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Title: Detection of Intratumoral Immune Cell Antigens in an Immunocompetent Glioblastoma Stem-Like Cell Model Using Substrate-Based Immunohistochemistry

Authors and Affiliations:

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Author Questionnaire

1. Microscopy: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **Yes**

Scope shots: 6.2.3, 8.2.3

Videographer: Please film the above-mentioned shots using the scope kit

2. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **NO**

3. Filming location: Will the filming need to take place in multiple locations? **NO**

4. Testimonials (optional): Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **NO**

Current Protocol Length

Number of Steps: 26

Number of Shots: 55 (2 Scope)

Interviews

Videographer: Obtain headshots for all authors available at the filming location.

Authors: Please note that **the questions will not appear on screen**. Please answer in **stand-alone full sentence with sufficient context**

INTRODUCTION:

~~REQUIRED: Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Sourav Chakraborty:** Our research focuses on cancer immunovirotherapy and understanding tumor-immune and virus-immune interactions to develop strategies that enhance antitumor immune responses.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Connor Howard:** Standardized chromogenic IHC protocols for GSC-derived glioblastoma are limited; our protocol enables reproducible single- and dual-antigen detection in FFPE tissues
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

- 1.3. **Sourav Chakraborty:** This protocol enables visualization and assessment of tumor-infiltrating immune cell-associated antigens and their spatial distribution within glioblastoma tissues.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.4. **Robert Newman:** Careful optimization of antibody compatibility, concentration, sequential staining, and chromogenic substrates is critical for specific signals and clear antigen discrimination.

- 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.5. **Checo J. Rorie:** Future studies can adapt this protocol to other tumor models and human specimens to investigate immune infiltration and treatment-associated changes.
- 1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Videographer: Obtain headshots for all authors available at the filming location.

Ethics Title Card

This research has been approved by the Institutional Animal Care and Use Committee at North Carolina Agricultural and Technical State University and Massachusetts General Hospital and Harvard Medical School

Authors: The Protocol states that the work followed Institutional Animal Care and Use Committee protocols 2004N000067 and LA24-0045, but does not name the committee that approved or the institution. Please confirm the wording for the Ethics Title Card, and tell us whether both institutions should be named

Please name both institutions.

Authors: Please check the ethics statement now. Please email our proofreading team (proofreading@jove.com) to make this change in the text manuscript as well

Protocol

***Videographer:** Please always record an audio slate while filming each shot mentioned in the shotlist.*

Authors: Commercial product names have been replaced with generic terms in the narration, following the editorial comment. For example, the xylene-free clearing agent, the endogenous alkaline phosphatase blocking solution, and the red and blue substrates. Please check if the narration is correct.

2. Deparaffinization and Rehydration of the Tissue Sections for Dual-Color Immunohistochemistry

Demonstrator: Sourav Chakraborty

2.1. To begin, obtain 5-micrometer-thick formalin-fixed paraffin-embedded mouse brain tumor sections [1]. Place the slides in a slide rack, and immerse the rack in about 400 milliliters of the xylene-free clearing agent 1 for 10 minutes in a chemical fume hood [2-TXT], and then in the clearing agent 2 for a further 10 minutes [3].

2.1.1. WIDE: Talent placing the FFPE sample slide on the table.

2.1.2. Talent placing the slide in a rack and immersing the slide rack in the xylene-free clearing agent 1 under a fume hood. **TXT: See text protocol for buffers and chromogenic substrates**

2.1.3. Talent removing the rack from solution1 and placing in solution 2.

Authors: Please provide an approximate volume of the clearing agent needed **400 mL**

2.2. Rehydrate the sections by immersing the rack sequentially in 100, 90, and 70 percent ethanol, for 5 minutes each [1]. Then, place the slides in distilled water for 5 minutes to complete the rehydration [2].

