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Immunohistochemistry for Tumor-Infiltrating Immune Cells After Oncolytic Virotherapy

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Abstract

Immunohistochemistry (IHC) is an integral laboratory staining technique, which is used for the detection of immune cells in mouse/human tissues or tumors. Oncolytic herpes simplex virus (oHSV) treatment or virotherapy of solid tumors results in antitumor immune responses and infiltration of a variety of immune cells into the tumor. Here, we describe a step-by-step chromogen/substrate-based single- and dual-color IHC protocol to stain immune cells in formalin-fixed, paraffin-embedded mouse glioblastoma (GBM) brain tumor sections after oHSV virotherapy. Tumor sections are deparaffinized with xylene, then gradually rehydrated using ethanol, followed by heat-mediated antigen retrieval using appropriate buffers. Tumor sections are incubated with primary antibodies, which detect a specific immune cell antigen, then incubated with peroxidase- or phosphatase-labeled secondary antibodies, followed by incubation with a color-producing substrate and color visualization (of immune cells) by light microscopy. The protocol described herein is also applicable to detect immune cells in other mouse and human tumors or organs after other forms of immunotherapy.

Keywords

Immunohistochemistry; Substrate-based immunohistochemistry; Dual-color immunohistochemistry; Oncolytic herpes simplex virus; Virotherapy; Immune cells; Antigen–antibody reaction; Antigen retrieval

1 Introduction

Oncolytic viruses are a distinct class of anticancer agents, which selectively replicate in and kill cancer cells without harming normal tissue, and often induce antitumor immune responses [1, 2]. Oncolytic herpes simplex virus (oHSV) has been genetically engineered for oncolytic activity and safety [3]. For example, T-Vec (talimogene laherparepvec) was recently approved as the first oncolytic virus in the USA for the treatment of advanced melanoma [3]. oHSV treatment induces an inflammatory reaction and attracts a variety of immune cells to infiltrate into the treatment site signifying an antitumor immune response [4, 5]. Tumor inflammation and immune cells in a tumor section can be detected or visualized by immunohistochemistry (IHC), a commonly used and integral laboratory

staining technique [6, 7]. This permits analysis of the distribution of labeled cells and quantification of cell numbers. The basic principle of IHC is that a reaction occurs between an immune cell antigen and an antibody conjugated with an enzyme (peroxidase, alkaline phosphatase, etc.) that can catalyze production of a colored substrate [8, 9], which is eventually visualized by light microscopy. Alternatively, the antibody can also be tagged to a fluorophore (fluorescein, rhodamine, etc.), and the antigen–antibody reaction is visualized by detecting the fluorophore under fluorescence microscopy (immunofluorescence) [10]. Fluorescence-based IHC is not stable for several months or years, since fluorophores continuously fade over time. Chromogen/substrate-based IHC has a major advantage over fluorescence-based IHC since staining quality can be preserved for years at room temperature without a fading problem. Herein, we describe a step-by-step chromogen-based single-/dual-color immunohistochemical protocol for staining tumor-infiltrating immune cells in a mouse glioblastoma (GBM) brain tumor model treated with oHSV-based immunotherapy. The strategies described herein are also applicable for other mouse/human tumors in the brain and in the periphery.

2 Materials

The materials required for single/dual IHC are listed below. Few specific materials/reagents that are exclusively used for double IHC in this review are indicated by asterisk (*). All steps with flammable reagents, such as xylene/ethanol, must be done inside a fume hood. All water for buffers/solutions should be ultrapure, MilliQ, or distilled.

