

EXERCISE

4

Immunocytochemistry

Note to Instructor: This protocol uses the “indirect” immunocytochemical method, that is, a primary antibody directed against the protein of interest is then tagged by a secondary antibody directed against the primary. While immunofluorescence (i.e., fluorescent tags such as rhodamine or fluorescein) yields less background, we have achieved readily-quantifiable results using secondary antibodies conjugated to alkaline phosphatase. The progression of immunostaining can be monitored using a standard phase-contrast inverted microscope (remember to remove the phase ring when attempting to observe immunostaining).

In this particular exercise, we visualize microtubules, which typically give a robust appearance when extracted. Alternatively, and especially for non-neuronal cells, one can visualize actin. Note that the microtubule extraction is not required for other antigens.

You will use the plates from the previous lab that were treated with medium containing 10% serum + dbcAMP.

PROTOCOL

Microtubule fixation and extraction

- Remove 1 mL of media from each plate.
- Add 1 mL of “microtubule stabilizing extraction solution” to the plate for 1 minute, then remove.
- Rinse once with PBS.

Fixation

- Fix with 1 mL of 4% paraformaldehyde for 5 min. at room temp.
- Rinse again with PBS.

“Blocking”

- Block with 1 mL 3% bovine serum albumin (BSA) in Tris-buffered saline for 15 min. at room temp. This prevents antibodies from sticking to essentially everything: antibodies exhibit low-affinity binding to almost everything, and high-affinity binding to their particular antigen. Blocking out-competes low-affinity binding, but not high-affinity, which increases our signal-to-noise ratio considerably.

Antibody binding

- Incubate with primary antibody (diluted 1:1000 in 1% BSA) for 45 mins. at 37°C.
- Remove the primary antibody (and save).
- Rinse 3x with PBS; 5 min. incubation at room temperature following each rinse.
- Incubate with secondary antibody (diluted 1:5000 in 1% BSA) for 45 mins. at 37°C; remove the secondary antibody.

- Rinse 3x with PBS, 5 min. incubation at room temp. each rinse.
- Rinse once in an alkaline buffer (pH 9; reason below).

Visualization

- Visualize by incubation with 1 mL of substrate solution BCIP/NBT.
- Stop the reaction AFTER VIEWED BY YOUR LABORATORY INSTRUCTOR by removing substrate solution and adding water.

The primary antibody that we will use is anti-tubulin.

Your secondary antibody is conjugated to alkaline phosphatase, and will yield a purple color when incubated with the substrate. The final rinse with an alkaline buffer is to maximize reactivity of alkaline phosphatase (so named because it prefers alkaline conditions).

Note which portions of your cells stain with which antibody. This can best be viewed by comparing bright-field microscopy (which will reveal only stained portions) versus phase-contrast (which will show the entire cell).

A good control would be to omit the primary antibody and do all the rest of the steps.

MATERIALS

4% paraformaldehyde

“Microtubule Stabilizing and Extraction solution”

PBS

5% BSA

Primary antibody (1:1000 in 1% BSA)

Secondary antibody (1:5000 in 1% BSA)

Alkaline Buffer (pH 9)

BCIP/NBT

- To make 4% paraformaldehyde:
 - Use 16% stock solution and dilute using water.
- To make Microtubule Stabilizing and Extraction solution:
 - Make: 60 mM Pipes (pH 6.9)
 - 10 mM EGTA
 - 2 mM MgCl₂
 - 1% Saponin
 - 10 μM Taxol
- PBS:
 - Dissolve 1 tablet into 200 mL distilled water.
- To make 5% BSA:
 - Dissolve BSA in TBS.
- To make primary antibody:
 - Dissolve into 1% BSA in TBS.

- Alkaline Buffer:
Dissolve: 100 mM Tris-Hcl
100 mM NaCl
5 mM MgCl₂
Adjust the pH of the solution to 9.
- BCIP/NBT:
Per 10 mL: Dissolve 33 μL BCIP + 66 μL NBT in 10 mL of alkaline phosphatase; this should be made fresh.

HINTS

- “Microtubule stabilizing extraction solution” can be stored at room temperature for several weeks.
- It is very important for the MT solution to be on the plate for only 1 minute.
- The secondary antibody and the BCIP/NBT color development solution need to be made on the day of use.
- Any cytoskeletal antibody can be substituted for anti-tubulin. The dilutions of both the primary and secondary are very important; changing the dilutions might yield much better results.
- The exposure time of the secondary antibody is crucial—increasing the incubation time may lead to over-staining.
- The primary antibody can be stored at -20°C and reused several times.

