Immunohistochemistry on Mouse Sections

Carrie Metzinger and Ellen Chang 2009

This protocol is for embryos which have been fixed in 4% paraformaldehyde.

All washes are carried out in a Coplin Staining Jar with 8 vertical slots for slides (Fisher 08-817) while all antibody incubations are in a humidified chamber.

Humidified chamber = Large glass staining dish (Fisher 08-813C) with 3 damp paper towels in the bottom, cell culture lid to separate slides from damp paper, and a lid sealed with parafilm.

Hydrophobic pen = Super PAP Liquid Blocker (Electron Microscopy Sciences #71310, or 71312)

Volume of liquid required to cover typical embryo section = 50 µl

DAY ONE

Deparaffinization/Rehydration (in fume hood)

- 3x Xylene, 5 minutes
- 2x 100% Ethanol, 10 minutes
- 2x 95% Ethanol, 10 minutes
- 2x dH_2O, 5 minutes

Antigen Unmasking

- In microwave oven, bring 10 mM sodium citrate, pH 6.0 to a boil in Coplin Staining Jar (No Lid!!) Approximately 45 seconds. Remove from microwave.
- Quickly immerse slides into the hot citrate solution (into vertical slots of jar).
- Place glass lid on top.
- Incubate slides in hot citrate solution on bench for 10 minutes.
- Remove lid and cool on bench in citrate solution for 30 minutes.

(Alternate Antigen Unmasking Technique For Bouin Fixed Embryos)

- Heat Water Bath to 90-100˚C
- Immerse staining jar containing 10 mM sodium citrate, pH 6.0 and slides into water bath with the glass lid on
- Incubate slides in water bath for 10 minutes
- Remove jar from water bath, remove lid and cool on bench for 30 minutes

(Optional Reducing Step)

- 1x dH_2O, 5 minutes
- Use hydrophobic pen to surround area to be stained
- In humidified chamber, add 25 µl denaturing buffer to each section.
- Add 25 µl 4 mM DTT to each section.
- Pipet liquid above each section up and down to mix solutions.

- Incubate in humidified chamber for 1 hour, at room temperature.
- Wrap humidified chamber in aluminum foil, so the chamber is dark.
- Add 40 µl 500 mM iodoacetate to each section.
- Pipet liquid above each section up and down to mix solutions.
- Incubate at 37°C for 30 minutes.
- Pipet off any remaining liquid and place in waste container.

Primary Antibody

- 3x dH_2O, 5 minutes
- 2x 3% H_2O_2, 10 minutes (to quench endogenous peroxidase activity in the embryo)
- 2x dH_2O, 5 minutes
- 1x TBS/0.1% Tween-20, 5 minutes
- Using hydrophobic pen, surround area to be stained

For NO phosphatase treatment proceed as follows:

- Block with blocking solution (see table) for 1 hour at RT in humidified chamber
- Take chamber into cold room
- Remove blocking solution by gently tapping slide on paper towel
- Add primary antibody diluted in blocking solution (see table), 50 µl per section
- Leave humidified chamber in cold room overnight

For phosphatase treatment proceed as follows:

- Rinse in 1XTBS
- Add phosphatase solution
- Place slides into humidified chamber
- Place chamber into oven for 3 hours at 30ºC
- Remove phosphatase solution by gently tapping slide on paper towel
- Rinse 1x in 1XTBS
- Block with blocking solution (see table) for 1 hour at RT in humidified chamber
- Take chamber into cold room
- Remove blocking, add primary antibody diluted in blocking solution (see table), 50 µl per section
- Leave humidified chamber in cold room overnight

DAY TWO (You can add DAB after any step: 2ºAb, ABC, or TSA)

Secondary Antibody

- In cold room, remove primary antibody and wash in 1X TBS/0.1% Tween-20
- At room temperature, 3x TBS/0.1% Tween-20, 5 minutes
- Re-outline sections with hydrophobic pen
- Add secondary antibody diluted in blocking solution (see table), 50 µl per section
- Incubate in humidified chamber at room temperature, 30 minutes
  - If using ABC kit, prepare ABC in eppendorf tube and pre-incubate at room temperature, 30 minutes.
- Remove secondary antibody by gently tapping slide on paper towel.
- 3x TBS/0.1% Tween-20, 5 minutes