2.2.1. Talent immersing the slide rack in 100% ethanol in a staining container

2.2.2. Talent immersing the slide rack in distilled water in a staining container, for 5 min.

3. Antigen Retrieval and Washing

Demonstrator: Connor Howard

3.1. Preheat a covered beaker containing freshly prepared 10 millimolar sodium citrate buffer, pH 6, in the microwave for 3 minutes [1]. Then place the slide rack into the buffer and microwave the sections for 15 minutes at the highest power [2].

3.1.1. WIDE: Talent placing the 2 L beaker holding the slide rack in 10 mM sodium citrate buffer into the microwave. **Author's NOTE: Using the microwave was filmed in 3.1.2.**

3.1.2. Talent opening the microwave and placing the slide rack in the buffer and closing the oven.

Authors: Please specify which of the 2 shots described here would you prefer, the microwave or the steam cooker on the shoot day. Or should we show both?

Response: Microwave only

3.2. Using heat-resistant gloves, remove the beaker from the microwave and cool it in ice-cold water for about 10 minutes [1]. Then, remove the slide rack, and rinse the sections twice in 1x DPBS (*D-P-B-S*) for 5 minutes each, using gentle, static washes [2].

3.2.1. Talent placing the hot beaker into a container of ice-cold water.

3.2.2. Talent transferring the slide rack from the beaker into a staining container of 1x DPBS.

Authors: Please specify which of the 2 cooling methods described here would you prefer, the bench cooling for 20 min or the ice-cold water bath for ~10 min, we can show only one of these. **Ice-cold water bath for 10 minutes**

3.3. During the wash, remove each slide individually and gently wipe around the tissue section with a lint-free wipe without touching the tissue [1]. Then, draw a liquid-repellent barrier around each tissue section with a hydrophobic barrier pen, and return the slide to the wash solution [2].

3.3.1. Close-up of Talent removing slide and wiping around the tissue section with a lint-free wipe.

3.3.2. Close-up of Talent drawing the hydrophobic barrier around the tissue section with the barrier pen.

3.3.3. Talent returning the slide to the wash solution.

4. Blocking

Demonstrator: Sourav Chakraborty

- 4.1. Once the barriers are complete, remove the slides from the staining container and place them flat in the humidified chamber [1]. Then, add 1 drop of the endogenous alkaline phosphatase blocking solution per section and incubate for 5 minutes [2].
 - 4.1.1. Talent removing the slides and placing the slides flat in the humidified chamber.
 - 4.1.2. Talent adding 1 drop of the endogenous alkaline phosphatase blocking solution to each section, and placing them aside.
 - 4.2. Rinse the slides in 1x DPBS in the staining container 2 times for 5 minutes each [1]. Then, place them flat in the humidified chamber, and add distilled water to the edges of the chamber [2].
 - 4.2.1. Talent rinsing the slides in 1x DPBS in a staining container.
 - 4.2.2. Talent place them flat in the humidified chamber and adding distilled water to the edges of the chamber.
- Authors:** I have added the word 'immediately' to the step 4.3 as per the NOTE given in the protocol
- 4.3. Immediately add 50 microliters of the 5 percent BSA blocking buffer to each section, and incubate for 30 minutes at room temperature with the chamber lid closed [1]. Then gently tap the slides onto absorbent paper to discard the blocking buffer [2].
 - 4.3.1. Talent adding 50 μ L of 5% BSA to each tissue section and keeping the sample aside. **Authors:** Instead of saying chamber lid on, can we say chamber lid closed? **YES**
 - 4.3.2. Talent gently tapping the slide on lint-free wipes to discard the BSA.
 - 4.4. Next, add one drop of 2.5% normal horse serum to each section and incubate for 30 minutes. The serum should match the host species of the alkaline phosphatase-conjugated secondary antibody [1]. Then, gently tap the slides on absorbent paper to discard the serum [2].
 - 4.4.1. Talent adding 1 drop of 2.5% normal horse serum to each tissue section and placing the sample aside.
 - 4.4.2. Talent gently tapping the slide on absorbent paper to discard the serum.

