Protocol for Immunocytochemistry

A. Seed adherent cells on 6-well tissue culture plates in a sterile tissue culture hood.

1. Sterilize glass coverslips by dipping them in 90% ethanol and carefully drying them over a flame for a few seconds.
2. Place each coverslip in sterile 6-well tissue culture plates (Figure 1).
3. Add 1-2ml of cell suspension over each coverslip in the 6-well plates.
4. Grow the cells at 37°C in a humidified CO_2 incubator until they are 50-70% confluent.
   Note: *The CO_2 level should be adjusted according to the type of medium used for growing the cells. Fibroblasts grown in High Glucose Dulbecco's Modified Eagle Medium with 10-20% fetal calf serum are maintained at pH 7.4 in a 10% CO_2 incubator.*

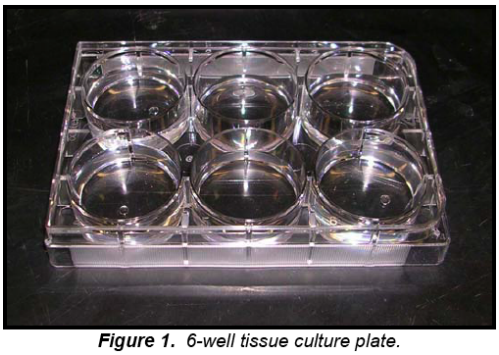


Figure 1. *6-well tissue culture plate.*

5. Aspirate the culture medium from each well and gently rinse the cells twice in PBS at room temperature. *Do not let the cells dry out.*

B. Fix the cells

1. Fix the cells by incubating them in 4% (v/v) paraformaldehyde in PBS for 20 minutes at room temperature.
2. Rinse the cells three times with PBS. The cells can be stored in 0.02% (w/v) sodium azide in PBS at 4°C for several days.
3. Proceed to Step C if the cells are to be heated in Antigen Retrieval Buffer, or Step D.
   Note*: Some of the antibodies work best if the cells are heated in Antigen Retrieval Buffer.*

C. Heat cells in Antigen Retrieval Buffer, if necessary

1. Preheat the Antigen Retrieval Buffer (100 mM Tris, 5% (w/v) urea, pH 9.5) to 95°C. This can be done by heating the buffer in a coverglass staining jar which is placed in a waterbath at 95°C.
2. Using a small pair of broad-tipped forceps, place the coverslips carefully in the Antigen Retrieval Buffer in the coverglass staining jar, making note of which side of the coverslips the cells are on (Figure 2).
3. Heat the coverslips at 95°C for 10 minutes.
4. Remove the coverslips from the Antigen Retrieval Buffer and immerse them, with the side containing the cells facing up, in PBS, in the 6-well tissue culture plates.
5. Rinse the cells 3 times in PBS.
6. Proceed to Step D.

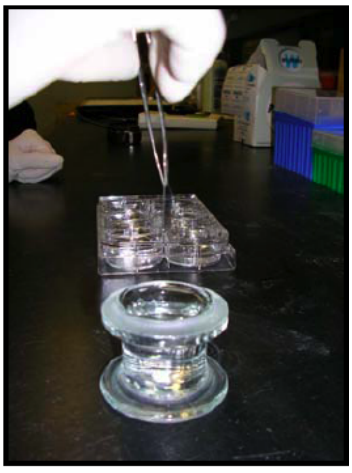


Figure 2. Coverglass Staining jar.