1. Slide staining set, preferably from polyoxymethylene (POM), including slide staining dishes, slide staining racks, and a power-coated metal housing.
2. Slide staining dish set rack containing a staining dish, lid, and a stainless steel rack.
3. Xylenes (Certified ACS).
4. * Xylene-free clearing solution.
5. 100% absolute ethanol.
6. 90% ethyl alcohol. Mix 450 mL absolute ethanol and 50 mL water.
7. 70% ethyl alcohol. Mix 350 mL absolute ethanol and 150 mL water.
8. Sodium citrate buffer. 10 mM sodium citrate, 0.05% Tween 20, pH 6.0. Dissolve 2.94 g Tri-sodium citrate (dihydrate) in 1 L water. Adjust pH with 1 N HCl. Add 0.5 mL Tween 20 and mix well. Store at 4 °C.
9. 1 mM EDTA, pH 8.0. Dissolve 0.37 g EDTA in 1 L water. Adjust pH with sodium hydroxide (NaOH). Store at 4 °C.
10. Tris–EDTA buffer. 10 mM Tris base, 1 mM EDTA solution, 0.05% Tween 20, pH 9.0. Dissolve 1.21 g Tris base and 0.37 g EDTA in 1 L water. Adjust pH with NaOH. Add 0.5 mL Tween 20 and mix well. Store at 4 °C.
11. 2 L glass beaker.

12. Plastic wrap.
13. 1× DPBS solution. Dissolve 95.5 g DPBS (Dulbecco's Phosphate-Buffered Saline) powder in 1 L water. Prepare 1× DPBS working solution for use by mixing 100 mL 10× DPBS and 900 mL water.
14. 1× DPBS/0.1% Tween 20. Add 1 mL Tween 20 in 1 L of 1× DPBS.
15. PAP Pen Liquid Blocker.
16. 3% H₂O₂ solution. Mix 20 mL 30% (w/w) hydrogen peroxide (H₂O₂) solution containing stabilizer and 180 mL water.
17. * Endogenous Peroxidase and Alkaline Phosphatase Blocking Solution.
18. Universal blocking buffer (5% BSA solution). Dissolve 1 g lyophilized bovine serum albumin (BSA) powder in 19 mL water. Store at 4 °C.
19. 10% horse serum blocking solution. Mix 1 mL normal horse serum and 9 mL water. Store at 4 °C.
20. 10% goat serum blocking solution. Mix 1 mL normal goat serum and 9 mL water. Store at 4 °C.
21. Primary antibodies for tumor-infiltrating immune cells (Table 1).
22. Humidified chamber.
23. HRP- and AP-conjugated secondary antibodies (Table 2).
24. DAB Substrate Chromogen System.
25. Red Alkaline Phosphatase (AP) substrate.
26. Blue AP substrate.
27. Hematoxylin solution. Mix 100 mL hematoxylin and 300 mL water.
28. Cover Glasses.
29. Xylene-based permanent mounting medium.
30. Xylene-free permanent mounting medium.

3 Methods

The protocol described below is designed to perform all steps at room temperature, except incubating brain tumor sections with primary antibody overnight at 4 °C in a humidified chamber. All steps with flammable reagents, such as xylene and ethanol, must be performed inside a fume hood.

