Video Article

Immunocytochemistry: Human Neural Stem Cells

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Abstract

Immunocytochemistry is a very powerful and fairly straightforward method for determining the presence, subcellular localization, and relative abundance of an antigen of interest, most commonly a protein, in cultured cells. This protocol presents an easy-to-follow series of steps that will enable researchers to conserve primary and secondary antibodies while getting high quality, reproducible qualitative and quantitative data out of their staining. There are two aspects of this protocol that help to conserve the volume of antibody necessary for staining. For one, the cells are grown on small, circular coverslips that are placed in wells of a tissue culture plate. After fixation, the cells on coverslips can be removed from the wells of the plate. For antibody staining, the coverslip with cells is inverted onto a small drop of antibody solution on parafilm and is covered with a second piece of parafilm to prevent drying. Using this method, only ~25 µl of antibody solution is needed for each coverslip (or sample) to be stained. This protocol describes immunostaining of human neural stem/precursor cells (hNSPCs), but can be used for many other cell types.

Video Link

The video component of this article can be found at https://www.jove.com/video/267/

Protocol

Day 1: Preparing German Glass Coverslips and Seeding Cells

Note: German glass coverslips are often preferable for culturing primary neural stem cells and neurons. We use the following cleaning protocol to improve cell adhesion and spreading. Position coverslips in a small rack that will allow liquid access to both sides of the coverslip. First, incubate coverslips in 1% Liquinox for 10 minutes, followed by 3 washes with deionized water. Make sure that no soap bubbles remain. Next, incubate slips in 1M HCl for 30 minutes, followed by 3 washes with deionized water. Dry coverslips at 65°C overnight. Transfer coverslips to a glass dish and autoclave to sterilize.

1. In a tissue culture hood, place sterile coverslips in an appropriately-sized well plate.
2. Coat coverslips with laminin for cell adhesion. Incubate coverslips in 10 µg/ml poly D-Lysine (PDL) in sterile water for 5 minutes at room temperature. Remove PDL and incubate coverslips in 20 µg/ml laminin in EMEM for 4 hours, or overnight, in a 37°C tissue culture incubator.
    1. *Note*: Refer to the **Passaging Human Neural Stem Cells** article (http://www.jove.com/index/Details.stp?ID=263) and the **Counting Human Neural Stem Cells** article (http://www.jove.com/index/Details.stp?ID=262) to learn how to resuspend and count hNSPCs.
3. Rinse laminin-coated coverslips with PBS and seed cells into each coverslip-containing well at a certain density (we often use an initial plating density of 100,000-300,000 cells/ml for cells that we are plating for immunostaining).
4. Place plate in 37°C tissue culture incubator and allow cells to adhere to coverslips (usually a minimum of 4 hours is required).

Day 2: Fixing, Permeabilizing, Blocking, and Adding Primary Antibody to Cells

Note: We use the following 4% paraformaldehyde fixative to preserve cell morphology. The recipe includes a pH transition to allow the paraformaldehyde to go into solution. We prefer this method rather than the use of heat to dissolve paraformaldehyde because higher temperatures can lead to the generation of formic acid, which can increase background staining when using fluorescence. Some components of the fixative help to preserve cytoskeletal structure ($MgCl_2$ and EGTA), while others help to maintain overall morphology (sucrose).

4% Paraformaldehyde Fixative for Cells

Reagents and steps:	**Amount for 100 mL of fixative:**
MilliQ water	75 ml
Paraformaldehyde (weigh out in the fume hood)	4 g
10N NaOH, stir and add dropwise until solution clears	as needed

10X PBS	10 ml
1M $MgCl_2$	0.5 ml
0.5 M EGTA	2 ml
Sucrose	4 g
6N HCl, titrate to pH 7.4	as needed
MilliQ water	bring to 100 ml
Store at 4°C for up to 1 week. Warm to 37°C before use	

