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PROTOCOL

Multi-parametric flow cytometry staining procedure for analyzing tumor-infiltrating immune cells following oncolytic herpes simplex virus immunotherapy in intracranial glioblastoma

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Abbreviations used: DPBS, Dulbecco's phosphate buffered salt solution; FC, flow cytometry; FMO, fluorescent minus one; GBM glioblastoma; MHC, major histocompatibility complex; oHSV, oncolytic herpes simplex virus

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ABSTRACT

Multi-color flow cytometry is a standard laboratory protocol, which is regularly used to analyze tumor-infiltrating immune cell subsets. Oncolytic herpes simplex virus has shown promise in treating various types of cancers, including deadly glioblastoma. Intracranial/intratumoral treatment with oncolytic herpes simplex virus expressing interleukin 12, *i.e.*, immunovirotherapy results in induction of anti-tumor immune responses and tumor infiltration of a variety of immune cells. Multi-color flow cytometry is employed to characterize immune cells in the tumor microenvironment. Here, we describe a step-by-step 11-color flow cytometry protocol to stain tumor-infiltrating immune cells in glioblastoma following oncolytic herpes virotherapy. We also describe a method to identify HSV-1 glycoprotein-B-specific $CD8^+$ T cells using fluorochrome-conjugated major histocompatibility complex multimers. The multimers carry major histocompatibility peptide complexes, which have the ability to interact and bind to T cell receptors present on the surface of T cells; allowing identification of T cells (*e.g.*, $CD8^+$) reactive to a desired antigen. This multimer staining can be used in conjunction with the multi-parametric flow cytometry staining. Brain tumor quadrants are harvested, minced, enzymatically digested, immune cells are isolated by positive selection, single cells are counted and blocked for Fc receptors, cells are incubated with dye and/or color-conjugated antibodies, and flow cytrometry is performed using a BD LSRII flow cytometer. The protocol described herein is also applicable to stain immune cells in other mouse and human tumors or in any desired tissues.

Keywords: flow cytometry, glioblastoma, immune cells, immunovirotherapy, oncolytic herpes simplex virus

BACKGROUND

Cancer immunotherapy using immune checkpoint blockade has recently revolutionized the treatment of various cancers [1,2]. However, checkpoint blockade immunotherapy has failed to reach its intended goals in a recent Phase III clinical trial in glioblastoma (GBM) [3], a lethal immunosuppressive cancer with no effective treatment available to date [4]. Oncolytic viruses, *e.g.*, oncolytic herpes simplex virus (oHSV), are an emerging therapeutic approach in the field of cancer immunotherapy [5-8] and have shown promise in treating cancer patients [9]. oHSV is a new and distinct class of anti-cancer agent, which preferentially infects and kills cancer cells, while sparing the normal cells/tissues, and often

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induces anti-tumor immune responses [5,10]. oHSV has been genetically engineered for its oncolytic activity and safety [11,12]. For example, oHSV T-Vec (talimogene laherparepvec) was recently approved as the first oncolytic virus in the US and Europe for the treatment of advanced melanoma [9,11]. oHSV treatment of any tumor, including intracranial GBM induces an inflammatory reaction and attracts a variety of immune cells into the treatment site to produce an anti-tumor immune response [13,14]. Brain tumor inflammation and infiltrating immune cells can be analyzed by multi-color flow cytometry (FC), a commonly used and integral laboratory staining technique in cancer immunology and immunotherapy [15,16]. This permits a detailed analysis of a complex immune population of cells in a short period of time. The basic principle of FC is that a reaction occurs between an immune cell antigen and an antibody conjugated with a fluorochrome, such as pacific blue, pacific orange, R-phycoerythin, fluorescein, allophycocyanin, *etc*. The fluorescently labelled immune cell components are excited by the laser to emit light at varying wavelengths, which are measured to determine the amount and type of cells present in a sample [15,16]. Tens of thousands of cells can be quickly examined and the data gathered are processed by computer software. However, processing tumor tissues and multi-color staining of immune cells involving more than two colors, *e.g.*, 11 colors can be challenging. This is especially true for tumors that are located inside the central nervous system, containing myelin and high level of cell debris, as opposed to tumors located outside the brain or from lymphoid organs, such as spleen, cervical lymph node, bone marrow, *etc*. [17]. Herein, we describe a simple step-by-step protocol describing sample preparation and 11-color staining of tumor-infiltrating immune cells in a mouse GBM brain tumor model after treatment with oHSV-based immunotherapy. Briefly, brain tumor quadrants are harvested, mechanically dissociated, enzymatically digested, single cell suspension is prepared, $CD45^+$ leukocytes are isolated, single cells are counted and blocked for Fc receptors, and subjected to 11-color FC staining. We also describe a method to identify HSV-1 glycoprotein-B (gB)-specific $CD8^+$ T cells using fluorochrome-conjugated major histocompatibility complex (MHC) multimers [18]. For multi-color staining, the cells are incubated with dye to stain and exclude dead cells from analysis, followed by incubation with color-conjugated antibodies for extracellular staining, fixation in paraformaldehyde, incubation with antibodies for intracellular staining, and FC is performed using a BD LSRII flow cytometer (**Fig. 1**). The described method is highly reproducible as demonstrated by several multi-color flow cytometry experiments [13,19]. The protocol described herein will also be applicable for other mouse/human tumors in the brain and in the periphery.

