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Protocol

Immunohistochemical Assessment of Immune Cells in Mouse Tumor Tissue

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This protocol describes a procedure for the evaluation of immune cells at the histological level. This technique preserves tissue architecture, making use of tissues that have been frozen in optimal cutting temperature (OCT) medium immediately after dissection and then cut with a cryostat into thin (~8 μm) sections. The tissue slices are transferred to microscope slides and, as described here, are fixed with either methanol or formaldehyde. The fixed tissue sections are then stained with antibodies directed against cell surface markers expressed by the immune cells of interest. The antibodies are detected with an avidin–biotin-based peroxidase system, and, after mounting, the sections can be stored and viewed for years.

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MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPE: Please see the end of this article for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Antibodies, primary and secondary (see Table 1)
Blocking solution (PBT + 10% goat serum)

The serum used for the blocking solution should be from the species in which the secondary antibody was raised.

DAB (3,3′-diaminobenzidine) peroxidase substrate kit (including Buffer Stock Solution, DAB stock solution, and Hydrogen Peroxide Solution; Vector Laboratories SK-4100)
Ethanol (95%, 100%)
Hematoxylin
Histomount mounting medium
Hydrogen peroxide (0.3% in ddH_2O)
Methanol (prechilled to −20°C) or formaldehyde (3%) <R>
Phosphate-buffered saline (PBS)
PBT (PBS + 0.1% Tween-20)

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TABLE 1. Antibodies used for immunohistochemical assessment of immune cells in mouse tumor tissue

Antibody	Clone	Species/isotype	Company	Catalog no.	Stock concentration	Staining dilution
CD3	KT3	RatIgG1, κ	AbD Serotec	MCA500GA	1.0 mg/mL	1:50
CD11b	M1/70	RatIgG2b, κ	BD Biosciences	550282	125 µg/mL	1:50
CD45	30-F11	RatIgG2b, κ	BD Biosciences	550539	62.5 µg/mL	1:50
F4/80	BM8	Rat IgG2a, κ	eBioscience	14-4801-82	0.5 mg/mL	1:50
Gr-1	RB6-8C5	RatIgG2b, κ	BioXcell	BE0075	Varies	15 µg/mL
Biotinylated goat anti-rat Ig	Polyclonal	Goat	BD Biosciences	559286	0.5 mg/mL	1:200

Slides with mouse tumor tissue and positive control tissue sections, frozen with OCT medium and stored at −80°C
Vectastain Elite ABC kit (including Reagents A and B; Vector Laboratories PK-6100)
Xylene

Equipment

Conical tubes (50 mL)
Coplin jar
Coverslips
Dry ice
Filter paper
Freezer set to −20°C
Hood
Humidified chamber
Microscope
Paper towels
Pens (slide-marking and hydrophobic)
Refrigerator set to 4°C
Slide boxes
Slide carrier
Slide holder
Slide staining chamber
Slide staining dishes, racks, and jars (e.g., EasyDip slide staining system; Ted Pella)
Slide tray (glass)
Tissue (lint-free)
Waste container (for DAB)

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METHOD

For this protocol, we recommend two controls: As a negative control, the secondary antibody should be used alone on the same tissue as the test samples. As a positive control, the primary and secondary antibodies should be used to stain spleen or another tissue known to contain the cells of interest.

Day 1: Tissue Preparation, Fixation, Blocking, and Primary Antibody Stain

1. Remove the frozen slides to be stained from storage at −80°C, and place them into a slide staining chamber.

   Keep the slides on dry ice while removing them from the freezer.

2. Place the slides in a hood to dry for ~10 min.
3. Label the slides with the pertinent sample information, and outline the tissue using a hydrophobic pen. Allow the hydrophobic circle to dry for ~5 min.

   From this point on, do not allow the slides to dry out.

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4. If the slides are to be fixed in methanol, immerse the slides in cold (−20°C) methanol in a Coplin jar for 10 min at −20°C. Immediately transfer the slides to PBT for washing in Step 6. Discard the fixative solution in the sink.
5. If the slides are to be fixed in formaldehyde, immerse the slides in 3% formaldehyde solution in a Coplin jar for 15 min at room temperature. Immediately move the slides into PBT for washing. Transfer the formaldehyde solution to a 50-mL conical tube and discard in the biohazard trash. Rinse the Coplin jar in ddH_2O.
6. Wash the slides twice for 2 min in PBT in a Coplin jar at room temperature.
7. Remove the slides from the PBT, place them in a staining chamber, and dry the hydrophobic outlines with lint-free tissue.
8. Block endogenous peroxidase activity by overlaying each slide with ∼200 µL of 0.3% hydrogen peroxide in distilled H_2O for 10 min at room temperature. After the incubation, drain the hydrogen peroxide solution onto a paper towel.
9. Wash the slides twice for 2 min in PBT in a Coplin jar at room temperature.
10. Remove the slides from the PBT, place them in the staining chamber, and dry the hydrophobic outlines with lint-free tissue.
11. Block the samples by overlaying each slide with ∼200 µL of blocking solution. Humidify the staining chamber, and incubate the slides in the humidified chamber for 30 min at room temperature.

