full spectrum cell analysis
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Immunohistochemical staining of formalin-fixed paraffin embedded tissues

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Materials

- Phosphate buffered saline (PBS) (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.76mM KH_2PO_4, pH 7.4)
- Clearing and rehydration reagents: Histoclear II, Ethanol 100%, 90%, and 70%
- Heat induced epitope retrieval (HIER) reagent: 10mM Sodium citrate buffer pH 6.0
- Antigen retrieval reagent: Proteinase K (20ug/mL in TE buffer, pH 8.0) or Trypsin (0.05% with 0.1% CaCl, pH7.8)
- Endogenous peroxidase blocking solution: 0.3% H_2O_2 diluted in ddH_2O
- Blocking reagent: 10% normal serum (species from which the secondary antibody is made) in PBS
- Primary antibody: Purified or biotinylated format (for 2-step protocol)
- Secondary antibody: Biotinylated (for 3-step protocol)
- Amplification reagent: Avidin- horseradish peroxidase (cat. 18-4100)
- Visualization reagents: H_2O_2 30% stock, Diaminobenzidine (DAB) 1% stock (Sigma chemical)
- Nuclear counterstain: Hematoxylin (Fisher Scientific)
- Mounting medium: Permount (Fisher Scientific)

Experimental Procedure

1. Paraffin-embedded specimens are cut and mounted on Superfrost plus slides. Slides are placed in plastic vertical slide holders.
2. Slides are heated for 20 minutes at 50-60°C in a dry oven to facilitate attachment of tissue and to soften the paraffin
   NOTE: Temperature should not exceed 60°C so as to not damage target antigens.
3. Remove paraffin and rehydrate tissue using the following slide wash/incubation sequence: Histoclear II 3 x 5 minutes, 100% Ethanol 2 x 5 minutes, 90% Ethanol 1 x 5 minutes, 70% Ethanol 1 x 5 minutes, ddH_2O 1 x 5 minutes. When moving the slides through the solutions, be sure to adequately mix the solutions and remove bubbles from collecting on the slides by dipping the slide holder with slides up and down in the solution several times. From this point on, it is critical that the tissue does not dry out as this will result in difficulty in interpretation of staining results.
   NOTE: If detecting an antigen with an epitope that is susceptible to crosslinking during fixation (resulting in decreased sensitivity of the primary antibody), antigen retrieval using heat with citrate buffer or protein digestion may be required. The antigen retrieval methods are dependent on the antigen of interest and must be optimized; incubation times are suggested starting points for optimization (see optional steps 4 or 5). We recommend trying both no antigen retrieval and either step 4 or 5 for detection of novel antigens.

4. Optional: Heat induced epitope retrieval (HIER). Completely submerge slides in excess antigen retrieval buffer and microwave till boiling. Continue boiling 15 minutes and then allow slides in citrate buffer to cool to room temperature (about 20 minutes).

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5. Optional: Antigen retrieval (protein digestion). Submerge slides in Proteinase K or Trypsin for 10-20 minutes at 37°C and then allow slides to cool to room temperature.
6. Gently wash slides by soaking in PBS (5 minutes/wash) using a coplin jar and orbital shaker set to low speed, repeat wash changing the solution twice.
7. If a peroxidase-catalyzed visualization (ex. DAB) will be used as the final step in the protocol, block endogenous peroxidase by submerging slides in 0.3% H_2O_2 for 15-40 minutes at room temperature
   NOTE: Individual tissues will require optimization of incubation times depending on the amount of endogenous peroxidase present in the tissue.
8. Gently wash slides 3X in PBS (5 minutes/wash) as described in step 6.
9. Block non-specific binding sites by completely overlaying the tissue with 10% normal serum blocking reagent (100uL-150uL/slide depending on the size of the tissue section) for 1 hour at room temperature. To limit evaporation of blocking reagent and to help evenly spread the blocking solution, gently overlay the tissue section with a piece of Parafilm cut to the dimension of the tissue. The Parafilm is not stretched and should only contact the blocking solution and not touch the tissue directly. Ensure that there are no air bubbles trapped underneath the Parafilm layer by gently lifting the edge of the Parafilm with forceps and allowing the air bubbles to disperse. Incubate slides in a humidified container to reduce the amount of evaporation.
10. Using forceps, gently lift and remove the Parafilm and submerge the slide into a coplin jar containing PBS and wash 1X in PBS (5 minutes/wash) as described in step 6.
11. Dilute the primary antibody (at manufacturer's recommended dilution) in blocking reagent and overlay the solution onto the tissue. Cover with Parafilm (as described in step 9) and incubate in a humidified container for 2 hours at room temperature.
    NOTE: Low abundance antigens may require primary antibody incubation times of up to 12 hours at 4°C.
12. Wash the slides 3X in PBS as described in step 6.

13. If using an unconjugated primary antibody, continue to step 13 (3-step protocol).
14. If using a biotinylated primary antibody, continue to step 15 (2-step protocol).

15. Dilute the biotinylated secondary antibody (at manufacturer's recommended dilution) in blocking reagent and overlay the solution onto the tissue. Cover the tissue with Parafilm (as described in step 9) and incubate in a humidified chamber for 1 hour at room temperature.
16. Wash the slides 3X in PBS as described in step 6.
17. Dilute the amplification reagent avidin- horseradish peroxidase (at recommended dilution) in blocking reagent and overlay the tissue. Cover the tissue with Parafilm and incubate for 30 minutes at room temperature in a humidified container (as described in step 9).
18. Wash the slides 3X in PBS (as described in step 6).
19. Prepare the visualization/development solution by adding 500 µL of DAB stock and 2.5 µL 30%H_2O_2, to 50 mL 1X PBS pH 7.4.
    NOTE: DAB is a suspected carcinogen, see manufacturer's recommendations for handling and disposal). Incubate slides in DAB until brown staining is detected, monitoring carefully using a brightfield microscope.
20. When development is complete, wash the tissue sections by immersing the slides into a coplin jar containing ddH_2O.

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21. If desired, nuclei can be counterstained using hematoxylin, again monitor until desired staining is achieved. Once nuclei are stained, immerse the slides into a coplin jar containing ddH_2O. Hold the coplin jar under a trickle of water to remove excess dye, but taking care not to damage the tissue section.
22. Dehydrate the tissue sections by incubating the slides in the reverse order of hydration: 3 x 5 min each: 70% ETOH, 90% ETOH, 100% ETOH, and 3 x Histoclear II (the reverse order of the wash-hydration sequence described in step 3).
23. Mount and coverslip using 3 drops of Permount. Allow slides to dry for 1-2 hours before visualizing using a brightfield microscope.

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