O'Brien Center For Benign Urologic Research
UW-Madison • UMASS Boston

Whole Mount IHC Protocol

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- *All steps should be conducted with gentle agitation on an orbital shaker, unless otherwise instructed.*
- *All incubations and washes are performed in 4-24 well culture plates*
- *The typical starting antibody concentration for whole mount IHC is half the concentration used for paraffin section IHC (if a 1:250 antibody dilution is used for paraffin section IHC, start with a 1:500 dilution for whole-mount IHC and optimize as needed)*
- *All buffers are free from sodium azide*

Tissue Preparation

1. Fix samples overnight in 4% PFA at 4°C
2. Wash in PBS at 25°C for 5 min (repeat 4X)
3. Dehydrate samples through a graded series of methanol : PBS and store in 100% methanol at -20°C.

IHC Staining

Day 1

1. Rehydrate through a series of MeOH / PBSTw at 25°C:
    A. 75% MeOH / 25% PBSTw for 10 min
    B. 50% MeOH / 50% PBSTw for 10 min
    C. 25% MeOH / 75% PBSTw for 10 min
    D. 100% PBSTx for 10 min (repeat 2X)
    E. PBS at 4°C with gentle shaking overnight

Day 2

1. Quick rinse in PBS
2. Block in block solution at 4°C with shaking overnight

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Day 3

1. Primary Antibody Step
    A. Dilute primary antibody at the optimized concentration in antibody dilution buffer and add to the sample wells
    B. Add water to the empty wells
    C. Cover culture plate, seal with parafilm to prevent evaporation, and incubate at 4°C for 72 hours with gentle agitation

Day 6

1. Quick rinse in PBS
2. Rinse in PBS at 25°C for 1 min (Repeat 5X)
3. Rinse in PBS at 25°C for 1 hr (Repeat 6X)
4. Secondary antibody step
    A. Dilute secondary antibody (1:500) in antibody dilution buffer and add to the sample wells
    B. Add water to the empty wells
    C. Cover culture plate, seal with parafilm to prevent evaporation, and incubate at 4°C overnight with gentle agitation

Day 7

1. Quick rinse in PBS
2. Rinse in PBS at 25°C for 5 min (Repeat 5X)
3. Rinse in PBS at 25°C for 1 hr (Repeat 6X)
4. Post-fixation step
    A. Add 4% PFA to the sample well
    B. Add water to the empty wells
    C. Cover plate, seal with parafilm, and incubate at 4°C overnight with gentle agitation

Day 8

1. Quick rinse in PBS
2. Rinse in PBS at 25°C for 5 minutes (Repeat 4X)s
3. Clear in Citifluor AF-1 at 4°C for a minimum of 3-5 days

Day 11

1. Image samples

End protocol

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MATERIALS

Phosphate Buffered Saline (PBS)

Makes 1L of 1X solution

- 1 packet of Dulbecco's Phosphate Buffered Saline (Modified, without calcium and without magnesium (MP biomedical # 1760420)
- Adjust volume to 1000 mL with H2O
- Adjust pH to 7.4
- Sterilize by autoclaving
- Store at room temperature for up to 1 week.

Tris Buffered Saline with Tween (TBSTw)

Makes 500 mL

- 0.02M Tris-HCl (Fisher BP153-1)
- 0.15M NaCl (Fisher BP358-212)
- 0.1% (v/v) Tween-20® (Fisher BP337-100)
- Adjust volume to 500 mL with H2O
- Adjust pH to 7.4

Blocking Reagent

Makes 100 ml of 10X solution

- 100 mM Maleic Acid (Sigma #M0375-500G)
- 150 mM NaCl (Fisher #S271-3)
- Adjust volume to 100 mL with H2O
- Adjust pH to 7.5. This is a strong buffer and will require several solid NaOH pellets to raise pH)
- Add 10% Blocking Reagent (Roche #11096176001). Microwave to dissolve (avoid boiling over, solution will be cloudy & viscous so watch carefully to ensure blocking reagent is completely dissolved)
- Aliquot 10 mL volumes into conical tubes & store at –20°C for up to 1 year.

Antibody dilution buffer

Makes 100 mL

- 1% Blocking Reagent (see recipe above)
- 5% serum from host species in which secondary antibody was made
- BSA Fraction V (Fisher #BP1600-100)
- Adjust volume to 100 mL with TBSTw (see recipe above)
- Aliquot 10 mL volumes into conical tubes & store at -20º C

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4',6-diamidino-2-phenylindole, dilactate (DAPI)

Makes 10 mL of a 300 µM stock solution of DAPI (This is a 1000X concentration)

- 0.0014 g DAPI dilactate (Invitrogen #D3571, FW 457.5)
- 10 mL N,N Dimethylformamide (Sigma #D4551-250mL)
- Prepare 100 µl aliquots and store at -20°C.
- To make working solution for IHC (a 1X solution containing 300 nM DAPI), dissolve the stock solution in PBSTw and apply it to microscope slides for 5 min, followed by several washes with PBSTw.

Notes

- *The powdered form of DAPI (from vendor) appears to be stable for many years when stored in dark at -20°C.*
- *The DAPI solution (dissolved in N,N Dimethylformamide) appears to be stable for at least 2 years when stored in dark at -20°C.*

Abbreviations

BSA = bovine serum albumin
DAPI = 4',6-diamidino-2-phenylindole
MeOH = Methanol
PFA = paraformaldehyde
PBS = phosphate-buffered saline
PBSTw = 1X PBS + 0.1% Tween-20
TBSTw = 1X TBS + 0.1% Tween-20

Reagents and Supplies

BSA, cat # BP1600-100, Fisher
Blocking reagent, cat # 11096176001, Roche
Citifluor, AF-1 reagent, cat #17970 Electron Microscopy Sciences
Goat Serum, Sigma G6767
Maleic acid, cat # M0375-500G, Sigma
Methanol, cat. # 148809, Fisher Scientific
Paraformaldehyde, cat # 101176-014, VWR
PBS, w/out Ca & Mg, MP Biomedicals powdered media, cat # ICN1760420, Fisher
Sodium chloride, cat # BP358-212, Fisher
Tween 20, cat # BP337-100, Fisher

Notes

- Primary antibodies raised in mouse should not be used to stain mouse tissues (we cannot overcome the excessive noise that results, even if the mouse primary antibodies successfully stain mouse tissue sections).
- Azide buffers have been shown to cause auto-fluorescence in imaging of whole-tissue samples, thus it is necessary to solely utilize azide-free buffers.
- Methanol has been shown to cause auto-fluorescence in imaging of whole-tissue samples, thus complete (we do overnight) rehydration of the tissues is necessary at the outset of the procedure.
- With regards to the blocking step, it has been demonstrated that the optimal incubation time for blocking is overnight, however, it is sufficient to block for a minimum of 4 hours if imaging background is not of concern.
- 0.1% Tween can be included in wash buffers if desired, however, TritonX should not be included.
- Tris-based buffers can be substituted for PBS in wash steps if desired

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