

Title:	Immunocytochemistry				
Protocol #:	1.1	Submitted:	18/06/10	Approved:	20/06/10
Category:	Cyto	Author(s): ¹	MVJ	Checked by:	AAH

Reagents:

1. Dulbecco's PBS
2. NaCl
3. KCl
4. CaCl₂
5. MgCl₂-6H₂O
6. Na₂HPO₄-H₂O
7. KH₂PO₄
8. PBS (without calcium and magnesium)
9. paraformaldehyde
10. normal donkey serum
11. PVA
12. DABCO
13. Hoechst (345/478)

Equipment

1. humid 37°C incubation chamber (this can be either the lids of a multi-well plate with moist kimwipes on the sides (if working with 2-3 slides) or else a larger plate that can be prepared by fixing a rail of two 1-ml pipettes for resting multiple slides on them)

Reagent Setup

1. Dulbecco's PBS
 - 139mM NaCl
 - 2.7mM KCl
 - 0.75mM CaCl₂
 - 0.48mM MgCl₂-6H₂O
 - 8.8mM Na₂HPO₄-H₂O
 - 1.48mM KH₂PO₄
2. 4% paraformaldehyde (PFA)
 - Dissolve paraformaldehyde in pre-warmed Dulbecco's PBS and then add one or two drops of 10N NaOH to dissolve any residual PFA
3. Blocking Buffer
 - 4% normal donkey serum in PBS
 - note: 1-4% BSA or commercial blocking buffers may also be used
4. Recipe for MOWIOL mountant
 1. Tris 0.2 M, pH 8.5 (MW: 121.14) prepared by adding 580 mg Tris to 24 ml water.

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2. Add 12 g of glycerol
 3. Add 12 ml of dH₂O
 4. Stir on a hotplate at no more than 55°C
 5. Add 4.8g of PVA in small batches and stir till entire contents dissolve
 6. Then add 2.5% (w/v) of DABCO
 7. Cool to RT
 8. Aliquot 1 ml into eppendorf tubes.
 9. Freeze at -20 °C and store in dark.
5. Hoechst (345/478) may be added prior to use at 10-20µg / ml. As an alternate to MOWIOI, Vectashield (4° fridge, histo lab) can be used.

Procedure:

1. **When your cells are ready for immunostaining, fix them in freshly prepared 4% paraformaldehyde. Another way is to have 16% paraformaldehyde made up and stored in 1ml Eppendorf tubes in the freezer room and make it to 4% with DPBS. In the latter case, make sure to pre-warm the 4% PFA before fixing the cells.**
Once cells are fixed for ~30 mins at room temperature, they can be used immediately for immunostaining. Alternatively, these can remain in 4% PFA in a moist container (sealed with parafilm) at 4°C. (Gluteraldehyde is another fixative that may be used (0.25% to 4% in 1X PBS). It cross-links proteins and generally retains their antigenicity.)
2. **Permeabilize cells at this point using chilled 50% methanol in 1X PBS. Preps should be incubated at room temperature in chilled methanol for 20 minutes.** Cells can also be permeabilized in 0.2% (v/v) TritonX-100 in 1X PBS. Generally this is performed for up to 5 minutes at room temperature immediately following fixation. Following permeabilization, the cells are usually washed 3X (5 min each) in 1X PBS prior to the blocking step. Always avoid using Methanol if you are staining cytoskeletal elements.
3. **Wash cells 3 times at room temperature (5 minutes each) in Dulbecco's PBS containing calcium and magnesium.**
Make sure that the washes contain generous volume of DPBS and that you have at least 3 good washes.
4. **Block for 30 minutes at room temperature in PBS blocking buffer.**
Although several commercially available blocking buffers are available (Thermo, Dako), most of the immunostaining uses 4% normal donkey serum for blocking. Make this fresh using 4% donkey serum (stored in 500µl aliquots at -20°C freezer room). You will need the blocking buffer to re-constitute primary and secondary antibodies.
5. **While the cells are blocking, set up a humid chamber for performing the antibody incubations.** The chamber can consist of absorbent paper/kimwipes, soaked in water and a plastic storage container with a lid.

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- 6. Dilute the primary antibody (or antibodies if performing multiple labeling) in PBS blocking buffer.** Use a hydrophobic marker / pap pen for marking the boundary of cells / sections so as to minimize the antibody volumes. Generally you should not need more than 50-100ul of diluted antibody solution per coverslip/section/cytospun spot of the slide.
- 7. Add diluted primary antibody and incubate at 4°C overnight.** This generally gives lesser background as compared to same concentration incubated for 1 hour at 37°C. However, you may incubate the primary antibody at 37°C for 1 hour or at room temperature for 3 hours based on your experience of the antibody to be used.
- 8. Wash cells 3 times at room temperature (5 minutes each) in Dulbecco's PBS containing calcium and magnesium.**
Make sure that the washes contain generous volume of DPBS and that you have at least 3 good washes.
- 9. Dilute secondary antibody in PBS blocking buffer.**
- 10. Incubate in secondary antibody in the humid incubation chamber for 1 h at 37°C or 3 hours at room temperature.**
- 11. Wash coverslips / slides/ labtek chambers with generous volumes of DPBS for 3 to 5 times. Your slides are now ready for mounting.**

Add a drop of mounting fluid; MOWIOL or Vectashield, containing Hoechst 33342. Mounting fluid should contain 10µl of (a 10mg/ml stock) Hoechst 33342 in 1 ml of mounting fluid. Hoechst 33342 is a nuclear stain that binds to the minor groove of DNA (absorbance max is at 340nm and emission max is at 450nm). Alternate nuclear counterstain includes DAPI (10mg/ml stock in 1X PBS), which also binds to the minor groove of DNA (359nm and emission max 461nm) and propidium iodide (10mg/ml stock in 1X PBS) that intercalates in the DNA (absorbance max 536nm and emission max 617nm).

