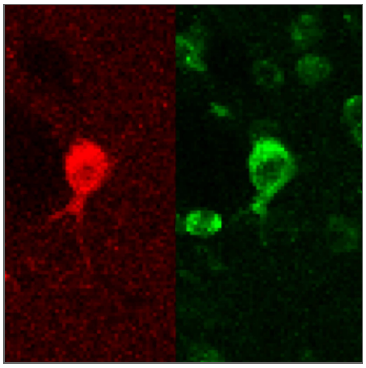

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Protocol status: Working
We use this protocol and it's working

Immunohistochemical fluorescent labelling and activity readout of striatal cholinergic interneuron activity

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ABSTRACT

This protocol delineates a method to identify choline acetyltransferase (ChAT)-expressing neurons and co-label these with a marker for global neuronal activity (phosphorylated-S6 Ribosomal Protein – pS6RP) in PFA perfusion-fixed 40 µm-thick mouse brain slices. Changes in the fluorescence of the pS6RP label are linked to changes in neuronal activity (Bertran-Gonzalez, J. et al. (2012)). Therefore, this immunohistochemical approach allows for the identification of cholinergic neurons alongside a readout of their activity. This protocol will focus on the use of this dual-labelling approach to target striatal cholinergic interneurons (ChIs) but may be applied to other brain regions or neurons of interest.

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MATERIALS

Reagents:

- Anti-Choline Acetyltransferase Antibody (Merck, RRID#AB_2079751)
- Donkey anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 568 (ThermoFisher Scientific, RRID#AB_2534104)
- Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™488 (ThermoFisher Scientific, RRID#AB_2535792)
- Hydrogen peroxide, 30% (VWR, CAS#7722-84-1)
- Methanol ≥99.9%(Sigma-Aldrich, CAS#67-56-1)
- Normal Donkey Serum (abcam)
- Paraformaldehyde prilled, 95%(Sigma-Aldrich, CAS#30525-89-4)
- Phosphate buffered saline (tablets)
- P-S6 Ribosomal Protein (S240/244) Rabbit Ab(Cell Signalling Technology, RRID#AB_331682)
- Sodium fluoride (ThermoFisher Scientific, CAS#7681-49-4)
- Triton™ X-100 (Sigma-Aldrich, CAS#9036-19-5)
- Vectashield® Plus Antifade Mounting Medium

Equipment:

- Adhesion slides, Polysine® (VWR, SKU#631-0107)
- Corning® Costar® TC-Treated Multiple Well Plates (Corning, SKU#CLS3513-50EA)
- Gilson pipettes (Gilson, SKU#FA10005M, FA10003M, FA10006M)
- Leica VT1000s Vibrotome (Leica Biosystems, SKU#8226-30-0007)
- MS 3.1 digital shaker (IKA, SKU#0003319000)
- Transfer pipette (VWR, SKU#612-1681)
- Wet Set Quick Dry Topcoat

BEFORE START INSTRUCTIONS

Note 1: pS6RP is a marker for neuronal activity, and will brightly fluoresce in tonically active neurons (e.g. striatal cholinergic interneurons). However, all neurons will express this marker and the stress induced by the perfusion-fixation process may lead to a confounding increase in expression in many neuron types. In terms of the tonically active ChIs, reducing stress in the perfusion step is ideal and lowers pS6RP signals from medium spiny neurons and other striatal neuronal populations.

Note 2: Fixation with PFA does not prevent Ser/Thr phosphatases from dephosphorylating proteins. Perfusion, fixation, slicing and staining should use cold solutions and equipment to prevent degradation of the pS6RP signal. Slicing and staining should occur as soon as possible to prevent signal loss. If this is not possible consider maintaining slices in antifreeze in a -20°C freezer. Maintaining slices in -4°C during the staining process as much as possible is ideal for maximal signal-to-noise. The inclusion of sodium fluoride (NaF) in solutions can help inhibit phosphatase activity, maintaining pS6RP phosphorylation states throughout the staining protocol.

Perfusion-Fixation

1 Habituate animal for 3 days prior to perfusion. Handle mouse, and simulate performing an intraperitoneal injection once a day (scruff and poke belly with sheathed needle & syringe).

2 On the day of the perfusion, anesthetize the mouse with an i.p. injection of pentobarbital (0.1 mL, 200 mg/ml). Perform a transcardial perfusion as normal, perfuse with ~5 mL PBS followed by ~5mL 4% cold PFA. Extract brain into chilled container with 4% PFA.

Note

Keep solutions chilled on ice.

3 Maintain brain in 4% PFA for 24 hours at -4°C.

4 Slice brain into 40 µm-thick slices.

Note

A Leica VT1000s vibrotome was used in this protocol. Chill well plates and fixed stage in -20°C freezer prior to slicing, and keep stage and buffer cold throughout slicing. We pack with ice.

Settings:
- Slice thickness: 40 µm
- Frequency: 10
- Speed: 2.5

5 Process slices for staining immediately after slicing, or store in anti-freeze at -20°C.

Note

Be sure to thoroughly wash the slices of anti-freeze prior to IHC labelling: 5x PBS with 0.1 mM sodium fluoride (PBS-NaF) washes, each for 10 minutes, plus overnight incubation in PBS-NaF at -4°C.

Primary Antibody Incubation

6 Wash sections 5 times in PBS-NaF, each time for 5 minutes.

7 Incubate for 5 min in PBS-NaF containing 3% H_2O_2 and 10% methanol (vol/vol).

8 Rinse 5 x 5 min in PBS-NaF.

9 Incubate for 20 minutes in 0.2 % Triton in PBS-NaF + 10% Donkey serum (DS).

10 Rinse 5 x 5 min in PBS-NaF.

11 Incubate overnight at 4°C in PBS-NaF containing 0.2% Triton, 3% DS, 1:300 rabbit anti phosphorylated ribosomal protein S6, 1:200 goat anti-ChAT.

Secondary Antibody Incubation

12 Wash sections in PBS-NaF 5 x 5 min.

13 Incubate for 60 minutes in 1:1000 Alexa Fluor 568 donkey anti-goat, 1:400 donkey anti-rabbit Alexa-488-coupled secondary antibody diluted in PBS-NaF + 3% DS.

14 Wash sections in PBS 5 x 5 min.

15 Mount slices with Vectashield.