    The "humidified chamber" is created by adding H_2O to the grooves between where the slides sit in the staining chamber.
12. Prepare primary antibody in blocking solution according to the dilutions indicated in Table 1.
13. Discard the blocking solution for all slides except the negative control, and dry the hydrophobic outlines. Add 100–200 µL of primary antibody diluted in blocking solution to each slide, and incubate overnight at 4°C.

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Day 2: Secondary Antibody Stain, Detection, and Counterstain

14. Wash the slides twice for 2 min in PBT in a Coplin jar.
15. Remove the slides from the PBT, place them in a staining chamber, and dry the hydrophobic outlines with lint-free tissue.
16. Dilute the secondary antibody (biotinylated polyclonal goat anti-rat antibody) in blocking solution, and overlay 150–200 µL of this diluted secondary antibody solution on each slide. Humidify the staining chamber, and incubate the slides in the humidified chamber for 40–60 min at room temperature.
17. During the secondary antibody staining, prepare enough Vectastain ABC Reagent for ∼150–200 µL per slide.
    i. Add 1 µL of Reagent A per 50 µL of PBS. Mix thoroughly by vortexing.
    ii. Add 1 µL of Reagent B to the mixture. Mix thoroughly by vortexing.
    iii. Allow the Vectastain reagent to stand for ∼30 min at room temperature before use.

    For example, for eight slides: To 1.6 mL of PBS, add 32 µL of Reagent A, followed by 32 µL of Reagent B.
18. At the end of the incubation with the secondary antibody stain, wash the slides twice for 2 min in PBS in a Coplin jar.

    From this point on, use only PBS, not PBT.
19. Remove the slides from the PBS, place them in the staining chamber, and dry the hydrophobic outline using lint-free tissue.

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20. Overlay each slide with 150–200 µL of Vectastain ABC Reagent. Humidify the staining chamber, and incubate the slides in the humidified chamber for 30 min at room temperature.
21. During the incubation with Vectastain ABC Reagent, prepare ~200 µL of peroxidase solution per slide.
    i. Add 1 µL of buffer stock solution to every 50 µL of distilled H_2O. Mix thoroughly by vortexing.
    ii. Add 2 µL of DAB stock solution and mix thoroughly by vortexing.
    iii. Add 1 µL of hydrogen peroxide solution and mix thoroughly by vortexing.

    For example, for eight slides: To 1.6 mL of ddH_2O, add 32 µL of buffer stock solution, followed by 64 µL of DAB, followed by 30 µL of hydrogen peroxide solution.
22. Wash the slides twice for 2 min in PBS in a Coplin jar.
23. Incubate the slides in peroxidase substrate solution until the desired stain intensity develops. Use a slide holder to observe the slides under a microscope in the dark.

    When using the secondary antibody alone as a negative control, incubate for the same duration as the experimental sections.
24. Rinse the sections by squirting them with ddH_2O over the DAB waste container, and place the slides in a glass slide tray filled with ddH_2O.
25. For the hematoxylin counterstain, filter the hematoxylin, and then transfer the filtered solution to a slide staining dish.

    Use gloves when working with hematoxylin; it will stain skin.
26. Transfer the slides to a slide carrier.
27. Immerse the slides in hematoxylin solution for 40–45 sec. Shake the slides occasionally to ensure even distribution of hematoxylin on tissue.
28. Place the slides back in ddH_2O. Shake them for ~2 sec, empty the bucket, and refill it with fresh ddH_2O. Repeat the rinsing process several times with fresh ddH_2O, until the rinse H_2O no longer turns blue.
29. Perform the next set of steps in a hood.
    i. Arrange six slide staining jars in sequence: two containing 95% ethanol, two containing 100% ethanol, and two containing xylene. Make sure all of the jars are filled enough to cover the specimens (in the Pella EasyDip slide staining system, up to the kink in the side).
    ii. Dunk the slides six times for 5 sec in the first jar of 95% ethanol.
    iii. Dunk the slides six times for 5 sec in the second jar of 95% ethanol.
    iv. Dunk the slides six times for 5 sec in the first jar of 100% ethanol.
    v. Dunk the slides six times for 5 sec in the second jar of 100% ethanol.
    vi. Dunk the slides six times for 5 sec in the first jar of xylene.
    vii. Dunk the slides six times for 5 sec in the second jar of xylene.

    Change the 95% ethanol in the jars with each staining session. Replenish the 100% ethanol jars with each staining session, and change every 2 wk. Change the xylene in the jars every 2 wk.
30. Allow the slides to dry at room temperature in the hood.

