
1. Formalin-fixed paraffin-embedded human tumor tissue blocks are received by the Biorepository and Tissue Research Facility (BTRF)
   • The BTRF removes any patient identifiers from blocks and performs 5 µm tissue sectioning
   • The BTRF deparaffinizes the sections and performs High pH TR Dako antigen retrieval for 20 mins at 97°C with 0 psi. The antigen retrieval time may be cut in half if the formalin fixation was weak for a particular specimen.
   • It is recommended to validate antibodies by immunohistochemistry before performing immunofluorescence.
   • It is recommended to have an adjacent section stained by hematoxylin and eosin for comparison.

2. Deparaffinized and antigen-retrieved slides are received from the BTRF in PBS.
   • Once slides are received, proceed with the protocol immediately. Do not allow slides to sit in PBS for an extended period of time.
   • Take note of the tissue fragility throughout staining procedure

3. Wash slides 2 × 5 min in PBS.

4. Block slides in a humid chamber for 1 hr at room temperature in blocking solution: 1× Western Blocking Reagent (Roche #11921673001) diluted in PBS + 0.1% Tween-20.
   • Humid chambers can be constructed with an inverted pipette-tip box containing a paper town saturated with water
   • 100–200 µl of blocking solution is sufficient for each slide
   • A piece of parafilm should be placed on each slide to ensure that the solution is evenly dispersed

5. Add primary antibody at the appropriate dilution in blocking solution overnight in a humid chamber at room temperature.
   • 100–200 µl of blocking solution is sufficient for each slide
   • A piece of parafilm should be placed on each slide to ensure that the solution is evenly dispersed

6. Wash slides 3 × 5 min in PBS.

7. Add secondary antibody at the appropriate dilution in blocking solution for 1 hr in a humid chamber at room temperature.
   • 100–200 µl of secondary-antibody solution is sufficient for each slide
   • 1:200 dilution of species-specific, highly-crosslinked Alexa-labeled antibodies from Molecular Probes; these fluorochromes do not need to be protected from ambient light during the staining process
   • A piece of parafilm should be placed on each slide to ensure that the solution is evenly dispersed

8. Wash slides 3 × 5 min in PBS.

9. Counterstain cells with 0.5 µg/ml DAPI and DyLight750-labeled wheat-germ agglutinin (1:350 dilution) in PBS for 5 min.
   • 100–200 µl of counterstaining solution is sufficient for each slide
   • A piece of parafilm should be placed on each slide to ensure that the solution is evenly dispersed
   • Slides may rest on the bench top at this time

10. Wash slides 2 × 5 min in PBS.

11. Incubate slides with 10 mM CuSO₄ in 50 nM NH₄Ac (pH 5.0; acidify with acetic acid) for 10 min.
   • CuSO₄ treatment dramatically reduces autofluorescence of sections, while only slightly diminishing the fluorescence of Cy- and Alexa-family dyes (Schnell et al. J. Histochem. Cytochem. 47:719 (1999))
   • Higher concentrations of CuSO4 reduced signal, whereas lower concentrations are less effective at quenching autofluorescence
   • Apply 100–500 µl of CuSO₄ treatment per slide and ensure tissue is completely immersed in solution.
• Slides may rest on the bench top at this time
12. Wash slides 5 min in PBS.
13. Carefully remove residual liquid on slide and mount with an antifade mounting medium.
  • We use 0.5% N-propyl gallate in 90% glycerol + 1x phosphate buffer (pH 8.0)
  • To reduce spherical aberration, use a minimal amount of cells suspended in mounting medium on each coverslip. We use 10–20 µl per 24 x 50 mm coverslip depending on tissue section size (allow to spread slowly across the entire surface of the coverslip once coverslip is mounted on slide).
  • Remove any drops of PBS on slide with a Kimwipe before mounting. These will dilute out the mounting medium and lower its refractive index. Careful not to disturb the tissue.
14. Seal the edges of the coverslip in clear nail polish and allow to air dry.
15. Remove residual salts from coverslip with a wet Kimwipe and air dry.
16. Store slides in 4°C until imaging.
  • Autofluorescence will be suppressed in mounted sections for a few days, but it will increase thereafter as Cu^{2+} ions diffuse out of the tissue
  • Slides may be imaged for up to one week