3.1 Single Immunohistochemistry (Chromogen-/Substrate-Based) for Formalin-Fixed Paraffin-Embedded Tumor Sections

1. Deparaffinize sections in xylene twice for 10 min (*see* Note 1).

2. Rehydrate sections with gradually decreasing concentrations of ethyl alcohol: 100%, 90% and 70% (5 min with each concentration) (*see* Note 1).
3. Leave the slides in water for 5 min.
4. For antigen retrieval, microwave sections in 10 mM sodium citrate buffer for 15 min using a microwaveable vessel or a glass beaker (*see* Notes 2 and 3).
5. After 15 min, cool tumor sections at room temperature for 20–25 min (*see* Note 4).
6. Rinse tumor sections in 1× DPBS twice for 5 min.
7. While running **step 6**, circle each tumor section with water-resistant Liquid Blocker or PAP pen (*see* Note 5).
8. Block endogenous peroxidase activity by dipping slides in 3% H₂O₂ solution for 5 min (*see* Note 6).
9. Rinse slides in 1× DPBS twice for 5 min.
10. Block nonspecific antigen–antibody binding by incubating tumor sections with 30–50 µL universal blocking buffer (5% BSA solution) for 30 min, followed by antibody-specific blocking buffer, i.e. 10% horse or goat serum, for another 30 min (*see* Note 7).
11. Incubate tumor sections with an appropriate primary antibody (optimally diluted in an appropriate blocking buffer; use 30–50 µL/section) overnight at 4 °C in a humidified chamber (*see* Notes 8 and 9).
12. Wash sections in 1× DPBS/0.1% Tween 20 three times for 5 min (*see* Note 10).
13. Incubate sections with appropriate secondary antibodies, such as HRP-conjugated anti-rabbit, anti-rat, or anti-mouse IgG (depends on the host origin of the primary antibody) for 30 min (*see* Notes 11 and 12).
14. Repeat washing as in **step 12**.
15. Incubate sections with a drop of DAB, prepared by adding 1 drop of chromogen in 1 mL of DAB substrate, until brown color develops (*see* Note 13).
16. Rinse slides in water for 1 min (*see* Note 14).
17. For counterstaining, dip slides in hematoxylin solution for 20 s (*see* Note 15).
18. Wash sections in water for 1 min, then in running tap water for another 5 min (*see* Note 16).
19. Dehydrate sections using gradually increasing concentrations of ethyl alcohol: 70%, 90%, and 100%, 5 min with each concentration (*see* Note 17).
20. Dip sections in xylene twice for 10 min (*see* Notes 17 and 18).
21. Take the slides out of xylene (one-by-one). Place the slides on an even surface in a fume hood (*see* Note 19).

22. Place one drop of xylene-based mounting medium on a glass cover slip (*see* Note 20).
23. Apply the cover slip on a tumor section and distribute the mounting medium throughout the section/slide by applying finger pressure (*see* Note 21).
24. Air-dry slides in the fume hood for at least an hour before light microscopy (*see* Note 22). See single IHC staining of immune cells in brain tumor sections in Fig. 1a.

3.2 Double Immuno-histochemistry (Substrate-Based) for Formalin-Fixed Paraffin-Embedded Sections

Substrate-based double immunohistochemistry is an easy process if both primary antibodies originated from different hosts (e.g., rat and rabbit). In most cases, both primary antibodies come from the same host (e.g., rabbit anti-CD68 for total macrophage and rabbit anti-pSTAT1 for M1-like macrophage; *see* Note 8; Fig. 1b), which makes this process more complicated since the same secondary antibody (e.g., HRP- or AP-conjugated anti-Rabbit IgG) is used. To solve this problem, we described previously a substrate-based double IHC protocol to stain both rabbit anti-CD68 and anti-pSTAT1 or rabbit anti-CD3 (total T cells) and anti-Ki67 (proliferating T cells) in the same tumor section [4]. Because it is a dual-color IHC, two distinct colors should be chosen to visually separate one color from the other by light microscopy. Here, we used blue color for CD68 or Ki67 and red color for pSTAT1 or CD3 for visualization by light microscopy. Briefly, the brain sections were incubated sequentially with primary antibodies (rabbit anti-pSTAT1 or -CD3; antibody), then secondary antibodies (AP-conjugated anti-rabbit IgG), followed by the development of red color using red alkaline phosphatase substrate. Next, the same brain sections were incubated with primary antibodies (rabbit anti-CD68 or -Ki67 antibody), then secondary antibodies (AP-conjugated anti-rabbit IgG), followed by the development of blue color using blue alkaline phosphatase substrate. The step-by-step substrate-based double IHC protocol is described below. Avoid xylene-based clearing agents/mounting media/alcoholic solutions while performing the following protocol (*see* Note 23).