5. Incubation with the First Set of Antibodies

- 5.1. Towards the end of the blocking step, dilute the first primary antibody in 2.5 percent normal horse serum [1-TXT].
 - 5.1.1. Talent diluting the rabbit anti-CD3 antibody 1:100 in 2.5% normal horse serum.
TXT: See Table 1 for the recommended antibody dilutions
- 5.2. Add approximately 50 microliters of the diluted first primary antibody to each tissue section [1]. Incubate the slides overnight, for about 16 hours, at 4 degrees Celsius in the humidified chamber [2].
 - 5.2.1. Talent adding ~50 µL of the diluted rabbit anti-CD3 antibody to each tissue section.
 - 5.2.2. WIDE: Talent placing the humidified chamber in the 4 °C refrigerator for ~16 h.
- 5.3. The next day, discard the primary antibody by gently tapping the slides on absorbent paper [1]. Wash the sections 3 times in 1x DPBS with 0.1 percent Tween 20, for 5 minutes each [2].
 - 5.3.1. Talent gently tapping the slide on absorbent paper to discard the primary antibody.
 - 5.3.2. Talent washing the slides by adding 1x DPBS with 0.1% Tween 20 in a staining container.
- 5.4. Place the slides flat in the humidified chamber, and add one drop of the alkaline phosphatase-conjugated anti-rabbit secondary antibody per section [1]. Then, incubate the sections for 30 minutes at room temperature with the chamber lid closed [2].
 - 5.4.1. Talent adding 1 drop of the AP-conjugated anti-rabbit IgG secondary antibody to each tissue section. **Authors: What dilution do you recommend? Undiluted**
 - 5.4.2. Talent closing the lid of the humidified chamber for the 30 min incubation at RT.

6. Detection of the First Antigen with the Red Substrate

Demonstrator: Dipongkor Saha

- 6.1. After incubation with the secondary antibody, wash the sections again 3 times in 1x DPBS with 0.1 percent Tween 20, for 5 minutes each [1]. During the final wash, freshly prepare the red substrate working solution [2].
 - 6.1.1. Talent washing the slides by adding 1x DPBS with 0.1% Tween 20.
 - 6.1.2. Talent mixing the red substrate working solution components.
- 6.2. Place the slides flat on the workbench against a white background [1]. Thoroughly mix the red substrate working solution, then add 50 microliters to each tissue section [2]. Incubate at room temperature until a red signal appears. Check the sections every 2 to 3 minutes against a white background or under a light microscope [3].
 - 6.2.1. Talent placing the slides flat on white absorbent paper on the workbench.
 - 6.2.2. Talent adding 50 μ L of the red substrate working solution to each tissue section.
 - 6.2.3. SCOPE: The red chromogenic signal being checked in the tissue section under the light microscope. **Authors:** Can you provide an image for this if our videographer cannot film on the day of the shoot?
- 6.3. As soon as the desired red signal appears, rinse the sections in 1x DPBS with 0.1 percent Tween 20 for 5 minutes [1].
 - 6.3.1. Talent immersing the slides in 1x DPBS with 0.1% Tween 20 in a staining container.

7. Incubation with the Second Set of Antibodies

Demonstrator: Sourav Chakraborty

- 7.1. Place the slides in the humidified chamber and add approximately 50 microliters of the second primary antibody to each tissue section [1]. Then, incubate the slides overnight, for about 16 hours, at 4 degrees Celsius [2].
 - 7.1.1. Talent placing the slide in a humidified chamber and adding ~50 μ L of the diluted rabbit anti-Ki67 antibody to each tissue section.
 - 7.1.2. WIDE: Talent placing the humidified chamber in the 4 °C refrigerator.
- 7.2. The next day, wash the sections 3 times in 1x DPBS with 0.1 percent Tween 20, for 5 minutes each [1]. Then, place the slides flat in the humidified chamber [2].
 - 7.2.1. Talent washing the slides in 1x DPBS with 0.1% Tween 20.