ABC Amplification

- Add pre-incubated ABC reagent, 50 µl per section
- Incubate in humidified chamber at RT, 30 minutes
- Remove ABC reagent by gently tapping slide on paper towel
- Wash 3x 1XTBS/0.1% Tween, 5 minutes

TSA Amplification

- Add Biotinyl Tyramide Reagent, 25 µl per section
- Incubate slides at RT, 10 minutes with gentle agitation on orbital shaker (set to 2)
- Remove Biotinyl Tyramide Reagent by gently tapping slide on paper towel
- 3x TBS/0.1% Tween, 5 minutes with gentle agitation on orbital shaker
- Add SA-HRP, 50 µl per section
- Incubate in humidified chamber at RT, 30 minutes
- Remove SA-HRP by tapping slide on paper towel
- 3x TBS/0.1% Tween, 5 minutes with gentle agitation on orbital shaker

DAB

- Prepare fresh DAB, 50 µl per section
- Add DAB
- As soon as slides develop, remove DAB (chemical waste disposal)
- Add a drop of dH_2O to stop reaction and then tap slide to remove water
- 2x dH_2O, 5 minutes

Dehydrate

- 2x 95% Ethanol, 10 seconds
- 2x 100% Ethanol, 10 seconds
- 2x Xylene, 10 seconds
- Mount coverslips with permoun

	Stage	Fixative	1°Ab, blocking solution	2°Ab biotinylated anti-rabbit, blocking solution
P-Smad CTerminal (Cell Signaling #9511L)	10.5	Bouin's	1: 100, 5% BSA	1:500, 5% BSA
	12.5 13.5 14.5	PFA	1:100, 5% BSA	1:500, 5% BSA
	17.5	PFA	1:100, 0.1%BSA/1% Goat Serum	1:2500, 2.5% Milk
P-Smad GSK3 (S210)	15.5	PFA	1:1500, 5% BSA	1:500, 5% BSA
	17.5	PFA	1:3000, 0.1%BSA/1% Goat Serum	1:2500, 2.5% Milk
P-Smad MapK (S214)	15.5	PFA	1:6000, 5% BSA	1:500, 5% BSA
	17.5	PFA	1:6000, 0.1%BSA/1% Goat Serum	1:2500, 2.5% Milk
Pericentrin	17.5	PFA	1:3000, 0.1%BSA/1% Goat Serum	1:2500, 2.5% Milk
	Stage	**Fixative**	**1°Ab**	**2°Ab biotinylated anti-goat** (vector labs BA5000)
CV-2 (R&D AF2299)	12.5 13.5 14.5	PFA	1:50, 5% Rabbit Serum	1:500, 5% Rabbit Serum
Chd (R&D AF758) *Use reducing step for better results.*	12.5 13.5 14.5	PFA	1:50, 5% Rabbit Serum	1:500, 5% Rabbit Serum

Notes:

- All antibodies included in this protocol require both the ABC kit and TSA kit, diluted in blocking solution
- It is recommended to use the serum from the animal in which the secondary antibody has been produced

Solutions

10X Tris Buffered Saline (TBS): For 1 Liter, add 24.2 g Tris base ($C_4H_{11}NO_3$) and 80g sodium chloride (NaCl) to 900 ml dH_2O. Adjust pH to 7.6 with concentrated HCl.

1X TBS/0.1% Tween-20: Wash Buffer. For 1 liter, add 100ml 10X TBS to 900ml dH_2O. Add 1 ml Tween-20 and mix.

10 mM Sodium Citrate Buffer: Antigen Unmasking Solution. For 1 liter, add 2.94 g sodium citrate trisodium salt dihydrate ($C_6H_5Na_3O_7 \cdot 2H_2O$) to 1 liter dH_2O. Adjust pH to 6.0.