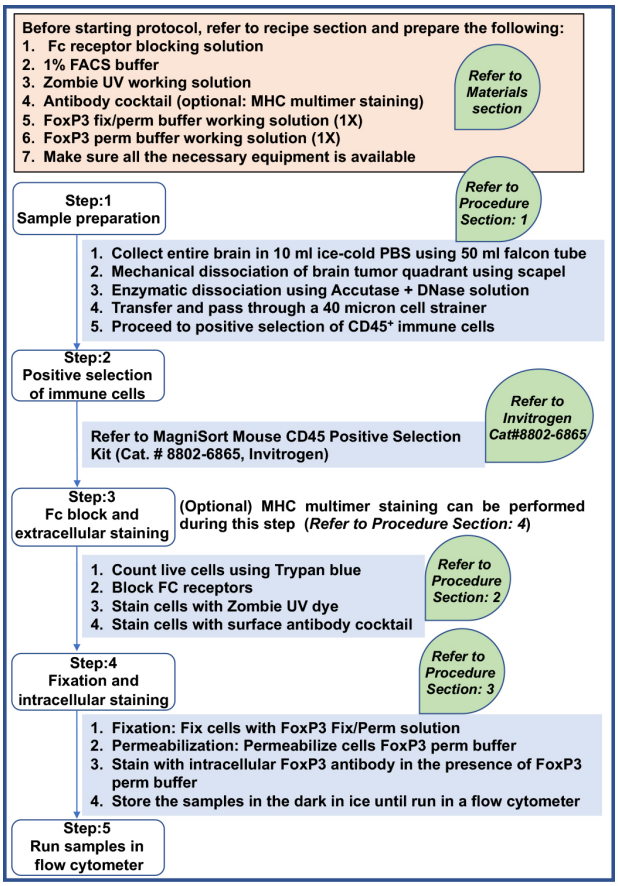

Figure 1. Schematic presentation of major steps involved in multi-parametric FC staining of intracranial GBM. 7 or 14 d after oncolytic virotherapy, brain tumor quadrants were harvested, mechanically dissociated, enzymatically digested, single cell suspension was prepared, $CD45^+$ leukocytes were isolated, single cells were counted and blocked for Fc receptors, and subjected to extracellular (optional: tetramer staining) and intracellular FC staining.

MATERIALS

Reagents

- ✓ Dulbecco's phosphate buffered salt solution (DPBS) (Corning, Cat. # MT21031CV)
- ✓ Accutase (Innovative Cell Technologies, Cat. # AT104)
- ✓ RQ1 RNase-Free DNase (Promega, Cat. # M6101)
- ✓ Heat inactivated fetal bovine serum (ThermoFisher Scientific, Cat. # 10082139)
- ✓ MagniSort mouse CD45 positive selection kit (Invitrogen, Cat. # 8802-6865)
- ✓ Zombie UV fixable viability kit (BioLegend, Cat. # 423107)
- ✓ TruStain FcX (anti-mouse CD16/32) antibody (BioLegend, Cat. # 101319,)
- ✓ Alexa Fluor 700 anti-mouse CD45 (BioLegend, Cat. # 103127)
- ✓ FITC anti-mouse CD45 (Cat. # 11-0451, Invitrogen)
- ✓ APC/Cy7 anti-mouse CD3ε (BioLegend, Cat. # 100329)
- ✓ PerCP/Cyanine 5.5 anti-mouse CD4 (BioLegend, Cat. # 100433)
- ✓ Brilliant Violet 510™ anti-mouse CD8a (BioLegend, Cat. # 100751)
- ✓ Brilliant Violet 605™ anti-mouse/human CD11b (BioLegend, Cat. # 101257)
- ✓ Alexa Fluor® 700 anti-mouse Ly-6G/Ly-6C (Gr-1) (BioLegend, Cat. # 108421)
- ✓ FITC anti-mouse CD69 (BioLegend, Cat. # 104505)
- ✓ PE/Cy7 anti-mouse CD152 (BioLegend, Cat. # 106313)
- ✓ Brilliant Violet 421™ anti-mouse CD274 (BioLegend, Cat. # 124315)
- ✓ PE/Dazzle 594 anti-mouse CD279 (BioLegend, Cat. # 109115)
- ✓ Alexa Fluor® 647 anti-mouse FOXP3 (BioLegend, Cat. # 126407)
- ✓ FoxP3 fix/perm buffer set (BioLegend, Cat. # 421403) contains 30 ml 4× concentrated FoxP3 fix/perm buffer (Cat. # 421401) and 25 ml 10× concentrated FoxP3 perm buffer (Cat. # 421402)
- ✓ HSV-1 glycoprotein B (gB) HSV-1-specific H-2K^b-HSV-1gB PE-conjugated (Immudex, Cat. # JD2670-PE)

Recipes

Fc receptor blocking solution For 10^6 cells, add 1.0 µg anti-mouse CD16/32 antibody in 100 µl DPBS, which is sufficient for one sample.

1% FACS buffer Add 5 ml heat-inactivated fetal bovine serum in 500 ml ice-cold DPBS solution. Mix well and store it at 4°C or keep it on ice while performing the procedure.

Zombie UV reconstitution and preparation of working solution Zombie UV kit (contains lyophilized powder of Zombie UV dye and DMSO) is stored at −20°C in the dark. Before starting step 2, equilibrate the kit to room temperature in the dark (cover with aluminum foil). Add 100 µl DMSO into one vial of Zombie UV dye for reconstitution and mix well by pipetting up and down.

