Oncogene Science

IMMUNOFLUORESCENT STAINING AND FLOW CYTOMETRY PROCEDURES

INTRODUCTION:

For immunofluorescence staining, target cells or frozen tissue specimens are first incubated with a monoclonal antibody or affinity-purified polyclonal antibody to the antigen of interest. Specifically bound antibody is visualized by incubation with a biotinylated second-step antibody against immunoglobulins of the relevant species (e.g. Biotinylated Goat Anti-Mouse IgG), followed by incubation with a fluorescent Streptavidin Conjugate (i.e. Streptavidin-Fluorescein, Streptavidin-Texas Red or Streptavidin-phycoerythrin). Alternatively, antigens may be visualized by the addition of a second step reagent (eg. Goat Anti-Mouse Ig) which has been directly conjugated with a fluorescent probe such as fluorescein, Texas-Red or phycoerythrin. For more rapid analysis, monoclonal antibodies directly conjugated with the fluorescent probe may by used in place of the two step processes described above. Although this method is more rapid it does not have the same degree of sensitivity as do the indirect methods. Once labeled, cells are analyzed either by flow cytometry or by using a fluorescent microscope equipped with fluorescein and rhodamine filters.

MATERIALS:

1. Equipment

- **Microscope equipped for epifluorescence,** with filters for fluorescein and/or rhodamine (Texas Red) and objectives of high numerical aperture (preferably oil immersion).
- Fluorescence activated cell sorter equipped with dual laser.

2. Solutions and Reagents

- Cryostat tissue sections (4 to 8 microns thick), or cultured cells grown on glass cover slips or slides.
- **OSI® Monoclonal Antibody or Affinity-Purified Polyclonal Antibody.**
- **Biotinylated or fluorescein, Texas-Red or phycoerythrin conjugates of Goat Anti-Mouse, Anti-Rat or Anti-Rabbit IgG.**
- **Fluorescein, Texas Red, or phycoerythrin, conjugated Streptavidin.**
- **Phosphate buffered saline (PBS):** In 1.0 liter distilled water, dissolve: 8.0 g sodium chloride; 1.3 g dibasic sodium phosphate; 0.2 g monobasic sodium phosphate. Adjust to pH 7.4.
- **PBS-FCS-NaN_3:** PBS with 5% (v/v) fetal calf serum, 0.1% NaN_3 (1mg/ml).
- **PBS-BSA-NaN_3:** PBS with 2% (w/v) bovine serum albumin, 0.1% NaN_3 (1mg/ml).
- **10% Normal Goat Serum** in PBS.
- **Fixatives as desired:** 1% formalin, 95% ethanol, 100% methanol, 100% acetone.
- **Aqueous Mounting Medium** (e.g. Hydromount, National Diagnostics) or 90% glycerol in PBS.
- **Subbing solution:** 0.3% (w/v) gelatin, 0.05% chromium potassium sulfate in distilled H_2O.

PROCEDURE:

1. Preparation of Slides

A. Cell Lines

- Grow cultured cells on sterile glass cover slips or slides overnight at 37°C.
- Rinse briefly with PBS.
- Fix as desired. Possible procedures include:
  - 10 minutes with 1% formalin in PBS (keep wet).
  - 5 minutes with -10°C methanol, air dry.
  - 2 minutes with cold acetone, air dry.

B. Frozen Tissue Sections

- Clean glass slides with 95% ethanol, treat with subbing solution and air dry.
- Cut 4 to 8 micron thick cryostat sections of tissue block (frozen in isopentane precooled in liquid nitrogen, embedded in OCT compound in cryomolds, and stored at -70°C).

- Allow frozen sections to come to room temperature (30 minutes).
- Fix slides in cold acetone for 10 minutes, keep refrigerated (or choose other fixation procedure).
- Rinse in three changes of PBS.

2. **Immunofluorescent Staining/Microscopy**

- Carry out incubations at room temperature in humidified chamber. Use suction to remove reagents after each step, but avoid drying of specimens between steps. Use sufficient reagent to cover the specimen (approximately 20 to 50 µl is usually adequate).
- Incubate specimens with 10% goat serum in PBS for 20 minutes to suppress non-specific binding of IgG (optional step).
- Wash with PBS.
- Incubate with primary antibody (OSI® Monoclonal Antibody or Affinity-Purified Polyclonal Antibody) for 60 minutes. Optimal antibody concentration for a given application should be determined by titration; usual range 3 to 30 µg/ml in PBS-BSA.
- Rinse with three changes of PBS.
- Incubate with biotin-conjugated second-step antibody (Biotinylated Goat Anti-Mouse IgG, Anti-Rat IgG or Anti-Rabbit IgG as required) for 45 minutes. Optimal antibody concentration for a given application should be determined by titration; usual range 2 to 20 µg/ml in PBS.
- Rinse with three changes of PBS.
- Incubate with Streptavidin-fluorescein or Streptavidin-Texas Red for 15 minutes in a dark chamber. Optimal Streptavidin conjugate concentration for a given application should be determined by titration. Usual range 1:100 to 1:400 in PBS.
- Wash extensively with PBS.
- Coverslip with aqueous mounting medium or 90% glycerol/PBS.
- Examine using a fluorescence microscope with appropriate filters. Store slides in the dark at room temperature (semi-permanent mounts), or 4° (glycerol/PBS).

3. **Immunofluorescent staining/Flow cytometry**

- Carry out incubations at 4°C.
- Add cells (2.5-5 x 10^5) in a final volume 0.05 ml to individual wells of a 96 well round bottom microtiter plate.
- Wash by adding 150 µl of PBS to the wells and centrifuging the cells at 1000 rpm for 2-3 minutes then carefully aspirate the wash buffer.
- Incubate with 50 µl of the desired primary antibody for 20 minutes. Optimal antibody concentration for a given application should be determined by titration; usual range 3-30 µg/ml in PBS-FCS-NaN_3.
- Wash three times with PBS-NaN_3.
- Incubate with 50 µl of the fluorescent conjugate second step (Goat anti-Mouse, Rat or Rabbit as required) for 20 minutes at 4°C in a dark chamber or by covering the microtiter plate with foil. Optimal concentrations should be determined for each application but using the cell concentrations and primary antibody concentrations suggested here a final concentration of 1-5 µg/ml in PBS-FCS-NaN_3 is sufficient.
- Wash three times with PBS-NaN_3.
- Fix the cells by adding 50 µl of a 2% solution of paraformaldehyde (pH7.4) to each well and incubating at 4°C for 20 minutes. Wash the cells extensively with PBS prior to analysis.
- If cells are not to be analyzed immediately then the cells may be stored at 4·C in the dark until needed. Prior to analysis the cells should be washed to removed residual paraformaldehyde and unbound florescent antibody.
- Cells may be analyzed on a flow cytometer equipped with a single laser for individual visualization of fluorescein or phycoerythrin or alternatively equipped with a dual beam laser for simultaneous visualization of fluorescein and phycoerythrin.
- Note: Cells may also be visualized using a fluorescent microscope equipped with a filter for fluorescein before flow analysis.

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