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NB300-223 Protocol

Immunostaining of cells in tissue culture protocol specific for Vimentin Antibody (NB300-223)

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Immunostaining of cells in tissue culture

The purpose of fixation is to denature the components of cells enough so that they stay on the dish and can be bound by antibodies, hopefully without destroying cellular morphology. Fixatives such as formalin, paraformaldehyde and glutaraldehyde chemically cross-link proteins, by binding to amino acid side chains. This chemical modification can also have the consequence of blocking antibody binding sites. Substances such as acetone and methonal are not true fixatives but are denaturants, which precipitate proteins without covalently modifying them. We routinely use a combination of mild formalin fixation followed by cold methanol for neurons, mixed neuron/glial cultures and most used cell lines. The formalin preserves the cellular morphology quite well, while the methanol further denatures the proteins of the cells and helps keep what is left of the cell adherent to the dish. For soluble proteins it may be necessary to skip the methanol step, but in this case you have to be very careful with the washing steps, as the cells tend to wash off the dish. Certain antibodies may be very sensitive to formalin fixation, so you may have to experiment a little, perhaps skipping that step. The following procedure work for antibodies to most cytoskeletal and signaling molecules. This procedure is good for cells in 6 well dishes or in 35mm tissue culture plates.

1. Draw the culture medium with aspirator and add 1 ml of
3.7 % formalin in PBS solution to the dish (from 10mls Fisher 37% formalin plus 90mls PBS, the Fisher formalin contains 37% formaldehyde plus about 1% methanol which may be relevant sometimes). Let sit at room temp for 1 minute (can add 0.1% Tween 20 to PBS used here and all subsequent steps to reduce background; probably best not to do this first time around, though, as it may extract your antigen or help wash your cells off the dish).
2. Take off the formalin/PBS and add 1ml of cold methanol (-20C, kept in well sealed bottle in fridge). Let sit for no more than 1 minute.
3. Take off methanol and add 1ml of PBS, not letting the specimen dry out. To block nonspecific antibody binding can add ~10ml (=1%) of goat serum (Sigma), and can incubate for 30 minutes. Then add antibody reagents. Typically 100ml of hybridoma tissue culture supernatent or 1ml of mouse ascites fluid or crude serum. Incubate for 1 hour at room temp. (or 37C for 30 minutes to 1 hour, or 4C overnight, exact time not too critical). Shake gently for well adherent cell lines (3T3, HEK293, etc.).
4. Remove primary antibody and replace with 1 ml of PBS. Let sit for 5-10 minutes, replace PBS and repeat twice, to give three washes in PBS.
5. Add 0.5 mls of secondary antibody. These are fluorescently labeled goat anti-chicken antibodies and are conjugated to ALEXA dyes and are from Molecular probes (Eugene Oregon, the ALEXA dyes are sulphonated rhodamine compounds and are much more stable to UV than FITC, TRITC, Texas red etc.). Typically make 1:2,000 dilutions of these secondaries in PBS plus 1% goat serum, BSA or non fat milk carrier. Incubate for 1 hour at room temp. (or 37C for 30 minutes to 1 hour, or 4C overnight). Shake gently for well adherent cell lines (3T3, HEK293, etc.).
6. Remove secondary antibody and replace with 1 ml of PBS. Let sit for 5-10 minutes, replace PBS and repeat twice, to give three washes in PBS.
7. Drop one drop of Fisher mounting medium onto dish and apply 22mm square coverslip. View in the microscope.