To prepare working solution, dilute Zombie UV dye at 1:100 in DPBS. For example, if there are 20 samples, prepare ~2 ml working solution by adding 20 µl of reconstituted dye into 1980 µl DPBS. Use appropriate amount of reconstituted Zombie UV dye in each sample, *e.g.*, since the surface antibody cocktail is 22.5 µl/sample (see **Table 1**), use 77.5 µl of Zombie UV solution to bring to a total volume of 100 µl per sample (see steps 2.3 and 2.4).

Antibody cocktail for surface antibodies For surface antibodies, see **Table 1**.

Antibody cocktail for fluorescent minus one controls Fluorescent minus one (FMO) includes all color-conjugated antibodies except one. For example, surface antibody cocktail for fluorescent minus CD3 (FMO-CD3) will include all colors listed above except 2.5 µl APC/Cy7 anti-mouse CD3ε, *i.e.*, surface antibody cocktail for FMO-CD3 will be 22.5 µl−2.5 µl = 20 µl. Similarly, prepare FMO-CD4, FMO-CD8a, FMO-CD11b, FMO-Ly-6G/Ly-6C, FMO-CD69, FMO-CD152, FMO-CD274 and FMO-CD279. FMO-FoxP3 includes antibody cocktail of all nine surface antibodies listed above (*i.e.*, 22.5 µl) except no use of intracellular FoxP3 antibody in step 3.5. FMO-PE should be included if tetramer staining (*i.e.*, step 4) is incorporated in extracellular staining protocol.

FoxP3 fix/perm buffer working solution (1×) Before the staining procedure (*i.e.*, prior to step 3.1), dilute 4× concentrated FoxP3 fix/perm buffer in DPBS to make 1× working solution. For example, add one-part (*i.e.*, 1 ml) FoxP3 fix/perm buffer (4×) to three parts (*i.e.*, 3 ml) DPBS.

FoxP3 perm buffer working solution (1×) Dilute 10× FoxP3 perm buffer in DPBS to prepare 1× working solution (required in steps 3.3–3.5). For example, add one-part (*i.e.*, 1 ml) FoxP3 perm buffer (10×) to nine parts (*i.e.*, 9 ml) DPBS.

MHC multimer (tetramer or dextramer) staining solution Prepare multimer staining solution by adding 10 µl of fluorochrome (PE)-conjugated MHC-I tetramers or dextramers against HSV-1 gB-specific H-2Kb-HSV-1 gB into 40 µl DPBS per test.

Equipment

- ✓ 15 ml conical centrifuge tubes (Fisher Scientific, Cat. # 12-565-269)
- ✓ 50 ml conical centrifuge tubes (Fisher Scientific, Cat. # 12-565-271)
- ✓ 1.7 ml microcentrifuge tubes (Fisher Scientific, Cat. # 07-200-534)
- ✓ Petri dishes (Fisher Scientific, Cat. # 08-757-100D)
- ✓ Disposable scalpel (Fisher Scientific, Cat. # 50-364-913)
- ✓ Sorvall ST 16R (Fisher Scientific, Cat. # 75-810-885)
- ✓ Sorvall Legend Micro 21R Microcentrifuge (Fisher Scientific, Cat. # 75-002-446)
- ✓ Nunc 2 ml serological pipets (Fisher Scientific, Cat. # 12-567-601)
- ✓ Nunc 5 ml serological pipets (Fisher Scientific, Cat. # 12-567-602)
- ✓ Nunc 10 ml serological pipets (Fisher Scientific, Cat. # 12-567-603)
- ✓ Digital water bath (Thomas Scientific, Cat. # 1198Q17)
- ✓ 40 µm cell strainer (Fisher Scientific, Cat. # 08-771-19)
- ✓ Hemocytometer counting chamber (Fisher Scientific, Cat. # 02-671-10)
- ✓ Hemocytometer cover glass (Fisher Scientific, Cat. # 12-519-10)
- ✓ Trypan blue stain (Sigma, Cat. # T-8154)
- ✓ Binoc inverted light microscope (Fisher Scientific, Cat. # 11-350-119)
- ✓ Round bottom 96-well microplates (Fisher Scientific, Cat. # 07-200-99)
- ✓ Aluminum foil (Fisher Scientific, Cat. # 01-213-101)
- ✓ LSR-II multi-color flow cytometer (BD Biosciences)

Table 1. Surface antibodies and the amount.

Surface antibodies (with listed vendor concentrations)	Amount[a]
APC/Cy7 anti-mouse CD3ε (0.2 mg/ml)	2.5 µl (or 0.5 µg)
PerCP/Cyanine 5.5 anti-mouse CD4 (0.2 mg/ml)	1.25 µl (or 0.25 µg)
Brilliant Violet 510TM anti-mouse CD8a (0.2 mg/ml)	2.5 µl (or 0.5 µg)
Brilliant Violet 605TM anti-mouse/human CD11b (0.2 mg/ml)	1.25 µl (or 0.25 µg)
Alexa Fluor® 700 anti-mouse Ly-6G/Ly-6C (0.5 mg/ml)	0.5 µl (or 0.25 µg)
FITC anti-mouse CD69 (0.5 mg/ml)	2 µl (or 1 µg)
PE/Cy7 anti-mouse CD152 (0.2 mg/ml)	2.5 µl (or 0.5 µg)
Brilliant Violet 421TM anti-mouse CD274	5 µl
PE/Dazzle 594 anti-mouse CD279 (0.2 mg/ml)	5 µl (or 1 µg)
Surface antibody cocktail/sample	22.5 µl

[a]Amount listed in this table for each antibody was used to stain 1 × 10^6 cells in 100 µl volume, as per manufacturer instructions. However, from our personal experience, the same listed amount was successfully used multiple times to stain up to 5 × 10^6 cells in 100 µl volume.

