

ANTIBODY STAINING ON DISCS (light microscope)

METHOD

DAY1

- 1) Dissect out eye discs in (*fresh*) 1X PBS (see NOTE 1).
- 2) Fix in PLP (see NOTE 2) for 10' then 20' (NOTE 2)
- 3) OPTIONAL: Return discs to 1X PBS and remove the peripodial membrane (see NOTE 3).
- 4) Wash discs 2X 10' in 1X PBS (see NOTE 4)
- 5) Permeabilize and block in 1X PBS, 10% goat serum, 0.1% Triton-X or 0.4% NP-40 for 30-60 minutes (NOTE 5).
- 6) Incubate O/N at 4° or at room temperature for 2-3 hours in primary antibody in permeabilization buffer (NOTE 6).

DAY2

- 7) Wash 7X 10' in permeabilization buffer (NOTE 7).
- 8) Incubate in 1 in 250-500 (or as appropriate) secondary antibody for 2-3hrs at room temp.

HRP SECONDARY

- 9) Wash 2X 10' in perm. buffer.
- 10) Wash 7X 10' in 1X PBS (NOTE 10).
- 11) Transfer the discs to 250 of 1X PBS and add 4 of 8% NiCl soln. Mix. Add 250 of 1mg/ml DAB (from -20°C freezer). Mix. Add 3 of 30% H₂O₂. Watch the color reaction under the scope. Leave for 5-30 minutes depending on the level of signal:background.
- 12) Stop the color reaction by transferring the discs to PBS.
- 13) Mount the discs in *FRESH* glycerol or embed them in plastic for sectioning as described below. For glycerol, put a small drop on the slide, transfer discs (fairly dry) to it, dissect off the head skeleton and orientate with the APICAL (convex) SURFACE UP. Place on coverslip and seal all around with clear nail polish (NOTE 13).

ABC ENHANCEMENT

Proceed from STEP 8) above using a biotin conjugated secondary made in the appropriate animal.

- 9) Wash 4X 15' in perm. buffer (NOTE 14)
- 10) Wash 4X 15' in 1X PBS
- 11) Incubate in pre-prepared vectastain mix (10ml 1X PBS w/ 2 drops (=10 approx.) of A mix and 2 drops of B mix: leave stationary for 30 minutes prior to use) at RT for hours or at 4°C overnight.
- 12) Wash 7X 15' in 1X PBS (NOTE 15).
- 13) Carry out the color reaction as in STEP 11) above and so on.....

FLUORESCENT SECONDARIES

These are particularly useful when you want to do double staining or confocal work.

STEP 8) will use an appropriate secondary conjugated to a fluorescent tag; incubate covered with foil. We have FITC (fluorescein=green) and TRITC (rhodamine=red) filters on the axiophot microscope.

9) Wash 7X 20' in 1X PBS (see NOTE 16) in the dark.

10) Mount the discs in vectashield mounting medium (kept in small black box at 4°) and keep slides in the dark at 4° until use. Look at them on the scope asap and photograph with high ASA film (1000-1600) from Bel Air.

TROUBLESHOOTING

1. There is no signal

Possible problems:

- i/ the 1° is dead or in too low a concentration or there are too many discs per well (see NOTE 6).
- ii/ the 2° is dead (check other 1° control) or you need a higher concentration.
- iii/ the fix is bad (check other 1° control and make a fresh batch) or it is destroying your epitope (try other fixes; see NOTE 2).
- iv/ background staining is obscuring the signal (see below).
- v/ If you left your slides mounted overnight before examining them it is possible that your signal has disappeared because of bad glycerol (see NOTE 13).
- vi/ If you have a very weak signal and not much background you might consider using ABC and Ni enhancement to amplify the signal.
- vii/ Make sure the discs are mounted with the apical side up.

2. There is very high background

This can be due to several things:

- i/ lower the concentration of the 2°
- ii/ wash more extensively, especially after the 2° .
- iii/ lower the concentration of the 1°.
- iv/ Consider removing the peripodial membrane if you see heavy apical staining of all cells.
- v/ If possible, you may want to affinity purify the antibody (especially if you are also seeing a lot of bands on the western).

3. Morphology looks bad / disc cracks on mounting

This is most likely due to a fix problem; make a fresh batch.

