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We use this protocol and it's working

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Immunohistochemistry (IHC) Whole-Mount Antibody Staining

Forked from Immunohistochemistry (IHC) Whole-Mount Antibody Staining

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ABSTRACT

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

MATERIALS

- 4% w/v PFA 4% w/v sucrose in PBS pH 7.3
- PBTr (Phosphate buffered saline + 0.5-0.8% Triton-X100)
- MeOH
- 1000X PK at -20°c at 10mg/ml
- Blocking solution: 10% normal goat serum (NGS), 1% DMSO, 0.5-0.8% Triton-X100 in PBS)
- PBT

Introduction

1 *Protocol based on Tom Hawkins' modified version of Jenny Regan's GFP protocol (forked)*

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 Paraformaldehydefix (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 bijous
2. **Wash -** Transfer to 1.5ml tubes, rinse 2x and wash 3 x 5mins PBS (on side)

Store at 4ºC 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) Dechorionate

4 1. Remove chorion, if still intact

(Day 1) Permeabilise

5 **For PFA:**

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

6 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

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

- Anti-ZIP14: Polyclonal rabbit
- 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

8
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) 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 1% low melting point agarose in PBS.

Keep at 4°C in the dark and image as soon as possible.

(Day 3) Additional Staining

11 Nuclear staining DAPI

For nuclear staining 4',6-diamidino-2-phenylindole (DAPI, 10 mg/mL) was added to one of the PBSTr washes at a concentration of 1:1000.

12 Phalloidin 647

Phalloidin is a highly selective bicyclic peptide used for staining actin filaments (also known as F-actin). It binds to all variants of actin filaments in many different species of animals and plants. Typically, phalloidin is used conjugated to a fluorescent dye, such as FITC, Rhodamine, TRITC or similar dyes, such as Alexa Fluor® 488 or iFluor 488.

Importantly, phalloidin is also pH sensitive: at elevated pH, a key thioether bridge is cleaved, and the phalloidin loses its affinity for actin. Phalloidin staining can be combined with antibody-based staining by adding the phalloidin conjugate during the primary or secondary antibody incubation step.

Phalloidin-iFluor 647

Troubleshooting

13 **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).

Antigen Retrieval
** Incubate in 150mM Tris-HCL pH9 for 15 min at 70oC.
WashinPBT2x10min,thendH202x5min.

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