Co-staining pPDH and cFos in free floating brain sections

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Solutions and Reagents

Wash buffer	0.2% Triton X-100 in PBS (PBST)
Blocking buffer	5% Normal Donkey Serum in PBST (or 5% serum from secondary antibody host species in PBST)
Antibody dilution buffer	2% Bovine serum albumin (BSA) in PBST
Primary antibody	pPDH rabbit monoclonal antibody (Cell Signaling mAb #37115), ***Optiona but highly recommended: cFos co-staining, cFos (C-10, Santa Cruz) mouse monoclonal***
Secondary antibody	Alexa fluor 488 Donkey anti-Mouse; Alexa fluor 647 Donkey anti-Rabbit
Mounting medium	Fluoromount-G or others

Methods

1. 30min-1h (or depending on experimental design) after behaviors or treatment, sacrifice the animal and perfuse the brain with cold PBS and 4% PFA. **Critical!** Isoflurane or other anesthesia will significantly increase pPDH level due to neuronal inhibition. **Immediately perfuse the animal after isoflurane** or other anesthetics. Do not let the animals sit in the isoflurane for more than a few minutes.
2. Postfix in 4% PFA overnight. Cut the PFA fixed brain into with preferred histology protocols. Post fixed sections can be stored at 4°C.
3. Incubate the sections in blocking buffer for at least 1 h with gentle agitation at RT.
4. While blocking, prepare primary antibody by diluting as 1:500 of both antibodies in antibody dilution buffer.
5. Transfer the sections in diluted primary antibody. Incubate overnight at 4°C with gentle agitation.
6. Wash three times in PBST for 15 min each.
7. While washing, prepare secondary antibody by diluting as 1:500 both antibodies in antibody dilution buffer.
8. Transfer the sections in diluted secondary antibody. Incubate for 1 hour at RT with gentle agitation.
9. Wash three times in PBST for 15 min each.
10. Transfer the sections in PBS with DAPI or other preferred counterstain. Incubate for 15 min at RT with gentle agitation.
11. Mount sections on slide with Fluoromount-G or other mounting media.
12. Standard imaging with confocal. Note: cFos will show nucleus staining. But pPDH signal will be in the cytoplasm and sometimes neurites. In some regions (BLA, LH, many brainstem areas), pPDH signal can fill the whole cells. In some regions (some cortical

regions) you may see cytoplasm signal with an empty nucleus. You may need a higher mag objective to resolve clearer signals (relative to cFos staining).