7.2.2. Talent placing the slides flat in the humidified chamber.

7.3. Add one drop of the same alkaline phosphatase-conjugated anti-rabbit secondary antibody per section [1]. Then, incubate the sections for 30 minutes at room temperature with the chamber lid closed [2].

7.3.1. Talent adding 1 drop of the AP-conjugated anti-rabbit IgG secondary antibody to each tissue section.

7.3.2. Talent closing the lid of the humidified chamber.

8. Detection of the Second Antigen with the Blue Substrate

8.1. After incubation with the secondary antibody, wash the sections 3 times in 1x DPBS with 0.1 percent Tween 20, for 5 minutes each [1]. During the final wash, freshly prepare the blue substrate working solution [2].

8.1.1. Talent washing the slides by adding 1x DPBS with 0.1% Tween 20.

8.1.2. Talent mixing the blue substrate working solution components.

8.2. Place the slides flat on the workbench against a white background [1]. Then, add 50 microliters of the blue substrate working solution to each tissue section [2]. Incubate at room temperature until a blue signal appears, checking the sections every 2 to 3 minutes [3].

8.2.1. Talent placing the slides flat on white absorbent paper on the workbench.

8.2.2. Talent adding 50 μ L of the blue substrate working solution to each tissue section.

8.2.3. SCOPE: The blue chromogenic signal being checked in the tissue section under the light microscope. . **Authors:** Can you provide an image for this if our videographer cannot film on the shoot day?

8.3. As soon as the desired blue signal appears, immerse the sections in 1x DPBS with 0.1 percent Tween 20 for 5 minutes [1]. Then, rinse the sections in distilled water for 1 minute [2].

8.3.1. Talent immersing the slides in 1x DPBS with 0.1% Tween 20 in a staining container.

8.3.2. Talent rinsing the slides in distilled water in a staining container.

9. Dehydration and Mounting of the Stained Sections

Demonstrator: Connor Howard

- 9.1. In a chemical fume hood, dehydrate the sections by immersing the rack sequentially in 70, 90, and 100 ethanol, for 5 minutes each [1]. Then, immerse the slides in the xylene-free clearing agent 1 for 10 minutes, and then in the clearing agent 2 for a further 10 minutes [2].
 - 9.1.1. Talent immersing the slide rack in 70% ethanol in a staining container.
 - 9.1.2. Talent immersing the slide rack in the xylene-free clearing agent 1.
- 9.2. Remove the slides from the second clearing agent and place them flat in the fume hood [1]. Add one drop of non-xylene-based mounting medium to an appropriately sized coverslip [2].
 - 9.2.1. Talent removing a slide from the xylene-free clearing agent II and placing it flat in the fume hood.
 - 9.2.2. Close-up of Talent placing 1 drop of the non-xylene-based mounting medium on the coverslip.
- 9.3. Quickly place the coverslip on the slide with the mounting medium facing the tissue sections [1]. Gently press the coverslip with the thumb to spread the medium evenly and remove any air bubbles [2].
 - 9.3.1. Close-up of Talent placing the coverslip on the slide with the mounting medium facing the sections.
 - 9.3.2. Close-up of Talent gently pressing the coverslip with the thumb. [We have filmed 9.3.1 and 9.3.2 combinedly]
- 9.4. Finally, air-dry the slides in the fume hood for at least 2 hours [1]. Evaluate and image the stained sections under a light microscope using a 20x objective [2].
 - 9.4.1. Talent placing the mounted slides flat in the fume hood to air-dry for at least 2 h.
 - 9.4.2. Shot of the sample being placed under the light microscope.

