

Indirect Immunofluorescence for Snap-frozen OCT-Embedded Tissues

I) NO Tissue Fixation.

- 1) Remove tissue and immediately place in OCT and snap freeze in liquid nitrogen.
- 2) Place the box in the cold while sectioning (Never let the slides warm up).
Store at -80C

II) Post fixation

- 1) Place slides directly into prechilled Methanol/Acetone (1:1) and fix the slides for 10 min in -20C. Rinse in water.
- 2) Immerse slides in **10mM Citric Acid Monohydrate buffer pH 6.0** (Fisher # A104-500) (1.36 g in 650ml H₂O (for 1 rack) or 1.88g in 900ml H₂O (for 2 racks)) and incubate in microwave. The solution will come to a boil in a few minutes. Let it boil for 6 minutes. (Set our microwave for 14 min for 650 ml or 17 min for 900 ml) *Don't let the slides go dry. This step enhances the availability of the antigen (i.e., by breaking aldehyde 'cross-links' and, in the case of BrdU immunohistochemistry, denatures dsDNA and releases it from histones effectively exposing BrdU)*
- 3) Remove from microwave and let cool in buffer 10'
- 4) Rinse in gently-running tap water 10'
- 5) Lie slides out in hybridization box. Wash with 1X PBS 2 X 5'
(Use PAP pen to restrict subsequent volumes of reagents applied to the sections).
- 6) Block with **Coultter 'Protein Blocker' Reagent** at RT. 30'
DO NOT RINSE; Pour excess blocker off and add diluted Ab, as described below.

III) Primary Antibody Incubation

- 1) Incubate sections with **primary antibodies** diluted in **PBT or Zymed antibody diluent**:(use water-soaked paper towels to create a moist atmosphere in the hybridization box)

O/N at 4°C

NOTE: stock is kept frozen in 10 µl aliquots (0.5mg/ml) at -80 °C. After thawing keep in at 4°C.

- 2) Rinse and then wash in PBS 3X 10'

2) Secondary antibody incubation

- 1) Incubate sections with appropriate **secondary antibody -Cy2 or -Cy3** (Jackson) diluted **1:600 in PBT or Zymed Diluent** in moist chamber in the dark

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2 hrs @ RT

- 2) Rinse and then wash in PBS using light-tight chambers 3 X 10'
- 3) Rinse briefly in ddH₂O
- 4) Remove excess water and let slides dry in the dark
- 5) Mount coverslip with **Kierkguard** mounting medium and seal with clear nail polish
- 6) Store slides at 4°C in a light-tight box until you are ready to look at them.

4% PFA per 50 ml:

- 1) Mix 2 g PFA (Fisher #04042), 6 L 10N NaOH, and 25 ml DEPC H₂O into 50 ml conical tube.
- 2) Heat to 60°C and place on rotator until PFA is dissolved. *Be careful not to overheat the solution.*
- 3) Chill before open the tube. Add 5 ml of 10x PBS and bring the volume up to 50 ml with DEPC H₂O .

For 50mL PBT Solution, add the following reagents in these amounts:

ddH ₂ O	43.5mL
10XPBS	5.0mL
10% BSA	0.5mL
10% Triton-X100	1.0mL

*Adjust to volume needed; store at 4°C for up to one week