1. Deparaffinize brain tumor sections twice for 10 min using xylene-free clearing solution.
2. Follow **steps 2–7** as in single IHC.
3. To block endogenous alkaline phosphatase, add one drop blocking solution per section and incubate for 5 min (*see* Note 24).
4. Rinse slides in 1× DPBS twice for 5 min.
5. Block nonspecific antigen–antibody binding by following **step 10** in the single IHC protocol.
6. Incubate tumor sections with an appropriate primary antibody (e.g., rabbit anti-pSTAT1 or anti-CD3 antibody diluted in horse serum; use 30–50 µL/section) overnight at 4 °C in a humidified chamber (*see* Note 8).
7. Wash sections in 1× DPBS/0.1% Tween 20 three times for 5 min (*see* Note 10).

8. Add one drop AP-conjugated anti-rabbit Ig per section and incubate sections for 30 min at room temperature in a humidified chamber (*see* Note 12).
9. Follow **step 7**.
10. Add 50 μ L red substrate per section and incubate until red color has developed (*see* substrate preparation in Note 25).
11. Wash sections in 1 $\times$ DPBS/0.1% Tween 20 for 5 min (*see* Note 10).
12. Incubate tumor sections with an appropriate primary antibody (e.g., anti-CD68 or Ki67; rabbit antibody diluted in horse serum; use 30–50 μ L/section) overnight at 4 °C in a humidified chamber (*see* Note 8).
13. Follow **step 7**.
14. Add one drop AP-conjugated anti-rabbit IgG per section and incubate sections for 30 min at room temperature in a humidified chamber (*see* Note 12).
15. Follow **step 7**.
16. Add 50 μ L blue substrate per section until blue color has developed (*see* substrate preparation in Note 26).
17. Wash sections in 1 $\times$ DPBS/0.1% Tween 20 for 5 min.
18. Wash sections in water for 1 min.
19. Dehydrate sections using gradually increasing concentrations of ethyl alcohol: 70%, 90% and 100% (5 min per concentration) (*see* Note 27).
20. Dip sections in xylene-free clearing solution twice for 10 min (*see* Note 27).
21. Take the slides out of clearing solution (one-by-one). Place the slides on an even surface in a fume hood (*see* Note 19).
22. Place one drop of xylene-free mounting medium on a glass cover slip (*see* Note 20).
23. Apply the cover slip on a tumor section and distribute the mounting medium throughout the slide by applying finger pressure (*see* Note 21).
24. Air-dry slides in the fume hood for at least an hour before light microscopy (*see* Note 22). See dual-color IHC staining of immune cells in brain tumor sections in Fig. 1b.

4 Notes

1. Place slides containing sections in the slide rack, then dip the slide rack into xylene/ethanol in a staining dish. Xylene/ethanol solutions can be stored in airtight glass containers at room temperature and reused many times for months/years. If dirt appears, filter them before use. Xylene/ethanol steps must be performed in a fume hood.