PROCEDURE

1. Harvesting and processing brain tumor samples and magnetic separation of immune cells

1.1. The whole brain bearing tumor is harvested from a sacrificed mouse in 10 ml ice-cold DPBS in a 50 ml falcon tube.

1.2. Decant all the content in a cell culture petri dish and excise the brain tumor quadrant around the needle track wound (site of tumor cell implantation) using a scalpel. Transfer the excised quadrant to a new cell culture petri dish.

1.3. Mechanical dissociation: Cut and mince the brain tumor tissue (tumor size is ~3–4 mm) with scalpel thoroughly for 1–2 min into fragments of about 0.5 mm and smaller.

1.4. Quickly re-suspend the minced tissues in 5 ml ice-cold DPBS and transfer all the contents to a 15 ml falcon tube. Keep the tube on ice.

1.5. Spin samples at 1500 rpm (or 400× *g*) using Sorvall ST 16R (Fisher Scientific, Waltham, MA) for 5 min at 4°C. Carefully decant off the supernatant to a new falcon tube labeled as "waste".

1.6. Enzymatic dissociation: Add Accutase (1 ml/tumor quadrant) + DNase I (10 units/ml; to avoid cell clumps) into the tube, re-suspend the pellet using 2 ml pipet, and incubate the solution for 10 min in 37°C digital water bath (Thomas Scientific, Swedesboro, NJ).

1.7. Gently triturate the solution with 2 ml pipet (3–5 times; it will make the remaining tissue pieces even smaller) and add 1% FACS buffer to the tube to a volume of up to 10 ml.

1.8. Transfer and pass this 10 ml cell suspension through a 40 micron cell strainer sitting on top of a new 50 ml falcon tube. Pass another 10 ml ice-cold DPBS through the strainer (in order to wash off any cells that may remain attached to the strainer), which brings a total volume of 20 ml single cell suspension.

1.9. Spin at 1500 rpm (or 400× *g*) using Sorvall ST 16R for 5 min at 4°C. Carefully decant off the supernatant to the waste tube. Re-suspend the cell pellet in ice-cold DPBS (1 ml DPBS/tumor quadrant) and count the total number of live cells present in single cell suspension.

1.10. Once single cell suspension is prepared, use MagniSort Mouse CD45 Positive Selection Kit (Cat. # 8802-6865, Invitrogen) to isolate $CD45^+$ leukocytes. To do that, follow 'Experimental Procedure Steps # 1–10' described in Invitrogen Cat. # 8802-6865. After positive selection, live cells are counted and purity is determined.

1.11. Transfer appropriate number of cells from each sample into new 1.7 ml tubes, including cells for FMO controls (see Notes 3 and 4 in step 1), and proceed to step 2.

TIPS/HINTS: (1) Perform the whole procedure at 4°C or on ice, except enzymatic digestion step; (2) Step 1

should be done in serum-free solution, since serum can counteract the activity of enzymatic digestion and Zombie UV staining in step 2.3. From our experience, maintaining cells in regular DPBS or serum-free solution during the whole procedure does not hamper the viability of tumor cells or immune cells; (3) In step 1.3, tumor tissue is soft and fragile, easy to mince; and (4) During enzymatic dissociation in step 1.6, stir the samples very gently in order to help in breaking cell-cell junctions.

CAUTION: (1) Perform steps 1.2 and 1.3 as quickly as possible in order to prevent the tissues from drying out; and (2) After each centrifugation, sometimes the pellet can be loose, so aspiration can be very risky since it may suck out the pellet.

NOTES: (1) For counting live cells in steps 1.9 and 1.10, mix 10 µl single cell suspension with 190 µl Trypan blue (Cat. # T-8154, Sigma). Take 20 µl of this solution in a hemocytometer and count only shiny cells, which are considered as live cells (the dead cells will be stained blue), in all four hemocytometer chambers under a light microscope. The total number of live cells/ml of suspension is counted as: total cells in four chambers/4 × dilution factor 20 × 10000; (2) Purity of the isolated cells in step 1.10 can be verified by staining with Alexa Fluor 700 anti-mouse CD45 (Cat. # 103127, Biolegend) or FITC anti-mouse CD45 (Cat. # 11-0451, Invitrogen); (3) If experimental protocol described in Saha *et al*. [13] is followed, 0.5 to 2 × 10^6 $CD45^+$ leukocytes can be isolated per brain tumor quadrant. If there are 1 × 10^6 $CD45^+$ cells/sample, there will be a total of 16 × 10^6 cells isolated from 16 mice (*i.e.*, mock group n = 8; oHSV group n = 8) brain tumor quadrants. Take half of the cells (*i.e.*, 0.5 × 10^6 cells/sample) from each of 16 samples into 16 new 1.7 ml tubes to perform 11-color staining. Mix together the other half of the cells from all 16 mice (*i.e.*, 0.5 × 10^6 cells/sample × 16 samples = total 8 × 10^6 cells), divide them equally into 10 separate 1.7 ml tubes, and use them for the preparation of FMO control for each individual color (except Zombie UV dye). As an example, fluorescent minus CD3 means staining cells with all colors except APC/Cy7 anti-mouse CD3 [13]. FMO control is especially helpful to identify a specific cell population in a given sample during computer analysis of flow cytometric data; (4) When a high volume of samples (*i.e.*, 50 to 100) are included in an experiment, the following staining procedure (described in steps 2–4) can be performed in a round bottom 96-well plate, instead of 1.7 ml tubes.

