General protocol Immunofluorescence

Protocols may need local adjustments

Paraffin-embedded sections

1. Rinse cells attached to cover slips twice with PBS, removing liquid by gentle aspiration in this and subsequent steps.

2. Fixation & permeabilization:

    a. Option I:
    b. Fix cells with 4% formaldehyde in PBS for 6 min at room temperature, then rinse briefly twice with PBS**.
    c. Permeabilize fixed cells with 0.2% Triton X-100 in PBS for 6 min.

    d. Option II:
    e. Fix/permeabilize cells in -20°C methanol for 6 minutes**.

    f. [**Note: At this stage, the procedure may be interrupted for long term storage. Coverslips immersed in PBS and stored at 4°C are good for several weeks.]

3. Wash cells briefly 3 times with PBS, then 2 times with PBS containing 5% BSA (blocking reagent).

4. Dilute primary antibody in PBS/5% BSA. Working quickly, aspirate area surrounding coverslip to dryness, then gently add 100 µl of diluted primary antibody to the coverslip, so that solution remains restricted to coverslip by surface tension. Incubate for 1 hour at room temperature in a moist environment to prevent drying.

5. Wash cells 3 times with PBS, then 2 times with PBS/5% BSA.

6. Dilute fluorochrome-coupled secondary antibody in PBS/5% BSA and apply as in step 5. Incubate 1 hr. at room temperature.

7. Wash cells 3 times with PBS, then mount coverslips to slides using antifade mounting medium.

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Considerations

- Adherent cells may be grown directly on coverslips or chambered slides; suspension cells may be adhered to coverslips via poly-L-lysine treatment.

- Care should be taken to use the highest quality primary and secondary antibodies in order to avoid non-specific labeling. Ideally the specificity of primary antibodies is confirmed via immunoblotting of cell extracts. A control immunofluorescence sample omitting the use of primary antibody will reveal the extent of non-specific signal generated by the secondary antibody.
- In case of high background, the use of less primary and/or secondary antibodies as well as increased or alternative blocking reagent can be considered. 10% serum in PBS is another useful blocking agent - use only serum that will not cross-react with secondary antibody.

- If the assay involves localization of a protein expressed from a heterologous promoter, then the researcher should keep in mind that overexpression of the protein may produce mislocalization and hence broader staining than expected from endogenous expression.

- Several approaches can be considered in cases of an unacceptably low signal. The immunofluorescence protocol itself may be altered: use increased amounts of primary antibody, extend the incubation of primary antibody to overnight at 4°C, or use a different fixation/permeabilization regimen (gluteraldehyde, acetone, others).

Acetone-fixed frozen tissues

1. Fix the tissue sections with a suitable fixative. One of the commonly used fixation methods for frozen tissue sections is to immerse the slides in pre-cooled acetone (-20°C) for 10 min.

2. Pour off the fixative and allow acetone to evaporate from the tissue sections for < 20 min at room temperature.

3. Rinse the slides in 300 ml of 10mM phosphate buffered saline (PBS) at a neutral pH for 2 changes, 5 min each.

4. Incubate the slides in 0.3% H2O2 solution in PBS at room temperature for 10 min to block endogenous peroxidase activity.

5. Rinse the slides in 300 ml PBS for 2 changes, 5 min each.

6. Optional: Add 100 µl blocking buffer (e.g. 10% fetal bovine serum in PBS) onto the sections of the slides and incubate in a humidified chamber at room temperature for 1 hour.

7. Drain off the blocking buffer from the slides.

8. Apply 100 µl an appropriately diluted primary antibody (in antibody dilution buffer, e.g. 0.5% bovine serum albumin in PBS) to the sections on the slides and incubate in a humidified chamber for 1 hour at room temperature or overnight at 4°C.

9. Rinse the slides in 300 ml PBS for 2 changes, 5 min each.

10. Apply 100 µl an appropriately diluted biotinylated secondary antibody (using the antibody dilution buffer) to the sections on the slides and incubate in a humidified chamber at room temperature for 30 min.

11. Rinse the slides in 300 ml PBS for 2 changes, 5 min each.

12. Add 100 µl pre-diluted Sav-HRP conjugates (using the antibody dilution buffer) to the sections on the slides and incubate in a humidified chamber at room temperature for 30 min (keep protected from light).

13. Rinse the slides in 300 ml PBS for 2 changes, 5 min each.

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14. Apply 100 µl DAB substrate solution (freshly made just before use: 0.05% DAB - 0.015% H2O2 in PBS) to the sections on the slides to reveal the color of the antibody staining. Allow the color development for < 5 min until the desired color intensity is reached.

15. Caution: DAB is a suspected carcinogen. Handle with care. Wear gloves, lab coat and eye protection.

16. Wash slides in 300 ml PBS for 2 changes 5 min each.

17. Optional: Counterstain slides by immersing sides in Hematoxylin (e.g. Gill's Hematoxylin) for 1-2 min.

18. Rinse the slides in running tap water for > 15 min.

19. Dehydrate the tissue slides through 4 changes of alcohol (95%, 95%, 100% and 100%), 5 min each.

20. Clear the tissue slides in 3 changes of xylene and coverslip using mounting solution (e.g. Permount). The mounted slides can be stored at room temperature permanently.

21. Observe the color of the antibody staining in the tissue sections under microscopy.

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Considerations

- For initial experiments, the user must titrate primary and secondary reagents so that staining with the secondary antibody alone yields no background while staining with primary and secondary antibodies yields strong, specific staining.

- Prepare the acetone fixative fresh and chill to -20°C.

- The protocol can be stopped after sectioning, at step 8, after which samples can be stored indefinitely in a slide box at -80°C.

- The protocol can also be paused at step 16, in which samples can be left to stain overnight at 4°C in the dark in a humidified chamber.

- If background is high with the secondary antibody, consider blocking with 2-10% normal serum from the host in which the secondary antibody was raised i.e. if a goat anti-rabbit antibody secondary demonstrates high background, consider blocking with 2-10% normal goat serum at the Sample Blocking Step.

- High background can also be mitigated by increasing the number and length of wash steps.

- Apply and aspirate buffers and solutions carefully so as not to detach the cells from their culture vessel, coverslip or slide.

- Take care to ensure that slides do not dry out by incubating with sufficient volumes and/or in a humidified chamber.