1. On the bench, remove the media and add prewarmed fixative. Incubate for 5-15 minutes (depending on the antigen to be detected, we usually use 10 minutes). Perform this and the following steps at room temperature.
2. Discard the fixative into an appropriate container, as many institutions handle paraformaldehyde as hazardous waste. Wash cells 3 times with PBS, 5 minutes each time. When removing solutions from the cells, be careful that suction does not dry out the cells. Also, always have the next solution on hand to add to cells before removing the solution that coats them so cells are not left to dry.
3. If the antigen of interest is inside the cell, the cell membrane must be made permeable to allow entry of the antibody. To permeabilize cells, use a fresh 0.3 % Triton X-100 solution in PBS. Pipette slowly because Triton is viscous, and make sure the detergent is completely dissolved in PBS before adding to cells. Permeabilize cells for 5 minutes, followed by washing the cells 3 times with PBS, 5 minutes each time.
4. The cells must be treated with a blocking agent to prevent non-specific binding of the antibody. We use bovine serum albumin (BSA) as a blocking agent. The blocking solution is made by dissolving 5% BSA into PBS (on a rocker or rotator because it takes time to dissolve) then filtering the solution with a 0.45 µm syringe filter. Block cells in 5% BSA/PBS solution for 1 hour at room temperature.

Dilute the primary antibody (which is hopefully specific for the protein of interest) to a pre-determined concentration in 1% BSA/PBS (prepared by dilution from 5%BSA/PBS). Place diluted primary antibody (30 µl per 25 mm coverslip, 20 µl per 18 mm coverslip, 15 µl per 12 mm coverslip) on a glass plate covered with parafilm. Pick up the coverslip with fixed cells, invert such that the cells face down, and lay it over the antibody solution so that the cells are in contact with the antibody. Place a second parafilm strip over the entire construct. Incubate at 4°C overnight.

Day 3: Washing, Secondary Antibody Incubation, Nuclear Stain and Mounting

1. Carefully remove the top parafilm strip and pick up the coverslip, invert it, and place it back into the plate with PBS in each well for washes so that the cells are facing up.
2. Wash cells 3 times with PBS, 5 minutes each time.
3. Choose a secondary antibody that will detect the primary antibody, for example, if the primary antibody was made in a rabbit, the secondary antibody should recognize rabbit IgG. Take care to also match the antibody isotype (IgG, IgM, etc). If using multiple primary antibodies, make sure they are from different species or isotypes, and plan to detect with different markers attached to the secondary antibodies. For example, you could use an anti-rabbit secondary coupled to a red fluorophore with an anti-mouse secondary coupled to a green fluorophore. Incubate cells in the secondary antibody solution diluted to an appropriate concentration in 1% BSA for 2 hours in the dark at room temperature, using the same technique as for primary (steps not shown in the video). The volume of secondary antibody needed for the incubation may be slightly larger than that for the primary antibody if the secondary is stored as a glycerol solution (40 µl per 25 mm coverslip, 30 µl per 18 mm coverslip, 25 µl per 12 mm coverslip).
    - *Note*: If you are using fluorescent molecules to visualize the secondary antibody, the sample needs to be protected from light once the secondary antibody has been added. Incubate in the dark, or in a foil covered plate.
4. Carefully remove the coverslips from the parafilm as described for the primary antibody. Wash cells 2 times with PBS, 5 minutes each time, followed by a 1 minute incubation in 2 µg/ml Hoechst nuclear stain diluted in PBS (if a nuclear counterstain is preferred). If the Hoechst stain is old and the signal is too dim, you can increase the incubation time and/or the concentration. After incubation with Hoechst, wash 1 time with PBS for 5 minutes.
5. The coverslips should be mounted on a slide with mounting media for visualization on a microscope. In cases in which a fluorescent molecule is used for visualization of the secondary antibody, the mounting media should contain agents to minimize photobleaching. We use Vectashield as a mounting media for fluorescence. Place an appropriate size drop of Vectashield on a microscope slide (12 µl per 25 mm coverslip, 6 µl per 18 mm coverslip, 3 µl per 12 mm coverslip).
6. Carefully pick up the coverslip and rinse the back with deionized water to remove salts (from the PBS). Dab off excess water on a kimwipe or paper towel, and lay on the drop of Vectashield with cells facing down. Place the coverslip on at an angle and allow to descend slowly to avoid trapping air bubbles. Label the slide with the date and any sample information.
7. Allow slides with coverslips to dry (in the dark) then suction off excess Vectashield from around the coverslip edge. The coverslip edges can now be sealed with nail polish, if desired (especially useful if coverslips will be viewed on an inverted microscope). We usually store our slides in a -20°C freezer.

Discussion

This protocol describes an immunostaining procedure for hNSPCs that minimizes the volume of antibody necessary and gives reliable cell staining. The procedure as described is best for intracellular antigens, but can be modified to stain cell surface molecules or to enhance staining of the cytoskeleton.

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