CRITICAL STEP: Step 1.3 is critical, because proper mechanical dissociation will determine the efficiency of enzymatic dissociation (in step 1.6) and result in obtaining a maximum number of single cells at the end of the procedure.

2. Blocking Fc receptors, Zombie UV staining (to exclude dead cells) and surface staining

2.1. Spin down the cells (prepared in step 1.11, *i.e.*, 16 sample tubes and 10 FMO controls for 10 antibodies) at 1800 rpm using Sorvall Legend Micro 21R Microcentrifuge (Fisher Scientific, Waltham, MA) for 5 min at 4°C. Carefully decant off the supernatant.

2.2. Re-suspend the cell pellet in 100 µl DPBS/Fc receptor blocking solution and incubate cells on ice for 10 min. Spin down and remove supernatant.

2.3. Add appropriate amount of Zombie UV dye to each tube. Re-suspend and incubate cells for 15 min at room temperature.

2.4. Add appropriate amount of antibody cocktail to each tube to bring to a total volume of 100 µl per sample. Re-suspend the pellet and incubate in the dark for 20 min at room temperature.

2.5. Add 100 µl FACS buffer to each tube and mix gently. Spin down and remove supernatant.

2.6. Repeat step 2.5 one more time and proceed to fixation and intracellular staining.

2.7. TIPS/HINTS: (1) In order to increase efficiency in timing, prepare fresh DPBS/Fc receptor blocking solution while running step 2.1; (2) Similarly, prepare antibody cocktail while running step 2.2, and this step can be extended to 15–20 min, if necessary. Since cells are not fixed, it is important to finish the procedure as quickly as possible; (3) Prepare antibody cocktail just before use, because some flow cytometry antibodies, such as APC/Cy7 and PE/Cy7 do not work well when cocktailed together for a long period of time. Similarly, Brilliant Violets antibodies also do not work well when cocktailed together for a long period of time, such as more than 2–3 h (personal communication with Amy Lee, a flow cytometry expert from BioLegend).

NOTES: (1) We are using an adapted BioLegend's 'no-wash' staining protocol, *i.e.*, no wash is required between step 2.3 Zombie UV staining and step 2.4 surface staining; (2) Amount of Zombie UV dye to be added in each

sample in step 2.3 depends on the volume of surface antibody cocktail to be added in step 2.4, and the end volume of Zombie UV dye/surface antibody cocktail is 100 µl. For example, if antibody cocktail is 22.5 µl, use 77.5 µl Zombie UV solution; (3) The antibody cocktail in step 2.4 includes nine surface antibodies (excludes Zombie UV dye and intracellular FoxP3 antibody).

3. Fixation, permeabilization and FoxP3 intracellular staining

3.1. Add 100 µl 1× FoxP3 Fix/Perm solution to each tube, re-suspend the pellet and incubate at room temperature in the dark for 20 min. Spin down (1800 rpm for 5 min, using Sorvall Legend Micro 21R Microcentrifuge) and remove the supernatant.

3.2. Wash once with 100 µl FACS buffer. Spin down and discard the supernatant.

3.3. Add 100 µl FoxP3 Perm buffer, re-suspend the pellet, and incubate cells in the dark for 15 min at room temperature. Spin down and discard the supernatant.

3.4. Again add 100 µl FoxP3 Perm buffer to each tube and re-suspend the pellet.

3.5. Add appropriate amount of intracellular FoxP3 antibody (*i.e.*, 2 µl or 1 µg from 0.5 mg/ml stock for 1 × 10^6 cells in 100 µl volume, according to manufacturer instructions) to each tube except fluorescent minus FoxP3 tube (FMO-FoxP3) and incubate in the dark for 30 min at room temperature. Spin down and discard the supernatant.

3.6. Wash two times with 100 µl FACS buffer. Spin down and discard the supernatant.

3.7. Re-suspend the pellet in 400 µl FACS buffer and store the samples in the dark in ice until analysis in a flow cytometer (*e.g.*, LSRII; BD Biosciences, San Jose, CA) using appropriate instrument settings.

TIPS/HINTS: Pellet may appear translucent and can be loose, so do not apply aspirator for removing waste supernatant.

NOTES: (1) This multi-color staining procedure requires single color compensation controls before running each individual sample in a Flow Cytometer. Follow eBioscience "Protocol: UltraComp eBeads" described in Cat. # 01-2222 for the preparation of single-color compensation controls for each individual color, except for Zombie UV. For Zombie UV compensation control, stain isolated immune cells by adding 100 µl Zombie UV working solution, followed by wash once with 100 µl FACS buffer, fix cells and wash with FACS buffer, and re-suspend the cells in FACS buffer. Maintain Zombie UV sample on ice in the dark until run in a Flow Cytometer. To save time, prepare Zombie UV single-color compensation control simultaneously while preparing other samples in step 2 and step 3. Prepare other 10 single-color compensation controls that require beads during 30 min incubation period in step 3.5; (2) Analyze samples as soon as the staining procedure is completed. However, conjugated colors in fixed samples can remain stable even for a week after staining, if they are maintained at 4°C in the dark.

CAUTION: The 4× concentrated FoxP3 fix/perm buffer (Cat. # 421401) contains paraformaldehyde, which is toxigenic and mutagenic. So, handle this solution with care wearing gloves, lab coat and face mask.