D. Permeabilize cells

1. Incubate the cells in 0.1% Triton X-100 in PBS for 15 minutes at room temperature.
2. Rinse the cells 3 times in PBS.

E. Block cells

1. Incubate the cells in 10% goat serum in PBS for 1 hour at room temperature.

F. Incubate cells in primary antibody/antibodies

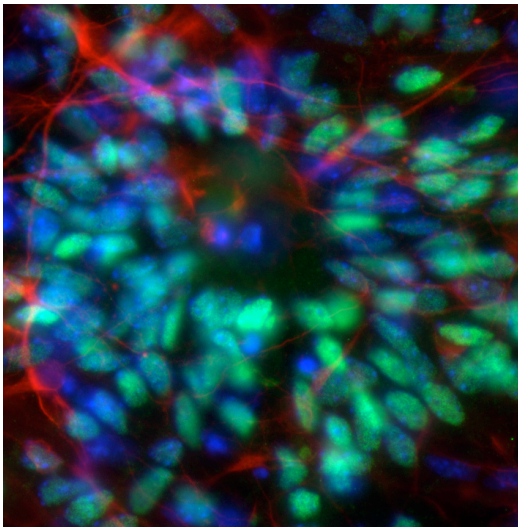
1. Dilute the primary antibody/antibodies to the appropriate concentration using 10% goat serum; the final volume should be sufficient to cover each coverslip (e.g. 0.5 ml-1 ml per coverslip).
   Note: *Optimal concentrations of primary antibody should be determined.*
2. Incubate the cells in the primary antibody/antibodies at 4°C, overnight, or at room temperature for 2 hours.
   Note**:** *Controls as described below may be included for the untested antibodies. There is variability incommercially available secondary antibodies, especially with respect to their ability to react with mouse monoclonal antibodies of all isotypes.*
   a) *Carry out the ICC protocol <u>without</u> adding the primary antibody and the secondary antibody. Any fluorescence seen is due to autofluorescence of the sample.*
   b) *Carry out the ICC protocol <u>without</u> adding the primary antibody. Any fluorescence seen is due to the secondary antibody binding nonspecifically to the sample.*
3. Rinse the cells in 1% goat serum in PBS 3 times for 10 minutes.

G. Incubate the cells in secondary antibody/antibodies

1. Dilute the fluorophore-conjugated secondary antibody/antibodies, away from light, in 10% goat serum. Be sure that the correct isotype-specific secondary antibody for each primary monoclonal antibody is used.
   Note: *A final concentration of 2-5* µ*g/mL of secondary antibody is suitable when analyzing fibroblasts with secondary antibodies from MitoSciences.*
2. Incubate the cells in the fluorophore-conjugated secondary antibodies for 2 hours at room temperature, away from light.
3. Rinse cells in 1% goat serum for 3x10 minutes, away from light.
4. Dilute DAPI to 300 ng/mL in 1% goat serum. Incubate the cells for 10 minutes in the diluted DAPI, away from light.

H. Mount coverslips and visualize under microscope

1. Label a microscope slide for each coverslip.
2. Add a drop of mounting medium to each slide.
3. Pick up each coverslip with a forceps and place it on the mounting medium, with the cell-side face down.
4. Apply nail polish or glue along the edges of the coverslips to seal them to the slides.
5. Visualize the cells using a fluorescence microscope equipped with the appropriate filters.



MATERIALS AND EQUIPMENT REQUIRED

Materials:

- Primary antibody/antibodies
- Fluorophore-conjugated secondary antibody/antibodies
- Cells of interest
- Tissue culture medium appropriate for the cells
- 6-well tissue culture plates (Fisher Scientific, www.fishersci.com, cat# 07-200-80)
- Glass coverslips
- Broad-Tipped Forceps, 4 1/2" long (Fisher Scientific, www.fishersci.com, cat# 10-300)
- Coverglass staining jar (Electron Microscopy Services, www.emsdiasum.com, cat# 72242-01)
- Microscope slides
- Paraformaldehyde (Electron Microscopy Services, www.emsdiasum.com, cat# 15713)
- PBS, pH 7.4 (8 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 140 mM NaCl, 2.7 mM KCl, adjust pH to 7.4 with NaOH)
- Antigen Retrieval Buffer (100 mM Tris, 5% (w/v) urea, adjust pH to 9.5 with HCl)
- 0.1% (v/v) Triton® X-100 in PBS
- Goat Serum (Invitrogen, www.invitrogen.com, cat# 16210-064)
- DAPI nucleic acid stain, 1 mg/mL
- Mounting medium
- Nail polish or glue (e.g. Duco Cement)

Equipment:

- Waterbath heated to 95°C
- Fluorescence microscope fitted with appropriate filters

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This guide is for quick reference only. Be completely familiar with the previous details of this document before performing the assay.