- 12. Seal with nail paint:** Avoid using nail paints with sparkles etc... the plain one in the lab is specifically used for this purpose.
- 13. View on fluorescence microscope or place in dark at 4°C for long-term storage.** Note: it is recommended to obtain images of the staining ASAP; paraformaldehyde fixation is reversible with time and fluorescent signals can fade off depending on the storage and anti-fade reagents used in the mounting fluid. However, in our experience, if slides are stored in a cool and dark place, staining can be visualized even after 2-8 weeks.

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To prep live mounts

Unlike fixed samples, live cells, which are adherent to coverslips, do not tolerate washes in PBS without calcium or magnesium. It is therefore suggested to supplement PBS with 0.2mM CaCl₂. This is a low enough calcium concentration that will not precipitate in the presence of phosphate salts. It is obvious to state that the permeabilization step be omitted. Mounting fluid should not be added to live cell studies.

Anticipated results

Fixation

Paraformaldehyde should be made fresh. The use of 4% paraformaldehyde as a fixative works for most antibodies and may be used up to 10% in some cases. A non-cross-linking fixative such as cold (-20°C) methanol can be used; due to its extraction of some lipids, methanol can destroy membranous organelles. PFA fixation does not have this effect on lipids and consequently better preserves organelle structure.

Controls

Secondary Antibody Alone

In order to control for the possibility of the secondary antibody cross-reacting with cells, it is necessary to set one coverslip aside and omit primary antibody incubation step. Incubate this control coverslip in PBS Blocking Buffer only for one or more hours. This control also helps eliminate signals that are due to autofluorescence of the cells, in the case of fluorescently tagged antibodies or endogenous peroxidase and phosphatase in the case of enzyme conjugated antibodies. If the background is high, it is recommended to analyze the cells without secondary antibody as well. This will help to determine whether the background is due to the secondary antibody or the cells themselves. If the cells show a lot of autofluorescence (esp. in the green channel), then use the proper controls to take the samples over to the META for emission fingerprinting.

Preimmune/Normal IgG

In addition to omitting the primary antibody, preimmune or “normal” IgG from the animal species in which the primary antibody was raised are sometimes incubated as a control. This control rules out the potential that any signal seen with the primary antibody is actually due to nonimmune IgG cross-reacting with antigen in the target cells.

Blocking

The PBS Blocking Buffer generally should contain normal serum from the host species that is used to generate the secondary antibodies. In theory,

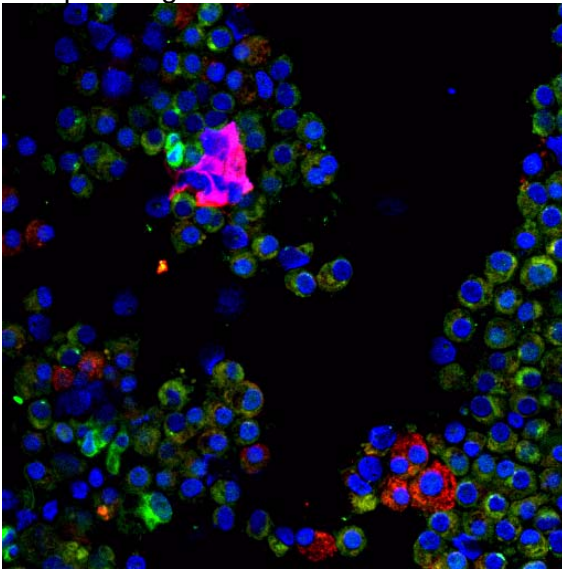
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if there are any host IgG molecules that can cross-react with the cells being studied, then the unlabeled/non conjugated IgGs present in the normal serum will cross-react during the blocking step.

For antibodies that are raised against specific peptides, it would be a good idea to block these antibodies with the specific as well as non-specific peptides so as to achieve more assuring controls. To block antibodies with peptides, use 4X by weight, the amount of peptide to antibody.

Antibody (1mg/ml): 5 μ l + Peptide (1mg/ml): 20 μ l + PBS 1X: 75 μ l \rightarrow Incubate O/N on a rotator at 4°C. Next day spin @14K for 2'. Remove the top 95 μ l, further dilute to appropriate working concentration and proceed for immunohistochemistry.

Sample image:



Note: In the quad staining above, the marker in magenta is actually saturated. Although this is an issue with the confocal imaging here, the selection of secondary antibodies is an important part of the protocol. Choose your secondary antibodies wisely. For example, it is better to select a 488 channel for an antigen that you expect to be weakly expressed. This allows you to visualize it better and puts it in a stronger channel for detection. However, needless to say, it should always be accompanied with all the IgG, secondary-only and dye-swap controls. If using META for emission fingerprinting, it is important to generate your own spectra from your cells. The same secondary antibody may generate a variant spectra in another cell type based on the scattering inside the specimen. For emission fingerprinting using (for example) the LSM Meta, it is necessary to have at least 6 different slides for a single quad labelling experiment: 4 with each of the 4 dyes, 1 with all of the 4 dyes and one unstained. Refer to the [linear spectral unmixing](#) protocol for further details.