3% Hydrogen Peroxide: For 100ml, add 10ml 30% H_2O_2 to 90ml dH_2O.
[Fisher Chemicals cat # H325-500]

5% BSA Blocking Solution: Dilute in 1X TBS/0.1% Tween-20. Make fresh or store O/N at 4ºC.
[Roche cat# 03 117 332 001]

5% Rabbit Serum Blocking Solution: Dilute in 1X TBS/0.1% Tween-20. Make fresh or store O/N at 4ºC. [Vector Labs cat# S-5000]

Primary/Secondary Antibodies: Dilute in Blocking Solution (See table)

ABC Reagent: 1:100 A + 1:100 B in 5% BSA (ex: For 1ml, 10µl A + 10µlB in 980µl 5% BSA.) Incubate this for 30 minutes at RT before use.
[Vector Labs: cat# Rabbit I_gG, PK-4001. cat# Goat I_gG, PK-4005.]

Biotinyl Tyramide: 1:50 in 1X Amplification Diluent (provided in PerkinElmer kit).

SA-HRP: 1:100 in 5% BSA.
[Perkin Elmer: TSA Biotin System, cat# NEL700A]

DAB: 1% NiCl, 10% DAB Substrate, 89% Peroxide Buffer
[Roche, cat# 11718096001]

Phosphatase: For 100µl solution, 10µl 10X Buffer
10µl 10X λppase
10µl 10X Mn
70µl water
[New England Biolabs, cat# P0753S]

1M DTT: For 1ml, add 0.15425g DTT to 1ml dH_2O. Make fresh.

4 mM DTT: For 1ml, add 4µl 1M DTT to 996µl dH_2O. Make fresh.

1M Tris-HCl pH 8.0: For 500ml, add 60.57g Tris base and fill to 500ml with nanopure H_2O. Adjust pH to 8.0 with HCl.

0.6M Tris-HCl pH 8.0: For 30ml, add 18ml 1M Tris-HCl pH 8.0 to 12ml nanopure H_2O.

Denaturing Buffer: For 1ml, add 250µl 0.6M Tris-HCl pH 8.0 to 750µl 8M Guanidium HCl [Sigma-Aldrich, cat# G9284-100ML]. Make fresh.

500 mM Iodoacetic Acid: Make and use this solution under the hood! For 8ml, add 0.7438g iodoacetate [Sigma-Aldrich, cat# I4386-10G] to 8ml dH_2O. Adjust pH to 8.5 with NaOH. Make fresh or store in the dark at -20°C.

References

Schmidl, M., Adam, N., Surmann-Schmitt, C., Hattori, T., Stock, M., Dietz, U., de Crombrugghe, B., Pöschl, E., and von der Mark, K. (2006). Twisted gastrulation modulates bone morphogenetic protein-induced collagen II and X expression in chondrocytes in vitro and in vivo. *JBC* **281,** 31790-31800

Ahn, K., Mishina, Y., Hanks, MC., Behringer, RR. and Crenshaw, EB. III. (2001). BMPR-IA signaling is required for the formation of the apical ectodermal ridge and dorsal-ventral patterning of the limb. *Development* **128,** 4449-4461.

Cell Signaling Technology protocol from Phospho-Smad1/5/8, Catalogue #9511.

Aitken, A., Learmonth, M. (2002). Carboxymethylation of cysteine using Iodoacetamide/Iodoacetic acid. The Protein Protocols Handbook **2**, 455-456.

Troubleshooting

This protocol generally works but…

Background in Secondary Antibody alone

- Try diluting secondary antibody
- Try reducing time in TSA (anywhere from 3 to 10 minutes)
- Try blocking in 2.5% Milk

Overall background

- Dilute primary antibody
- Remove ABC or TSA/SA-HRP steps
- Try blocking in 5% goat serum and diluting primary antibody in 0.1%BSA/1% goat serum
- Longer peroxide treatment

No staining at all

- Longer heat treatment
- Trypsin or pepsin treatment before heat treatment