CRITICAL STEP: Continuous presence of FoxP3 Perm Buffer from steps 3.3 to 3.5 is critical to maintain the cell permeability to intracellular antibodies.

4. Tetramer staining to determine antigen-specific $CD8^+$ T cells (an optional step during surface staining in step 2)

NOTES: While the extracellular and intracellular staining can be utilized to differentiate immune cell subsets, detection of antigen-specific T cells can be imperative to evaluate the effectiveness of oHSV therapy. Tetramer staining can be utilized to determine whether oHSV therapy can produce viral antigen and/or tumor antigen-specific T cells. Here, we describe tetramer staining for HSV-1 glycoprotein B (gB)-specific $CD8^+$ T cells after treating tumors with oHSV. Below are the steps that can be followed to successfully integrate tetramer staining in the extracellular flow cytometry staining protocol.

4.1. Follow steps as described above till step 2.3, and then continue as below.

4.2. After step 2.3, spin samples at 1800 rpm, 5 min at 4°C. Gently decant off the supernatant.

4.3. Re-suspend each sample in 50 µl of tetramer solution and incubate samples in the dark for 45 min at 4°C. Spin down and remove supernatant.

4.4. Re-suspend cells in 100 µl 1% FACS buffer. Spin down and remove supernatant. Repeat this wash step once more time.

4.5. Continue to step 2.4.

NOTES: (1) If tetramer staining is included: use 100 µl Zombie UV in step 2.3 (instead of 77.5 µl); add 22.5 µl surface antibody cocktail in 77.5 µl DPBS (instead of Zombie UV) to bring to a total volume of 100 µl per sample; and prepare single color compensation control for PE and FMO control (FMO-PE); (2) The PE color is compatible with other 11 colors listed above; (3) During multimer staining, it is important to match the MHC haplotype with mouse strain [20]. For example, if protocol involves C57BL/6J mice, use multimers with H2-Kb or H2-Db but not H2-L haplotypes. A complete list of MHC haplotype matches for each mouse strain can be found here [21]; Multimers are most often used to identify T-cell populations in conjunction with the anti-CD8 and anti-CD4 antibodies. Both anti-CD8 and anti-CD4 antibodies can affect tetramer staining [20]. Therefore, it is recommended that cells should be stained with multimer before staining with other extracellular antibodies.

ANTICIPATED RESULTS

This manuscript mainly focuses on describing step-by-step protocols of brain tumor sample preparation and 11-color staining that should be applicable to any given multi-color compatible flow cytometer. Because different Flow Cytometry cores employ different types of flow cytometers and since each flow cytometer has different instrument settings to analyze samples, detailed flow cytometric analysis is not included in this manuscript. However, the excitation/emission wavelengths and filter set used for each fluorophore is shown in **Table 2**, which will help the readers in designing multiplex flow experiments. Because this multi-color FC protocol involves single-color compensation controls and appropriate FMO controls for each individual color, any given number of samples can easily be compensated and analyzed in a Flow Cytometer using appropriate instrument settings (*e.g.*, FSC/SSC settings and fluorescence detector voltages; see **Table 3**), as we demonstrated previously [13,19]. An example of sequence and gating for multi-parametric FC (*e.g.*, 10-color) of individual cell subtypes can be seen in Saha *et al*. [13]. Inclusion of FMO control for each individual color in multicolor FC helps to separate any specific type of immune population from a pool of immune cells during flow cytometric analysis (see an example in **Fig. 2**). Inclusion of tetramer staining before extracellular staining procedure can lead to successful detection of HSV-specific $CD8^+$ T cells infiltrated into the tumor (**Fig. 3**).

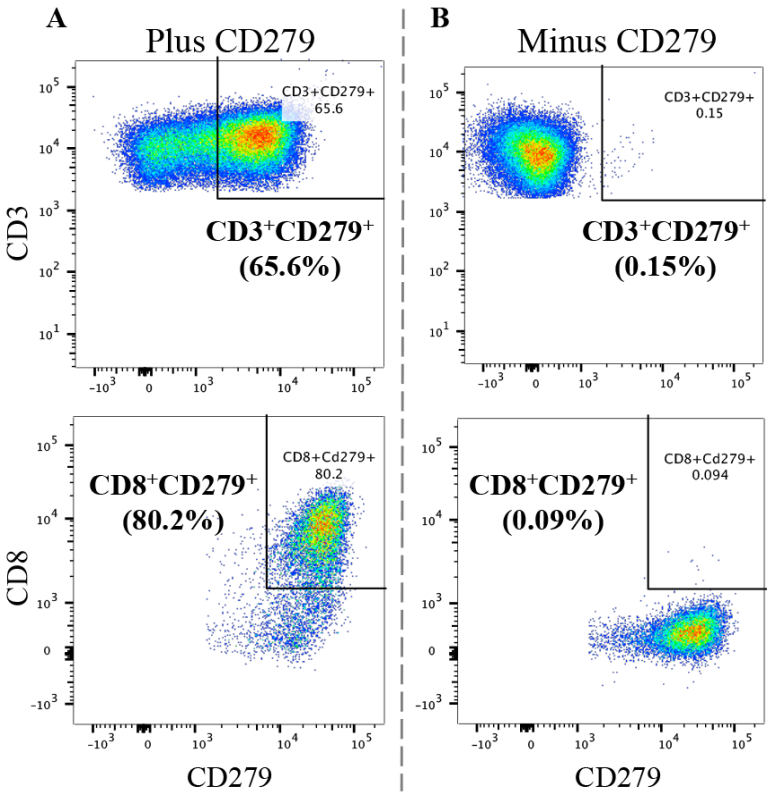

Figure 2. Representative 2D dot plots illustrating all color staining including CD279 (A) or excluding CD279 (FMO-CD279) (B). 7 d after oncolytic virus treatment, tumors were harvested and isolated tumor-infiltrating immune cells were subjected to multi-color flow cytometry staining with (A) or without (B) staining for CD279. Gating for $CD3^+CD279^+/CD8^+CD279^+$ in (B) helps to separate $CD3^+CD279^+/CD8^+CD279^+$ population in (A).