Authors: Please confirm the scope objective we should state here **20X**

Results

10. Results

- 10.1. The single-color protocol detected both surface [1] and intracellular antigens as distinct brown signals, while preserving tissue morphology with minimal background staining [2].
 - 10.1.1. LAB MEDIA: Figure 1. *Video editor: Please sequentially emphasize the CD3, the CD4, and the CD8 panels*
 - 10.1.2. LAB MEDIA: Figure 2. *Video editor: Please sequentially emphasize the Ki67 and the GranB panels*
- 10.2. The dual-color protocol detected two antigens within the same tissue section. CD3-positive T cells stained red, Ki67-positive cells stained blue, and arrows indicate double-positive proliferating T cells [1].
 - 10.2.1. LAB MEDIA: Figure 3, top image. *Video editor: Please emphasize the black arrows marking the colocalized cells*
- 10.3. Co-staining for pSTAT1 (*p-stat-1*) in red and CD68 in blue was indicative of M1-like tumor-associated macrophages within the same section [1].
 - 10.3.1. LAB MEDIA: Figure 3, bottom image. *Video editor: Please emphasize the black arrows marking the colocalized cells*
- 10.4. The same workflow co-detected the neuronal marker NeuN (*new-N*) in red and green fluorescent protein in blue, which likely indicates that the tumor cells differentiate into cells carrying a neuronal marker [1].
 - 10.4.1. LAB MEDIA: Figure 4. *Video editor: Please emphasize the black arrows in the region labeled TUMOR AREA*

1. Deparaffinization
 Pronunciation link: No confirmed link found
 IPA: /diːˌpærəˈfɪnəˈzeɪʃən/
 Phonetic Spelling: dee·pair·uh·fin·uh·ZAY·shuhn

2. Rehydration
Pronunciation link: <https://www.merriam-webster.com/dictionary/rehydration>
IPA: /,ri:har'dreɪʃən/
Phonetic Spelling: ree·hy·DRAY·shuhn
3. Immunohistochemistry
Pronunciation link: <https://www.howtopronounce.com/immunohistochemistry>
IPA: /,ɪmjənoʊ,hɪstoʊ'kɛmɪstri/
Phonetic Spelling: im·yuh·noh·his·toh·KEM·ih·stree
4. Formalin
Pronunciation link: <https://www.merriam-webster.com/dictionary/formalin>
IPA: /'fɔ:rməlɪn/
Phonetic Spelling: FOR·muh·lin
5. Paraffin
Pronunciation link: <https://www.merriam-webster.com/dictionary/paraffin>
IPA: /'pærəfɪn/
Phonetic Spelling: PAIR·uh·fin
6. Xylene
Pronunciation link: <https://www.merriam-webster.com/dictionary/xylene>
IPA: /'zaɪ,lɪ:n/
Phonetic Spelling: ZY·leen
7. Ethanol
Pronunciation link: <https://www.merriam-webster.com/dictionary/ethanol>
IPA: /'ɛθə,nɔ:l/
Phonetic Spelling: ETH·uh·naal
8. Antigen
Pronunciation link: <https://www.merriam-webster.com/dictionary/antigen>
IPA: /'æntɪdʒən/
Phonetic Spelling: AN·tih·juhn
9. Millimolar
Pronunciation link: No confirmed link found
IPA: /,mɪlɪ'moʊlər/
Phonetic Spelling: mil·ih·MOH·ler
10. Sodium citrate
Pronunciation link: <https://www.merriam-webster.com/dictionary/sodium%20citrate>
IPA: /'soʊdiəm 'sɪ,treɪt/
Phonetic Spelling: SOH·dee·uhm SIT·rayt
11. DPBS
Pronunciation link: No confirmed link found
IPA: /,di:pi:bi:'ɛs/
Phonetic Spelling: D·P·B·S
12. Hydrophobic
Pronunciation link: <https://www.merriam-webster.com/dictionary/hydrophobic>
IPA: /,haɪdrə'foʊbɪk/
Phonetic Spelling: hy·druh·FOH·bik