    If necessary, the procedure can be stopped here until the following day.
31. Mount the slides with Histomount solution; use glass coverslips and avoid air bubbles. Allow ~30 min for the slides to dry. They can be stored in slide boxes for years.

    Samples of immunohistochemical images are shown in Figure 1. See Troubleshooting.

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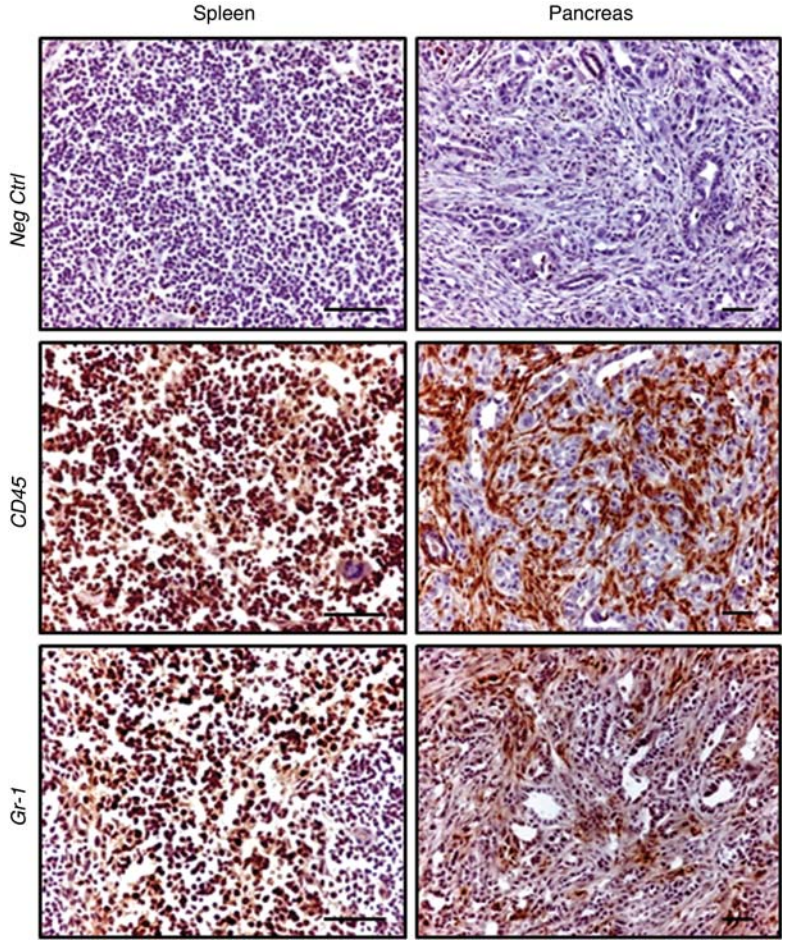

FIGURE 1. Sample immunohistochemical images of spleen and pancreas tissue, each from a pancreatic tumor-bearing mouse, stained for CD45 and Gr-1. Neg Ctrl, negative control (secondary antibody alone). Scale bars, 50 µm (note the differences in magnification between the two tissues).

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TROUBLESHOOTING

Problem (Step 31): There is a lack of color balance between counterstaining and specific staining.

Solution: It can be difficult to obtain the right balance between counterstaining and specific staining (Steps 23 and 27). Each stain must be optimized on its own, which typically requires varying the actual incubation time and using this precise time for each sample for any given experiment.

Problem (Step 31): There is unwanted background staining of noncellular material.

Solution: This problem can often be overcome by switching to immunofluorescent staining, which requires fluorochrome-labeled antibodies and retitration of the antibodies. Background staining and optimal titration of antibodies might vary from tissue to tissue.

Problem (Step 31): Tissue architecture is poor on OCT sections.

Solution: An alternative approach that better preserves tissue architecture is to fix the tissue immediately upon dissection and embed it in paraffin for sectioning. Staining procedures will then require antigen retrieval steps, and antibodies will have to be retitrated for this approach (Bayne et al. 2012).

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DISCUSSION

This protocol is very useful for determining the spatial location of the main immune subpopulations in the tumor microenvironment. Quantification is more tedious but can be performed manually or automatically with appropriate equipment and software. Findings using these approaches have been published (Clark et al. 2007; Beatty et al. 2011; Bayne et al. 2012).

RECIPE

Formaldehyde (3%)

1. Prepare monobasic sodium phosphate buffer by dissolving 4.22 g of sodium dihydrogen orthophosphate hydrate ($NaH_2PO_4 \cdot H_2O$) in 250 mL of ddH_2O. Adjust the pH to ~7.4.
2. In a hood, immediately before use, prepare 3% (v/v) formaldehyde by adding 1.5 mL of 37% (w/v) formaldehyde solution to 48.5 mL of monobasic sodium phosphate buffer.

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