2. Antigen retrieval step: This is a critical step for the success of this protocol. Sodium citrate buffer, 1 mM EDTA and Tris–EDTA buffer are the three most popular antigen retrieval solutions. Which one to use depends on the source of the primary antibody. It is advised to follow vendor instructions first regarding the selection of antigen retrieval solutions. However, sodium citrate buffer can be used as a default buffer solution for almost all primary antibodies (listed in Table 1) described in this protocol.
3. Antigen retrieval step: Use a 2 L microwaveable glass beaker (*see* Subheading 2) for boiling the sections. Do not fill the beaker with more than 600–800 mL retrieval solution. Before placing slides into retrieval buffer solution, first prewarm the solution by microwaving for 3 min. Afterward, place the rack holding the slides into a prewarmed buffer solution, cover the glass beaker with plastic wrap (make a few holes in the wrap to allow for evaporation during boiling), then boil for at least 15 min. Be sure to monitor for evaporation, watch out for boiling over during the procedure, and do not allow the slides to dry out.
4. The cooling process at room temperature can take time. To speed up the process, place tap water in a plastic bucket with some ice, and place the beaker in ice-cold tap water. This may take around 10 min for cooling.
5. Circling sections with liquid blocker is critical. If one slide has more than one section, this prevents spillover of primary/secondary antibodies/other reagents applied on one section to an adjacent section. Even if there is only one section/slide, circling the sections with a liquid blocker is needed to hold the antibodies/reagents on the tumor sections.
6. Endogenous peroxidase may react with the substrate during the color development stage (*see step 8*) and can evoke some unnecessary background. To avoid this, it is necessary to block endogenous peroxidase activity. However, blocking solution should not be applied for more than 5 min. Some protocols may require blocking alkaline phosphatase instead of endogenous peroxidase (*see* dual IHC protocol).
7. The type of blocking buffer to be used (to reduce nonspecific reactions between antigens and antibodies, and eventually unwanted background) depends on the source of secondary antibodies. For example, secondary antibodies anti-rabbit IgG and anti-mouse IgG are produced in horse, so 5–10% horse serum should be used as blocking buffer. Similarly, secondary antibody anti-Rat IgG is produced in goat, so 5–10% goat serum should be the blocking buffer. Most IHC secondary antibody kits come with ready-to-use 2.5% blocking buffer, which can be directly applied (one drop/section) on tumor sections. However, it is always better to have regular horse or goat serum at hand, so that users can prepare appropriate dilutions (e.g., 5–10%) as necessary. If a secondary antibody kit is obtained from a particular vendor, horse or goat serum should also be obtained from the same vendor.

8. Primary antibodies are listed in Table 1 with their appropriate dilution in appropriate blocking buffer. All antibodies listed detect mouse immune cell antigens (Fig. 1), and several of them are also reactive to other species. For more information, check antibody datasheets available at the vendor's site using the listed catalogue numbers.
9. Although many primary antibodies may perform well if incubation is done at room temperature for 30–60 min, it is usually better to incubate overnight at 4 °C. Duration of overnight incubation can vary from 12 h to 24 h, and this variability does not affect antibody performance or staining quality.
10. The wash time is arbitrary. Each wash time can be extended from 5 min to 10–15 min depending on the level of nonspecific staining background that may develop during color development (*see step 15*). Washing should be gentle (no rocking or movement is required) in order to avoid losing sections from the slide.
11. See the list of HRP-labeled secondary antibodies in Table 2. If the primary antibody is made in rabbit, rat, or mouse, then HRP-conjugated anti-rabbit, anti-rat, or anti-mouse IgG, respectively, must be used as secondary antibodies.
12. Apply one drop/section, however, if intensity of the staining (i.e. background) is high, it can be reduced by diluting secondary antibodies 1:1 in PBS.
13. Color development either by DAB or other chromogenic system needs careful observation to minimize the level of background. For some antibodies (anti-CD3, anti-CD4, anti-CD8, anti-CD68, anti-Ki67, etc. listed in Note 9), color development may take 20–60 s. This can be observed by the naked eye by placing the slide on a white background. As soon as color appears, the chromogenic reaction has to be stopped immediately by dipping the slides in water in order to avoid excessive background (*see step 16* in Single IHC). For other antibodies (e.g., anti-Cleaved Caspase 3), color development can take 2–5 min. Sometimes color cannot be seen by the naked eye (e.g., if very low number of immune cells present in the tumor, thus less chromogenic reaction or a good choice of primary antibody and low number of immune cells present in the tumor resulting in no background reaction), so slides can be placed under a light microscope to observe color development.
14. Slides can be kept in water as long as 1 h if necessary, and this should not affect the staining quality.
15. Counterstaining is done in order to better visualize the stained tumor-infiltrating immune cells. If sections have stronger background color (brown), hematoxylin staining can be done for as long as 1 min or even undiluted hematoxylin can be used for 20 s.
16. While washing slides with running tap water, place the sections facing opposite to the running water to avoid directly hitting the sections.
17. Use the same alcohol/xylene solutions applied in **steps 1 and 2**. Use a fume hood.

