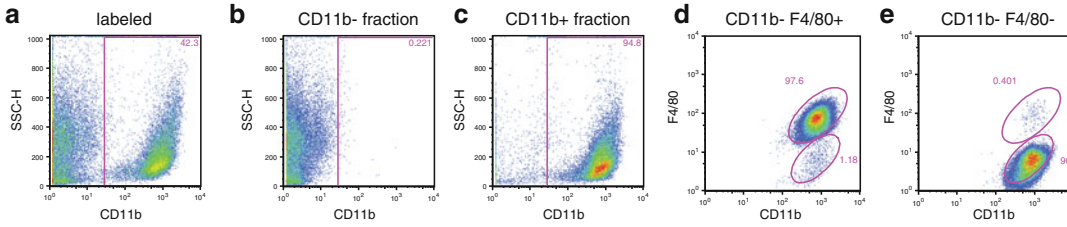

Fig. 3 Dot plots of murine mammary gland tumor samples collected during the pre-enrichment procedure for myeloid immune cells by magnetic cell sorting and after the isolation by flow cytometry-based sorting. In this example, F4/80+ and F4/80– cells were isolated from the tumor. (**a**) Panel **a** depicts the dot plot and gating of CD11b+ myeloid cells before the magnetic column pre-enrichment. (**b**) and (**c**) show the CD11b– and CD11b+ fractions, respectively, obtained after the magnetic column pre-enrichment procedure. The comparison between (**a**) and (**c**) shows the enrichment of CD11b+ cells by the magnetic column. (**d**) and (**e**) show the purity of F4/80+ and F4/80– cells, respectively, after isolation by flow cytometry-based sorting

4 Notes

1. Researchers can adjust cutting speed, blade force, and blade travel on the tissue chopper. We have good experience with maximum cutting speed and blade force, and 5–10 μm blade travel. As an alternative to the tissue chopper, researchers can use razor blades, scissors, or scalpels to chop the tumors into very small fragments.
2. We have optimized the digestion mix for mammary tumors. For other tumor types, different composition of the enzyme digestion mix and different incubation time might be needed. An example for prostate cancer and subcutaneous B16 melanoma can be found in [13].
3. If the sample is not properly digested, it is also possible to add trypsin at the final concentration of 1.5 mg/mL to the digestion mix. Trypsin might cleave some surface markers that can be of interest for sorting or other type of analysis. For this reason, before proceeding with the experiment, it should be established if the marker of interest is cleaved by trypsin.
4. We have good experience with the MACS cell separation reagents from Miltenyi Biotec. However, researchers can also purchase magnetic separation columns from other vendors.
5. This protocol describes an indirect method for magnetically labeling the cells of interest. Researchers should select the Micro Beads based on the fluorochrome conjugated to the antibody used for column enrichment. For example, if the antibody is conjugated with allophycocyanin (APC), researchers are advised to use anti-APC Micro Beads.

6. MACS separators are strong magnets with holders for columns. Since there are different column sizes, researchers should match the correct separator with the correct column holder (*see* **Note 7**).
7. Based on the number of expected magnetically labeled cells, researchers should select the correct column capacity. For example, LS columns from Miltenyi Biotec can hold up to 10^8 magnetically labeled cells from up to 2×10^9 total cells. Make sure to match the correct MACS separator format (*see* **Note 6**).
8. We have good experience with the pan-myeloid marker CD11b conjugated to APC for column enrichment. However, it is also possible to use other markers for immune cells, like CD45, or CD3 for the isolation of lymphocytes.
9. According to the immune cell populations of interest, researchers should design their own antibody combinations. The choice of the fluorochromes conjugated to the antibodies is dependent on the flow cytometer used. Make sure that the flow cytometer contains the right lasers and detectors to excite and read the fluorochromes. An overview of our frequently used antibody combinations can be found in Tables 1 and 2.
10. We have good experience with BD LSRII Flow Cytometer with DIVA software (BD Biosciences, USA). Other machines can be used as long as they can detect the antibody combination used (*see* **Note 9**).
11. We have good experience with BD FACSARIA II sorter with DIVA software (BD Biosciences, USA). Other machines can be used as long as they can detect the antibody combination used (*see* **Note 9**).
12. Alternatively, we also have good experience using the MACSmix™ tube rotator from Miltenyi Biotec located in an incubator at 37 °C.
13. Round up the number of counted cells and use this value to calculate the amount of antibodies, sorting buffer or Micro Beads in order to ensure that all cells are labeled. For example, if the researcher counts 1.2×10^6 cells, round it up to 2×10^6.
14. Prior to use, resuspend the Micro Beads by vortexing.
15. Scale up the volume of sorting buffer according to the total number of cells.
16. Miltenyi Biotec provides the correct plunger together with the column.
17. This is the optimal concentration for cell sorting for the BD FACSARIA II in our facility. We suggest asking your FACS facility for optimal cell concentration. In case of very few cells, do not add less than 500 µL of AutoMACS™ Rinsing Solution in order for the sorter to have enough volume.

18. Use AutoMACS™ Rinsing Solution for sorting because it is colorless. Phenol red in medium may interfere with fluorochromes.
19. Cells easily attach to the wall of polystyrene tubes. To avoid this, polypropylene tubes are recommended for collection of sorted cells. Researchers should choose the size of the polypropylene tube accordingly to the tube holder of the sorter machine.

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