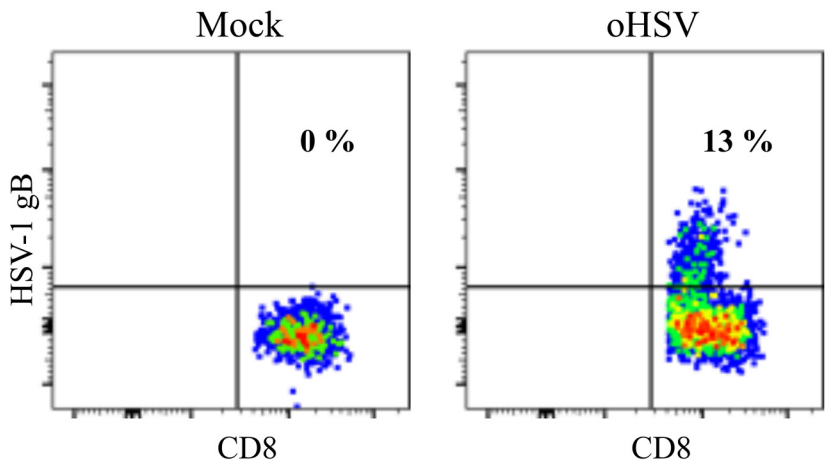

Figure 3. Representative 2D dot plots showing HSV-1 protein gB-specific CD8⁺ T cells infiltrated into the tumor after oHSV treatment. 14 d after oncolytic virus treatment, tumors were harvested and isolated tumor-infiltrating immune cells were subjected to tetramer staining to determine HSV-1 gB-specific CD8⁺ T cells.

Table 2. Emission/excitation wavelengths and filter sets for each fluorophore used during flow cytometric analysis.

Fluorophores	Laser (excitation wavelength)	Emission maximum (nm)	Filter sets
APC/Cy7	Red (633 nm)	785	780/60 (755LP)
Alexa Fluor® 700	Red (633 nm)	719	730/45 (685LP)
Alexa Fluor® 647	Red (633 nm)	668	670/30
PerCP/Cyanine 5.5	Blue (488 nm)	695	695/40 (685LP)
FITC	Blue (488 nm)	519	530/30 (505LP)
PE/Cy7	Blue (488 nm)	785	780/60 (735LP)
PE/Dazzle 594	Blue (488 nm)	610	610/20 (600LP)
Brilliant Violet 510	Violet (405 nm)	510	585/42 (550LP)
Brilliant Violet 605	Violet (405 nm)	605	675/25 (595LP)
Brilliant Violet 421	Violet (405 nm)	421	450/50
Zombie UV	Ultraviolet (355 nm)	459	450/50

Table 3. FSC/SSC settings and fluorescence detector voltages used during compensation and flow cytometric analysis in a given experiment.

Parameter	Voltage
FSC	375
SSC	272
APC/Cy7	685
Alexa Fluor® 700	745
Alexa Fluor® 647	625
PerCP/Cyanine 5.5	757
FITC	446
PE/Cy7	709
Brilliant Violet 510	580
Brilliant Violet 605	650
Brilliant Violet 421	401
Zombie UV	368

OHSV-IL12 treatment of brain tumors induces local tumor inflammation and tumor immune cell infiltration [13,19], which includes: (1) increased tumor infiltration of T cells ($CD3^+$, $CD3^+CD8^+$); (2) reduction of immune suppressive regulatory T cells ($CD3^+FoxP3^+$, $CD3^+CD4^+FoxP3^+$); (3) increased T cells/regulatory T cell ratio ($CD8^+/CD4^+FoxP3^+$); and (4) reduction of GBM tumor cells [13,19]. This multiparametric FC protocol is reproducible, as we demonstrated previously for brain tumors [13], melanoma [18] and spleen [19]. We anticipate that this strategy should be applicable to study antitumor immune response to other tumors in the brain and in the periphery after other oncolytic virotherapy or other forms of immunotherapies.

TROUBLESHOOTING

Possible problems and their troubleshooting solutions are listed in **Table 4**.

Table 4. Troubleshooting table.

Step	Problems	Causes	Suggestions
1.2, 1.3	Excessive cell death	Tissue left at room temperature Enzymatic dissociation for extended periods	Always keep tissue on ice Enzymatic dissociation should be performed only for 10 min
2.4	No extracellular staining	Inappropriate antibody dilution Inadequate incubation time	Use recommended dilution of antibody Use recommended incubation conditions
3.2	No intracellular staining	Intracellular staining performed using FACS buffer	Permeabilization buffer should be used for intracellular staining
3.7	Spillover of fluorophores	Flow cytometry instrument not setup appropriately	Run recommended Cytometry Setup and Tracking (CST) beads Run compensation controls
4.1	No tetramer staining	Mis-match MHC haplotype	Refer to mouse alloantigen database [21]