NOTES

1) I usually do the dissection in a glass dissecting dish or on a sylgard plate with Ted Pella forceps. This (and all subsequent PBS) can be substituted for by freshly made 0.1M phosphate buffer (pH 7). Either way, you don't want to leave the dissected discs in buffer for more than 10-15 minutes. If your staining is very weak or fuzzy, shorten this step to a minimum. Fix them in small batches if necessary.

2) After dissecting the discs transfer them into a very small drop of the buffer. Fill the rest of the glass well with *freshly made* PLP. After 10 minutes transfer to just the fix. This prefix avoids the discs getting curled in a blob as you dunk them into fix; it is strongly recommended to avoid hassles when you come to mounting. Opinions vary on this, but I think it is a good idea to do the fixing ON ICE and sometimes for as long as 10 + 35 minutes (but not more). If this fix doesn't work other possibilities include 4% formaldehyde in 1X PBS or PEMS (see Ashburner lab manual p. 372). In some unusual cases (e.g. if the antigen is sequestered in vesicles) you may need to put detergent in the fix to get good staining (see -Rho in Freeman et al. (1992) for example).

3) This is necessary when your staining is very weak or obscured by peripodial membrane staining. I use the OOO insect dissecting pins from the storeroom on a sylgard dish to do

this. Sometimes it helps to leave as much cuticle on the head skeleton as you can as it leaves you with more to hold on to.

4) Some protocols do this wash with 0.1% Triton-X or 0.4% NP-40 detergent. In my experience this doesn't make a big difference; PBS alone is also OK. Use the eppendorf baskets in the fisher deep well plates. In this, and all subsequent washes, check that the discs are submerged by adding a few more drops of liquid to the baskets.

5) Choice of detergent: NP-40 is the harshest and probably a good one to try first. Some protocols use 0.1% Triton-X, which is a bit milder and will preserve the tissue better. But basically follow whatever choice the provider recommends.

6) This is done in nunc plates in order to save antibody. Each well holds about 10 volume. Try not to put more than about 3-5 pairs of discs per well. No shaking necessary. The concentration of antibody will be rather specific. If you are trying bleeds for a new antibody you might want to test a series 1 in 10, 100 and 1000. If you have done a western, try a 1° concentration 1/10th of what you used to see a clear signal on the western.

7) Washing should be done in large volumes in the baskets with shaking (about speed 2.5 on rotating shaker). Times and amounts are flexible depending on how much background you are getting.

8) The choice of secondary depends on the animal the primary antibody was produced in. For example, for RAT -Elav primary you should use a goat/donkey -RAT secondary. This may be conjugated to HRP (for brown/black DAB staining), to a fluorescent tag like FITC or rhodamine, or to biotin if you wish to do an ABC amplified staining. We usually order our secondaries from Jackson
In cases where your signal is weak, the length of incubation in the secondary CAN make a difference (I did a control for this).

10) The use of detergent in the wash will apparently inhibit the color reaction slightly, so in the case of disc staining (but not ovary or embryo) I like to transition to PBS and do most of the washing in just buffer. The detergent will help remove excessive background if this is a problem for you and obviously, the more washes you do the better, especially after the secondary.

11) NiCl enhances the stain to give a blue-black instead of rust color and is optional. When you add it to the buffer it will form a white cloudy precipitate. This is not a problem, just make sure you mix it around before adding the H₂O₂. If you see no signal, it is possible the hydrogen peroxide is bad. You can get fresh H₂O₂ from the storeroom; just add a fresh 3 . Typical times for the color reaction are 5-10 minutes, but if there is very weak background you might want to leave it for up to 30 minutes if you cannot see a signal.

13) BE WARNED. If the glycerol is bad, your staining may disappear overnight completely. If at all uncertain you can get fresh glycerol in the storeroom. Some people in the lab have used aquamount. I find this is more difficult to handle and tends to dry out eventually.

14) + 15) +16) In all these steps do as much washing as you can bear, remembering that the ABC reaction enhances background as much as the signal, and that fluorescent secondaries tend to give more signal than HRP 2°'s.

SOLUTIONS

10X PBS

80g NaCl
2g KCl
14.4g Na₂HPO₄
2.4g KH₂PO₄
in 800ml ddH₂O,
bring up to 1L,
no pHing should be necessary
pH ~ 7.4

PLP FIX