Protocol

Immunofluorescence staining

Version 1.0

Background This protocol is suitable for immunofluorescence staining of tissue sections with most antibodies. It is considerably more sensitive than most published protocols, and is suitable for use in Quantitative immunofluorescence (QIF) microscopy in accordance with the requirements of Mosedale et al (1996) J Histochem Cytochem 44:1043. Antibodies do differ, however, in the optimum incubation conditions: for example some antibodies work better at 37ºC than 4ºC, or in the presence of 0.5% tween-20. The protocol below contains the incubation conditions that we have found to be optimal for the majority of antibodies, but we recommend testing a variety of conditions when working with antibodies for the first time. Where we have optimised a particular antibody for use in immunofluorescence, the conditions appear on our datasheet for that antibody and those conditions should be substituted for those herein.

The following protocol can be used for multiple labelling (simultaneous detection of multiple antigens on the same section) utilising secondary antibodies coupled to different fluorophores. However, it is essential to validate the specificity of the secondary antibodies used IN EACH EXPERIMENT.

Almost all of our work has been with frozen sections, and this method may not be optimal for staining other types of sections (e.g. formaldehyde fixed or paraffin-embedded). Formalin, in particular, reduces antigenicity of sections by crosslinking the positively charged residues that are usually at the core of high affinity epitopes. Thus, while formalin gives better retention of tissue structure than acetone fixation, it reduces sensitivity of antibody detection by 2-10 fold.

Procedure

Section preparation

1. Embed fresh tissue in embedding medium and freeze in liquid nitrogen. Store tissue blocks at -70˚C.

2. Warm tissue blocks to temperature of microtome (-20 to -25˚C). Cut 4 µm sections using a cryotome. Use a motor-driven cryotome to ensure sections are all of identical thickness if performing quantitative work.

3. Collect sections onto poly-L-lysine coated slides (slides dipped in 0.01% (w/v) poly-L-lysine solution for about 10s and left to dry overnight at room temperature. Stored at -20˚C and warmed to room temperature prior to use.)

4. Fix sections in ice-cold acetone for 90s. Leave to dry (takes 10-30 minutes, may be speeded up with cold hair-dryer). Store in slide boxes (ideally inside sealed plastic bags) at -20˚C until required.

Antibody Staining

1. Warm sections to room temperature before opening bag. Do not freeze-thaw sections, so sections must be stored in individual bags for each experiment.

2. Draw around underneath each section with a marker pen. Trace this line from above (i.e. around section on same side as section) with PAP-pen. Try an ensure that the area enclosed by the PAP-pen round each section is of approximately similar.

3. Rehydrate/block sections in phosphate-buffered saline (PBS: 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$) +3% IgG-free bovine serum albumin (Sigma A-2547) for 30 minutes at room temperature.

4. Remove excess block solution by dabbing around section carefully with a tissue (NOTE: the section is never completely dried out) and apply 25 µl primary antibody (depends on area of slide within PAP-pen) in PBS+3% BSA and leave overnight (15-17 hours) in a humid box at 4˚C (may vary, depending on the optimization of the antibody).

5. Remove remaining primary antibody by drying round the section as much as possible and replace with several drops (c. 150 -250 µl) of PBS). Leave for 3 minutes. Repeat twice more, for a total of three washes.

5. Dry around section as much as possible and apply 25 µl of secondary antibody in PBS + 5% serum (from the same species as the host of the secondary antibody) and leave for 4-6 hours at room temperature. It is advisable for multiple labelling to have all secondary antibodies raised in the same species. We routinely use minimum-cross reactivity antibody raised in donkey (available from Jackson Immunoresearch).

6. Repeat wash as in step 4.

7. Swill each slide gently in a beaker of MilliQ water, being careful not to dislodge the section. Remove and blot dry as much as possible (careful of section!). Allow to dry. Apply 1 drop of mounting medium per section and a coverslip. Cover each corner with nail varnish to hold coverslip in place and view. Sections may be viewed immediately, but for quantitative work leave until the next day, as fluorescence intensity increases somewhat over the first 12 hours after mounting. Always store stained sections at -20ºC in boxes containing silica gel prior to viewing, as the antibody complexes can dissociate at room temperature.

KEY POINTS:

* For quantitative work it is imperative that at every stage every slide is treated exactly the same – this includes wash times (to within a few seconds). If you have more than about 12 slides do them in two batches – but now be careful that antibody incubation times do not vary!

* For ALL multiple labelling experiments it is important to confirm that the secondary antibodies do not bind to each other or to the inappropriate primary antibody before doing your experiment.

Materials

PAP-pen	Agar Scientific cat # L4197
Embedding medium	Bright Instruments "Cryo-M-bed"
Cryotome	Bright OTF (motor drive model produces more consistent sections)
Slides	BDH Superfrost slides (low iron clear glass, washed, cleaned and polished; cat # 406/0169/02)
Poly-L-lysine solution	Sigma, cat # P-8920
Mounting medium	Agar Scientific Citifluor AF-1, cat #A1320

Source References

Mosedale et al. (1994) J Histochem Cytochem 44:1043

References where we have used this protocol

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DISCLAIMER

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