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References

1. Topalian SL, Taube JM, Anders RA, Pardoll DM (2016) Mechanism-driven biomarkers to guide immune checkpoint blockade in cancer therapy. Nat Rev Cancer 16: 275-287. doi: 10.1038/nrc.2016.36. PMID: 27079802
2. Yang Y (2015) Cancer immunotherapy: harnessing the immune system to battle cancer. J Clin Invest 125: 3335-3337. doi: 10.1172/JCI83871. PMID: 26325031
3. Filley AC, Henriquez M, Dey M (2017) Recurrent glioma clinical trial, CheckMate-143: the game is not over yet. Oncotarget 8: 91779-91794. doi: 10.18632/oncotarget.21586. PMID: 29207684
4. Omuro A, DeAngelis LM (2013) Glioblastoma and other malignant gliomas: a clinical review. JAMA 310: 1842-1850. doi: 10.1001/jama.2013.280319. PMID: 24193082
5. Saha D, Ahmed SS, Rabkin SD (2015) Exploring the antitumor effect of virus in malignant glioma. Drugs Future 40: 739-749. doi: 10.1358/dof.2015.040.11.2383070. PMID: 26855472
6. Bommareddy PK, Silk AW, Kaufman HL (2017) Intratumoral approaches for the treatment of melanoma. Cancer J 23: 40-47. doi: 10.1097/PPO.0000000000000234. PMID: 28114253
7. Bommareddy PK, Shettigar M, Kaufman HL (2018) Integrating oncolytic viruses in combination cancer immunotherapy. Nat Rev Immunol 18: 498-513. doi: 10.1038/s41577-018-0014-6. PMID: 29743717
8. Saha D, Martuza RL, Rabkin SD (2018) Oncolytic herpes simplex virus immunovirotherapy in combination with immune checkpoint blockade to treat glioblastoma. Immunotherapy 10: 779-786. doi: 10.2217/imt-2018-0009. PMID: 30008259
9. Andtbacka RHI, Kaufman HL, Collichio F, Amatruda T, Senzer N, et al. (2015) Talimogene laherparepvec improves durable response rate in patients with advanced melanoma. J Clin Oncol 33: 2780-2788. doi: 10.1200/JCO.2014.58.3377. PMID: 26014293
10. Saha D, Wakimoto H, Rabkin SD (2016) Oncolytic herpes simplex virus interactions with the host immune system. Curr Opin Virol 21: 26-34. doi: 10.1016/j.coviro.2016.07.007. PMID: 27497296
11. Bommareddy PK, Peters C, Saha D, Rabkin SD, Kaufman HL (2018) Oncolytic herpes simplex viruses as a paradigm for the treatment of cancer. Annu Rev Cancer Biol 2: 155-173. doi: 10.1146/annurev-cancerbio-030617-050254.
12. Jhawar SR, Thandoni A, Bommareddy PK, Hassan S, Kohlhapp FJ, et al. (2017) Oncolytic viruses—natural and genetically engineered cancer immunotherapies. Front Oncol 7: 202. doi: 10.3389/fonc.2017.00202. PMID: 28955655
13. Saha D, Martuza RL, Rabkin SD (2017) Macrophage polarization contributes to glioblastoma eradication by combination immunovirotherapy and immune checkpoint blockade. Cancer Cell 32: 253-267. doi: 10.1016/j.ccell.2017.07.006. PMID: 28810147
14. Saha D, Wakimoto H, Peters CW, Antoszczyk SJ, Rabkin SD, et al. (2018) Combinatorial effects of VEGFR rinase inhibitor axitinib and oncolytic virotherapy in mouse and human glioblastoma stem-like cell models. Clin Cancer Res 24: 3409-3422. doi: 10.1158/1078-0432.CCR-17-1717. PMID: 29599413
15. McKinnon KM (2018) Multiparameter Conventional Flow Cytometry. Methods Mol Biol 1678: 139-150. doi: 10.1007/978-1-4939-7346-0_8. PMID: 29071679
16. Young YK, Bolt AM, Ahn R, Mann KK (2016) Analyzing the tumor microenvironment by flow cytometry. Methods Mol Biol 1458: 95-110. doi: 10.1007/978-1-4939-3801-8_8. PMID: 27581017
17. Pösel C, Möller K, Boltze J, Wagner D, Weise G (2016) Isolation and flow cytometric analysis of immune cells from the ischemic mouse brain. J Vis Exp: 53658. doi: 10.3791/53658. PMID: 26967380
18. Bommareddy PK, Aspromonte S, Zloza A, Rabkin SD, Kaufman HL (2018) MEK inhibition enhances oncolytic virus immunotherapy through increased

tumor cell killing and T cell activation. Sci Transl Med 10: eaau0417. doi: 10.1126/scitranslmed.aau0417.

19. Cheema TA, Wakimoto H, Fecci PE, Ning J, Kuroda T, et al. (2013) Multifaceted oncolytic virus therapy for glioblastoma in an immunocompetent cancer stem cell model. Proc Natl Acad Sci USA 110: 12006-12011. doi: 10.1073/pnas.1307935110. PMID: 23754388

20. Wooldridge L, Lissina A, Cole DK, van den Berg, A. (Hugo) , Price DA, et al. (2009) Tricks with tetramers: how to get the most from multimeric peptide-MHC. Immunology 126: 147-164. doi: 10.1111/j.1365-2567.2008.02848.x. PMID: 19125886

21. BioLegend. Mouse alloantigens. [Cited January 11, 2018]. Available from: http://www.biolegend.com/media_assets/support_resource/BioLegend_Mouse_Alloantigens.pdf.