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# Immunofluorescence Staining Protocol

## Description

Immunofluorescence staining or immunocytochemistry is a laboratory technique to detect specific proteins or antigens expression in cells with the aid of selective probes that can be visualized under a fluorescence microscope. This protocol outlines a two-step ICC process, employing a primary antibody targeting a specific antigen and a secondary antibody labeled with a fluorescent probe. The following procedure has been adapted for 3D bioprinted samples containing cells encapsulated in a hydrogel, which have already been prepared (ie. paraffin or frozen).

## Materials Needed

- Prepared paraffin or frozen-sections
- Xylene (Sigma-Aldrich, Cat # 534056)
- Ethanol
- 10 mM Sodium Citrate buffer
- 1 × PBS
- Triton X-100 (Thermo-Fisher Scientific, Cat #A16046-AE)
- Primary antibody
- Secondary antibody with fluorophore
- Serum for the secondary antibody
- Cover glass
- Mounting medium, e.g., Fluoromount-G
- PAP pen
- Dry oven
- Centrifuge tubes
- Serological pipettes
- Staining jar
- Staining tray/chamber
- Micropipette and tips
- Nail polish
- Fluorescence/Confocal microscope

## Procedure

**For paraffin sections:**
For frozen sections, skip to number 10

1. Incubate sections in xylene #1 for 5 minutes.
2. Transfer sections into xylene #2 and soak for 5 minutes to completely remove the paraffin.
3. Soak sections in 100% Ethanol #1 for 5 min.
4. Transfer sections into 100% Ethanol #2 for 5 minutes to remove xylene.
5. Perform serial rehydration by soaking the sections in 90%, 70%, 50%, and 30% Ethanol for 5 minutes in each wash.
6. Soak sections PBS #1 for 5 minutes.
7. Soak sections PBS #2 for 5 minutes.
8. Rinse sections in PBS.
9. Perform **antigen retrieval**:

   9.1 Add 10 mM Sodium Citrate buffer in a staining jar and put the microscope slides in the jar, making sure the slides are fully covered by the buffer.
   9.2 Place the jar in the water bath and heat up the water to reach boiling temperature (98°C-100°C). Incubate the samples for 10 minutes after the water starts boiling.
   9.3 Cool the samples in the buffer to room temperature.

   Proceed to Step 10 after antigen retrieval.

 

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**For frozen sections:**

10. Bake slides in an oven set to 37 °C for 30 minutes.
11. Circle the areas for staining on the slides using a PAP pen.
12. Soak slides in PBS for 30 min for rehydration in a staining jar.
13. Take slides out and gently shake off excess PBS on Kimwipes.
14. Add 100-200 µL 0.1v/v% Triton X-100 solution and incubate for 10 min to permeabilize the cells. *If staining the cell surface marker/protein, the permeabilization step can be skipped.
15. Remove the Triton X-100 and add 100-200 µL of blocking buffer (10% serum used for the secondary antibody) onto the staining areas and incubate in a humidified chamber at RT for 30 minutes.
16. Gently shake off blocking buffer from the slides.
17. Add 100-200 µL of primary antibody (1:200 diluted in PBS) to the staining areas and incubate in a humidified chamber for 2 hours at room temperature or overnight at 4 °C.
18. Gently shake off antibody solution on Kimwipes.
19. Soak the slides in PBS for 5 minutes . Repeat three times for a total wash of 15 minutes. Use fresh PBS for each wash.
20. Add 100-200 µL diluted secondary antibody (1:500 diluted in PBS) at RT for 1 hour.
21. Gently shake off antibody solution on Kimwipes.
22. Soak the slides in PBS three times, for 5 minutes each time. *Use fresh PBS for each wash.
23. Gently shake off excess PBS on Kimwipes.
24. Add ~20 µL DAPI mounting medium on the slides and mount with glass coverslips. Store mounted slides in the staining box to protect from direct light. *Apply the media and coverslip carefully to avoid air bubbles.
25. Allow medium to fully dry before imaging.
26. To prolong the life of fluorescent slides, use Nail Polish to seal the slides along edges of the coverslip. Store sealed slides in the storage box.
27. Proceed to imaging with fluorescence microscope.

**Notes:**

1. Always check to have the correct primary and secondary antibody pair/combination to ensure antibody species specificity.
2. Primary with different species domains for the secondary antibody can be mixed and do double/triple staining.
3. When adding blocking buffer and antibody solutions, hold the micropipette almost horizontally to gently add the liquid onto the tissue area dropwise within the circled area. Avoid directly adding the liquid on tissue sections.

 

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## Sample images

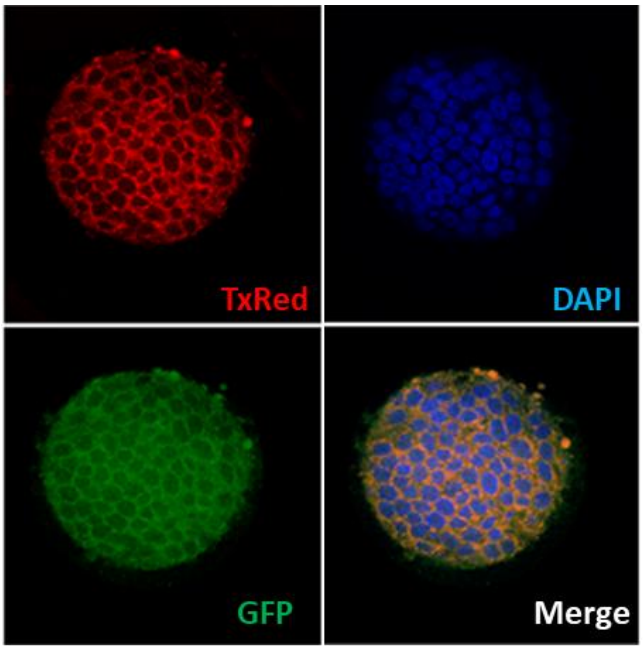


**Fig.1. MCF-7 breast cancer cells cultured in PureCol® hydrogel. Staining was performed on a 10-µm section of the paraffin-embedded collagen gel.**

 