18. Sections can be kept in xylene longer than 10 min. Keeping sections in xylene longer during the second wash may be required while performing the mounting step (**step 21** in single IHC).
19. Never let the sections dry in the xylene step, which can lead to fragile brain tissue sections before mounting. Finish mounting quickly within 30 s–1 min.
20. The size of the glass cover slip depends on the number of sections present on a slide and the distance between the sections. In general, a standard microscope slide (75 × 25 mm) containing three or four sections can be easily covered by a 50 × 50 mm size cover glass. A 25 × 25 mm size cover slip can easily cover a single mouse brain tumor section.
21. Bubble removal: while covering the section with a cover slip containing Cytoseal XYL, bubbles may form, which can be removed by applying finger pressure.
22. These permanent mount slides can be stored at room temperature for years while preserving the staining quality.
23. Blue substrate is partially soluble in xylene. It is used in **step 16** of dual-color IHC protocol.
24. A blocking solution that can block both endogenous peroxidase and alkaline phosphatase activity was chosen for this protocol, since alkaline phosphatase (AP)-conjugated anti-rabbit IgG secondary antibody is included in the double IHC protocol. H₂O₂ can be replaced by this solution in single IHC in **step 8**.
25. Prepare red substrate working solution preparation according to the manufacturer's instructions (50 µL/section). Prepare just before use and mix well. Discard the leftover solution after use. Color development can take 20–30 min of incubation at room temperature.
26. Prepare blue substrate working solution according to the manufacturer's instructions (50 µL/section): Prepare just before use and mix well. Discard the leftover solution. Color development may take 20–30 min of incubation at room temperature.
27. Alcohol and clearing solutions can be reused. Follow Notes 17 and 18.

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Fig. 1.

(a) Single IHC staining of tumor infiltrating immune cells in mouse GBM. Representative images (10× magnification) are shown. Brown indicates positivity (anti-CD4, -CD8, -CD68+20% brightness correction). Scale bar = 100 μm. (b) Dual-color IHC staining of total macrophage (CD68; blue color) and M1-like macrophages (pSTAT1; red color) in mouse GBM. A representative image (20× magnification) is presented. Scale bar = 100 μm

Table 1

List of primary antibodies with their sources and dilutions

Primary antibodies	Source	Cat. no.	Dilution	Blocking buffer
Rabbit anti-CD3	Abcam	ab5690	1:100	Horse serum
Rat anti-CD4	eBioscience	14-9766-80	1:200	Goat serum
Rat anti-CD8a	eBioscience	14-0808-80	1:100	Goat serum
Rabbit anti-granzyme B	Abcam	ab4059	1:150	Horse serum
Rabbit anti-CD68	Abcam	ab125212	1:100	Horse serum
Rabbit anti-Ki67 (for proliferating immune cells by double IHC)	Abcam	ab16667	1:100	Horse serum
Rabbit anti-PD-L1	Abcam	ab205921	1:400	Horse serum
Rabbit anti-cleaved caspase-3 (Asp175) (for apoptotic immune cells by double IHC)	Cell Signaling	9661	1:100	Horse serum
Rabbit anti-phospho-stat1 (Tyr701)	Cell Signaling	9167	1:100	Horse serum
Rabbit anti-iNOS	Abcam	ab15323	1:100	Horse serum
Mouse anti-T-bet/Tbx21	Abcam	ab91109	1:100	Horse serum
Rat anti-MHC class II	Abcam	ab25333	1:150	Goat serum
Rabbit anti-F4/80	Abcam	ab111101	1:100	Horse serum
Rat anti-CD34	Abcam	ab8158	1:150	Goat serum

Table 2

List of secondary antibodies with their sources and dilutions

Secondary antibodies	Source	Cat. no.	Dilution
HRP anti-rat IgG	Vector Lab	MP-7444	One drop/section
HRP anti-rabbit IgG	Vector Lab	MP-7401	One drop/section
HRP anti-mouse IgG	Vector Lab	MP-7402	One drop/section
AP anti-rabbit IgG	Vector Lab	MP-5401	One drop/section