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🌐 Immunohistochemistry (IHC) Whole-Mount Antibody Staining

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ABSTRACT

Immunohistochemistry (IHC) Whole-Mount Antibody Staining protocol @FishFloorUCL

Introduction

- 1 *Protocol based on Jenny Regan's GFP protocol, modified by Tom Hawkins*
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Fix and dehydrate

- 2 Depending on the antigen**, you should either fix in PFA or TCA. Always start with PFA (option 1). TCA (option2) can be better for older embryos >36hrs

Either:

(Option 1) Fix in PFA

1. **Fix** - Fix in sweet Paraformaldehyde fix (4% w/v PFA 4% w/v sucrose in PBS pH 7.3) for 3 hrs at room temperature or 4°C overnight
2. **Wash** - After fixing, transfer to 1.5 mL tubes and rinse 2x, and wash 3 x 10 mins in PBTr (Phosphate buffered saline + 0.5-0.8% Triton-X100) on a shaker
3. (Optional) Dissect brains at this point
4. **Dehydrate** - Transfer to MeOH
 - 1 X 5 mins in 50% MeOH / 50% PBTr
 - 2 X rinse in 100% MeOH

Store at 20°C for at least 30mins or for up to 6 months (good point to leave overnight)

Or:

(Option 2) Fix in TCA

1. **Fix** - 2% TCA in PBS for exactly 3 hrs at room temperature in 5ml bijoux
2. **Wash** - Transfer to 1.5ml tubes, rinse 2x and wash 3 x 5mins PBS (on side)

Store at 4°C for a week but add 20mM Azide if storing for longer (to prevent mould). In azide, PBS embryos should keep for a month.

(Day 1) Rehydrate

- 3 This step applies to PFA fixed, MeOH stored embryos only**

- 1 x 5mins 50% MeOH / 50% PBTr
- 3 x 5mins PBTr

(Day 1) Permeabilise

- 4 For PFA:**

1. **Digest** at room temperature, with the tube lying on its side (no agitation) (use a 22°C

incubator in high summer or deep winter)

Store PK at -20°C at 10mg/ml – This is 1000x stock

ProteinaseK (PK) digestion times vary with embryo age. Digest according to the table below

Developmental Stage	PK Treatment
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Up to tailbud	no PK
2 - 10ss	quick rinse in 1X PK
10 - 15ss	1 min 1X PK
16 - 26ss	2 min 1X PK
24 hpf	15 min 1X PK
30 hpf	20 min 1X PK
36 - 48 hpf	30 - 40 min 1X PK
2.5 dpf	30 - 40 min 1.5X PK
3 dpf	30 - 40 min 2X PK
4 dpf	30 - 40 min 3X PK
5 dpf	30 - 40 min 4X PK

Note: If the embryos are dissected, reduce the incubation time or avoid this step.

2. Rinse 3x PBTr

3. **Post fix** in 4% PFA for 20 min at room temperature on gentle shaker

4. **Wash** 3x 5min PBTr

For TCA:

1. Rinse embryos 3 x 5 mins PBS to remove azide.
2. Prechill trypsin* solution (0.25% in PBS) and 5mL per tube of PBTr on ice until cold.
3. Incubate embryos in trypsin in ice for 5-10 mins (according to age 36hpf - 5dpf) may need longer for older embryos, depending on the trypsin batch, titrate upon first use.
4. Rinse 2x in cold PBTr then 3x10mins in cold PBTr, then bring to RT

*Trypsin stock is 2.5% (10X)

(Day 1) Block

- 5 Incubate in IB for at least 1 hour at room temp on shaker. (IB: 10% normal goat serum (NGS), 1% DMSO, 0.5-0.8% Triton-X100 in PBS)

- For 1ml IB: 100 mL NGS, 10 mL DMSO, 0.89 mL PBTr
- For 5ml IB: 500 mL NGS, 50 mL DMSO, 4.45 mL PBTr.

(Day 1) Apply Primary Antibody

6 Incubate in IB + primary antibody overnight at 4°C on shaker

- AntiGFP: Polyclonal rabbit α -GFP from AMS Biotechnology (cat #TP401) gives great results. Use 1/1000. AMS Biotechnology, 185A/B Milton Park, Abingdon, Oxon OX14 4SR, UK.
- Titrate other antibodies upon first use. Monoclonals generally at between 1:100 and 1:1000 Supernatants at 1:5 - 1:50

(Day 2) Wash

- 7
1. Remove the primary antibody (primary antibody can be kept at 4°C for reuse within a week)
 2. Rinse 3x in PBT.
 3. Wash at least 4 x 30min in PBTr on shaker.

