Blocking Protocol LM/EM

Author: Thomas E. Phillips, Ph.D
University of Missouri, Columbia 05/26/06

This version (09/22/06) is modified by Shiv for the use in MIF-UIUC

Store all antibodies in solution with 50% glycerol at -20 C to prevent freeze-thaw damage (if you do not have non-frost free freezer in your lab). Alternatively we make 5 or 10, µl aliquots and store at -80 C. Gold conjugates are kept at 4 C.

We recommend the use of the Alexa fluorochromes for confocal and most widefield fluorescence work. For routine use, we recommend buying your own stocks from www.Invitrogen.com

For LM & EM immuno block for at least 30 min. Most immunocytochemists think 30 min is more than enough (some only use 5-10 min). There is at least one publication showing longer times can be beneficial. I have gone as long as overnight but that can weaken EM thin sections.

Too much blocking protein can interfere with antibody binding.

One strategy is to block in a more concentrated solution and then incubate your primary and secondary antibodies in a more diluted blocking buffer.

HWB = HEPES WASH BUFFER (PBS can generally be substituted)
70 mM NaCl
30 mM HEPES
2 mM CaCl2
pH 7.4

GBB2 = GOAT BLOCKING BUFFER 2 STOCK
(For EM immuno using gold conjugated goat antisera)
5% BSA
5% normal goat serum
0.1% cold water fish gelatin
0.1% Micr-O-Protect
in HEPES wash buffer, pH 7.4

GBB3 = GOAT BLOCKING BUFFER 3 STOCK
(For LM immuno using goat antisera conjugated with fluorescent probes)
5% BSA
5% normal goat serum
0.1% Micr-O-Protect
in HEPES wash buffer, pH 7.4

INCUBATION BUFFERS:

0.1% BSA + 0.1% Micr-O-Protect in HWB
or
1.0% BSA + 0.1% Micr-O-Protect in HWB
or
0.1% BSA-c + 0.1% Micr-O-Protect in HWB
or
1.0% BSA-c + 0.1% Micr-O-Protect in HWB

GENERAL COMMENTS ON REAGENTS:

If you want to use Bovine Serum Albumin in your blocking buffer, it is important to use an IgG free preparation. I like the one from Jackson Immuno www.jacksonimmuno.com). #001-000-162.

It is common for the blocking buffer to include some normal serum from the species your secondary antibody is made in. So if your secondary antibodies are Goat anti-rabbit or Goat anti-mouse, you can include 1-5% normal goat serum. Normal goat serum can be obtained from Jackson Immuno (#005-000-121).

Cold Water Fish Skin Gelatin can be included in the blocking buffer. This is more common for immunogold labeling work. It can reduced background but too much is reported to reduce the real labeling efficiency. You can buy 40% CWFS Gelatin from Electron Microscopy Sciences (www.emsdiasum.com).

Acetylated BSA (BSA-c) is an acetylated, linearized form of BSA. The acetylation increases the net negative charge on BSA. Linearization exposes hydrophobic binding sites. BSA-c is recommended by some for immunogold work. You can buy 10% BSA-c from Electron Microscopy Sciences (www.emsdiasum.com).

Tween-20 is a non-ionic surfactant that is used to prevent background binding to hydrophobic sites. It is available from Sigma or Fisher of many other sources.

Micr-O-protect is a relatively non-toxic biocide (anti-microbial) that is effective against bacteria, yeast and fungi but is less toxic to mammalian cells than azide. It is Catalog #1-585-720 from Roche (http://www.roche-applied-science.com/cfm/country_id_a.jsp). They recommend using it in solutions with high protein concentrations at 0.4 to 1.0% and down to 0.1% in solutions with low protein levels.

SAMPLE LM IMMUNOSTAINING PROTOCOL

All steps must be done in a humidity chamber to prevent evaporation! You can use a Petri dish containing wet cotton or a layer of water with the slide held above the water by something like a bottle cap.

Block in 50 mM Glycine in HWB for 30 min to block free aldehyde groups (use this step only for aldehyde fixed tissues that haven't been rinsed in glycine/HWB yet). Block 30 min in Blocking Buffer (e.g., GBB2)

Brief rinse in incubation buffer (e.g., 0.1% BSA-c)

Incubate in primary antibody at 1-10 ,g/ml in incubation buffer. For LM work, we use 1 hr to overnight incubations – almost always we go overnight at room temperature.

Wash 3x with HWB
Incubate in secondary antibody at 1-10 ,g/ml in incubation buffer. For LM work, we use 1 hr to overnight incubations. We generally use 1 ,g/ml of Alexa conjugated secondary antibodies for 4 hrs at room temperature.

Wash 3x with HWB
Coverslip with Mowiol. Store at 4 C in the dark until viewed. Variants – we sometimes use only BSA for the blocking buffer. This would be true if we were staining using lectins or a secondary made in an unusual species (e.g., chicken or sheep).

SAMPLE EM IMMUNOSTAINING PROTOCOL
Only use sections on Nickel grids. If the sections are on mesh grids, I immerse them so they get labeled on both sides. If they are on Formvar or Carbon/Formvar coated grids, I float them on drops. You need 20-50 ul drops for each step. All incubations need to be done in a humidity chamber because evaporation at any stage will give you terrible background.

Block in 50 mM Glycine in HWB for 30 min to block free aldehyde groups (use this step only for aldehyde fixed tissues that haven't been rinsed in glycine/HWB yet). Block 30 min in GBB2 (i.e., 5% BSA + 5% NGS + 0.1% CWFS gelatin)

Brief rinse in incubation buffer (e.g., 0.1% BSA-c)

Incubate in primary antibody at 1-10 ,g/ml in incubation buffer. For EM work, we use 1 hr to overnight incubations at room temperature. The incubation time varies widely depending on the antibody but about half I do overnight and others for only 2-4 hrs to keep background low. I generally work at higher concentrations (i.e., 10 ,g/ml) than I do for LM.

Wash 3x with HWB
Incubate in gold conjugated secondary antibodies at 1:10 to 1:40 dilutions. I generally use 1:10 or 1:20 dilutions. I do this step for 1 hr to overnight but generally for 2-4 hrs at room temperature. I try to use 10 nm gold conjugates (much higher binding efficency than 15 or 20 nm conjugates. Not as good as 6 nm gold but a lot easier to see!). I generally buy my gold conjugates from Electron Microscopy Sciences (www.emsdiasum.com) and store them at 4 C for at least 1 year before I see a fall off in labeling.

Wash 3x with HWB
Wash 3x on a dH2O.
Counterstain with uranyl acetate and lead citrate as appropriate for the sample. This step varies widely with the resin and sample prep.

Some people like to incubate the grids on a drop of aldehyde fix after the last wash steps to keep from losing gold during the uranyl and lead staining steps but I am not convinced it does anything in most or all cases.

If you have any questions, please call Shiv at 217-333-1214