13. Endogenous
Pronunciation link: <https://www.merriam-webster.com/dictionary/endogenous>
IPA: /ɛnˈdɑːdʒənəs/
Phonetic Spelling: en·DAH·juh·nuhs
14. Alkaline
Pronunciation link: <https://www.merriam-webster.com/dictionary/alkaline>
IPA: /ˈælkəˌlaɪn/
Phonetic Spelling: AL·kuh·line
15. Phosphatase
Pronunciation link: <https://www.howtopronounce.com/phosphatase>
IPA: /ˈfɑːsfəˌteɪs/, /ˈfɑːsfəˌteɪz/
Phonetic Spelling: FOSS·fuh·tays / FOSS·fuh·tayz
16. BSA
Pronunciation link: No confirmed link found
IPA: /ˌbiː ɛs ˈeɪ/
Phonetic Spelling: B·S·A
17. Serum
Pronunciation link: <https://www.merriam-webster.com/dictionary/serum>
IPA: /ˈsɪrəm/
Phonetic Spelling: SEER·uhm
18. Conjugated
Pronunciation link: <https://www.merriam-webster.com/dictionary/conjugated>
IPA: /ˈkɑːndʒəˌgeɪtɪd/
Phonetic Spelling: KON·juh·gay·tid
19. Antibody
Pronunciation link: <https://www.merriam-webster.com/dictionary/antibody>
IPA: /ˈæntɪˌbaːdi/
Phonetic Spelling: AN·tih·baa·dee
20. Anti-rabbit
Pronunciation link: No confirmed link found
IPA: /ˌæntɪˈræbɪt/
Phonetic Spelling: an·tee·RAB·it
21. IgG
Pronunciation link: No confirmed link found
IPA: /ˌaɪ dʒiː ˈdʒiː/
Phonetic Spelling: eye·G·G
22. Chromogenic
Pronunciation link: <https://www.howtopronounce.com/chromogenic>
IPA: /ˌkroʊməˈdʒenɪk/
Phonetic Spelling: kroh·muh·JEN·ik
23. Ki67
Pronunciation link: No confirmed link found
IPA: /ˌkiː ˌsɪksti ˈseɪvən/
Phonetic Spelling: kee·SIX·tee·SEV·uhn

24. Intracellular
 Pronunciation link: <https://www.merriam-webster.com/dictionary/intracellular>
 IPA: /ˌɪntrəˈsɛljələr/
 Phonetic Spelling: in·truh·SEL·yuh·ler
25. Morphology
 Pronunciation link: <https://www.merriam-webster.com/dictionary/morphology>
 IPA: /mɔːrˈfɑːlədʒi/
 Phonetic Spelling: mor·FAH·luh·jee
26. Colocalized
 Pronunciation link: No confirmed link found
 IPA: /ˌkoʊˈloʊkəˌlaɪzd/
 Phonetic Spelling: koh·LOH·kuh·lyzd
27. pSTAT1
 Pronunciation link: No confirmed link found
 IPA: /ˌpiːˈstæt wʌn/
 Phonetic Spelling: pee·STAT·one
28. CD68
 Pronunciation link: No confirmed link found
 IPA: /ˌsiː diːˈsɪks tiːˈeɪt/
 Phonetic Spelling: C·D·SIX·tee·ayt
29. Macrophages
 Pronunciation link: <https://www.merriam-webster.com/dictionary/macrophage>
 IPA: /ˈmækrəˌfeɪdʒɪz/
 Phonetic Spelling: MAK·ruh·fay·jiz
30. Tumor-associated
 Pronunciation link: No confirmed link found
 IPA: /ˈtuːmə əˌsoʊsiˈeɪtɪd/
 Phonetic Spelling: TOO·mer·uh·SOH·see·ay·tid
31. NeuN
 Pronunciation link: No confirmed link found
 IPA: /ˈnuː ɛn/
 Phonetic Spelling: NEW·N
32. Fluorescent
 Pronunciation link: <https://www.merriam-webster.com/dictionary/fluorescent>
 IPA: /flʊˈresənt/, /flɔːˈresənt/
 Phonetic Spelling: floo·RESS·uhnt / flaw·RESS·uhnt
33. Neuronal
 Pronunciation link: <https://www.merriam-webster.com/dictionary/neuronal>
 IPA: /nɜːˈroʊnəl/
 Phonetic Spelling: noo·ROH·nuhl