(Day 2) Block Endogenous Peroxidases

8 *Unnecessary with fluorescent-conjugated antibodies OR < 24hr embryos*

1. Wash 5 mins in 50% MeOH/PBTr
2. Wash 10 mins in 100% MeOH
3. Incubate in MeOH peroxide for 10 mins at room temperature (1 mL MeOH/10ml 30% H₂O₂)
4. Wash 5 mins 50%MeOH/PBTr
5. Wash 5 mins 50% MeOH/PBTr

(Day 2) Apply Secondary Antibody

9 Incubate in IB + secondary antibody overnight at 4°C on shaker
If you are using a fluorescently tagged antibody, keep tubes in the dark

Secondary depends upon primary antibody and detection method

- **For fluorescence:** Molecular Probes (www.probes.com) Alexa Fluor 488 goat α -rabbit IgG is good (cat # A-11034). Use at 1:200.
- **For non-amplified HRP detection:** Sigma (www.sigmaaldrich.com) goat α -rabbit IgG (whole molecule) peroxidase conjugate is good (cat # A-6154). Use at 1:200.
- **For amplified HRP detection:** Vector labs (www.vectorlabs.com) biotinylated anti-mouse IgG (#BA-9200) or anti-rabbit IgG (#BA-1000) are both great.

(Day 3) Wash

- 10
1. Rinse 3 x in PBSTr
 2. Wash at least 4 x 30 min on shaker

If you are using a fluorescently tagged antibody, keep tubes in the dark

Fluorescently stained embryos are now ready to image.

1. Rinse out Triton with PBS.
 2. Either transfer to 75% glycerol (+AF1 CITIFLUOR) (through 25% and 50% glycerol/PBS) and mount. Or keep in PBS and mount in Agarose.
- Keep at 4°C in the dark and image as soon as possible.

(Day 3) DAB Detection

11 See below for amplification for non-amplified detection (HRP conjugated secondary antibodies)

1. Make up 1 mL DAB solution** per tube of embryos.
2. Transfer embryos to a 24-well plate.
3. Remove PBT and add 500µl DAB solution. Incubate for 20 min.
4. Add 1 mL of 0.3% H₂O₂ per 1 mL of the remaining DAB solution.
5. After the 20 mins is up, swap solution for 500µl DAB+ H₂O₂

Monitor reaction closely (can take 1min or 2hrs+, usually around 20-40 mins).

- More H₂O₂ can be added if it is slow.
- Always change the whole solution for a DAB solution with more H₂O₂ i.e. do not just add more H₂O₂ to the solution with the embryos in it as it will cause some embryos to blacken instantly.
Try 1 µl 0.6% per mL then 0.9% etc... this can go up to 6%, but this will usually cause background very high up, and you'd be better off using more or a better primary antibody.

DAB stock: Sigma D5637; 25mg/500µl dH₂O. Keep aliquots at -20°C and thaw immediately before use.

****DAB solution:** 500µl stock in 30ml PBS, 250µl in 15ml PBS, 125µl in 7.5ml PBS, 16.6µl per 1ml PBS

(Day 3) 'ABC' Amplification

12 For amplified detection when using biotinylated secondary antibodies.

1. Prepare ABC reagent: to 5ml of PBTr add 2 drops of vectorlabs elite 'A' reagent***, vortex.
2. Allow to settle. Open the tube and pop any bubbles at the top of the tube and then add 2 drops of vectorlabs elite 'B' reagent, vortex and leave to stand for 20 mins.
3. Incubate embryos in ABC reagent for 3-4 hours at room temperature on shaker or overnight at 4°C on shaker.
4. Wash as above (3 rinses then 4 x 30mins) and follow DAB detection section above

***Vectorlabs (www.vectorlabs.com) can provide basic ABC kit ('elite')(containing only A and B reagents Cat# PK-6100) or kits containing biotinylated antibodies and A & B reagents and blocking serum can also be bought (Cat #s anti-rabbit IgG: PK-6101 anti-mouse IgG: PK-6102). The non-'elite' kit is probably fine too (use only one drop each of A and B, the elite probably makes not much of a difference anyway).

(Day 3) Wash

- 13
 1. Wash 3 x 10min in PBS.
 2. If embryos were not raised in PTU then here they can be bleached. For bleaching mix fresh bleach using 1 mL 10% w/v KOH 0.4ml 30% H₂O₂ topped up to 12.5 mL. Place embryos in solution and monitor. Bleaching usually takes about 45 mins. After which, rinse with PBS several times. If bleaching not required then proceed to glycerol step below. BLEACHING IS NOT ADVISABLE WITH FLOURESCENT ANTIBODIES (for obvious reasons, although I have never tried it so it may be OK)
 3. Transfer to 75% glycerol (through 25% and 50% glycerol/PBS). Store at 4°C until imaging.

Troubleshooting

14 Methanol

Some antibodies are particularly sensitive to MeOH. Try omitting the dehydration step.

Detergent

Some antibodies are particularly sensitive to detergent. Try swapping PBSTr for PBS.

Antigen retrieval

Although fixation is essential for preserving tissue morphology, this process can also reduce the detectability of proteins by IHC, due to the formation of chemical modifications. Antigen retrieval is an approach to reduce or eliminate chemical modifications. This step should occur just before PK treatment (after rehydration/washing).

Protocol for antigen-retrieval from <https://www.aveslabs.com/blogs/protocols/citrate-buffer-antigen-retrieval-protocol>