**Chromogenic cFos Immunohistochemistry Protocol (frozen tissue; updated July, 2015)**

**Experimental info Reagent info (manufacturer, source)**

Researcher(s): 1° antibody: 1:500 Rabbit-anti-phospho-cFos (CST, 5348)

Experiment: 2° antibody: 1:200 biotinylated Goat-anti-rabbit (Vector, PK-6101)

Animal #(s): PBS (0.01M); 10% Triton X; goat serum

**Procedure: Day 1 (date )**

1. Tissue preparation
   1. dry slides using a slide warmer
   2. fix tissue in NBF, 10 minutes, room temperature, gentle agitation; **start time:**
2. Washes
   1. PBS, 5 minutes, room temperature, gentle agitation; **start time**:
   2. PBS, 5 minutes, room temperature, gentle agitation; **start time**:
   3. PBS, 5 minutes, room temperature, gentle agitation; **start time**:
3. Non-specific tissue blocking
   1. 25 ml/box, 1 hours, room temperature, gentle agitation; **start time**: ;
      * **Vserum: l** = Vtotal  l x 0.02 serum
      * **VTriton X: l** = Vtotal  l x 0.001 10% Triton X
      * **VPBS: l** = Vtotal l– Vserum  l – VTriton X  l
4. 1° antibody incubation
   1. encircle tissue with hydrophobic wall (via PAP pen)
   2. 200 l /slide, 48 hours, 4°C, gentle agitation, *dark,* with water dish in box; **start time**: ;
      * **Vserum: l** = Vtotal  l x 0.02 serum
      * **VTriton X: l** = Vtotal  l x 0.005 10% Triton X
      * **V1° Ab : l** = Vtotal  l x 0.0015 1° antibody
      * **VPBS: l** = Vtotal l– Vserum  l – V1° Ab  l – VTriton X  l

**Procedure: Day 2 (date )**

1. Washes
   1. PBS, 5 minutes, room temperature, gentle agitation; **start time**:
   2. PBS, 5 minutes, room temperature, gentle agitation; **start time**:
   3. PBS, 5 minutes, room temperature, gentle agitation; **start time**:
2. 2° antibody incubation
   1. 200 l /slide, 1 hour, room temperature, gentle agitation; **start time**: ;
      * **Vserum: l** = Vtotal  l x 0.015 serum
      * **V2° Ab : l** = Vtotal  l x 0.005 2° antibody
      * **VPBS: l** = Vtotal l– Vserum  l – V2° Ab  l
3. ABC reagent (**prepare 30 min into 2° antibody incubation**)
   1. 200 l /slide, 1 hour, room temperature, gentle agitation; **start time**: ;
      * **VA: l** = Vtotal  l x 0.02 Reagent A
      * **VB : l** = Vtotal  l x 0.02 Reagent B
      * **VPBS: l** = Vtotal l– VA  l – VB  l
4. Washes
   1. PBS, 5 minutes, room temperature, gentle agitation; **start time**:
   2. PBS, 5 minutes, room temperature, gentle agitation; **start time**:
   3. PBS, 5 minutes, room temperature, gentle agitation; **start time**:
5. DAB staining (**work under hood)**
   1. 500 l /slide; add to wells at 20 seconds intervals and allow 6 minutes for development;
      * **Vbuffer solution: drops** = VdiH2O  ml x 0.4 buffer stock solution
      * **VDAB solution : drops** = VdiH2O  ml x 0.8 DAB stock solution
      * **Vhydrogen peroxide: drops** = VdiH2O ml x 0.4 hydrogen peroxide
      * **VdiH2O: ml**
6. Washes
   1. PBS, 5 minutes, room temperature, gentle agitation; **start time**:
   2. PBS, 5 minutes, room temperature, gentle agitation; **start time**:
   3. PBS, 5 minutes, room temperature, gentle agitation; **start time**:
7. Mounting and image
   1. Coverslip slides with non-DAPI mounting medium
   2. Image under brightfield