Staining brain homogenates with NeuN+ in *Lampropholis delicata*

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Procedures

Tissue Collection

1. Anaesthetise the animal via intraperitoneal injection of 10µL 10mg/mL Alfaxan.
2. Euthanise the animal via decapitation with surgical scissors. Collect head and store it in cold PBS until dissection. Remove brain following established protocols and store resultant tissue sample in cold PBS on ice.

Homogenates Preparation

1. For those regions that need collagenase (e.g.: Optic tecta):
    a. Transfer the tissue to a 1.5mL centrifuge tube containing 100µL of 125U/mL of pre-warmed collagenase (type II) in 1X PBS.
    b. Incubate for 30-min at 32°C

2. For those regions that do not need collagenase (e.g.: Olfactory bulbs) / after digesting with collagenase:
    a. Mash the tissue through a 100µm mesh filter using the rubber stopper of an insulin syringe.
    b. Rinse through the mesh filter with 1mL of cold 1X PBS.
    c. Centrifuge the samples at1000 RFC for 10 minutes, remove supernatant, and resuspend in 500µL 1x PBS

Intracellular and Nuclear Staining

1. Fix the samples with 10% Neutral-Buffered Formalin (NBF): add NBF to sample at 1:10 to reach a final concentration of 1% NBF. Incubate at 32°C for 20min to fix.
2. Do PBS wash: centrifuge samples at 800-1200xg for 10min to pellet cells. Remove the supernatant. Resuspend the pellet in 200µL warm 1X PBS.
3. Permeabilize cells by adding pre-warmed digitonin to all samples and controls. Incubate at 32°C for 20min.
4. Do PBS wash: centrifuge samples at 800-1200xg for 10min to pellet cells. Remove the supernatant. Resuspend the pellet in 500µL warm 1X PBS.
5. Add 1:100 NeuN solution and incubate all night at 4°C.
6. PBS wash: centrifuge samples at 800-1200xg for 10min to pellet cells. Remove the supernatant. Resuspend the pellet in 500µL warm 1X PBS.

To optimize dye concentration, we'll make a preliminary experiment where:

Volume sample (µL)	Volume 1:100 solution dye (µL)	Total volume (µL)
100	0	100
95	5	100
90	10	100
80	20	100
70	30	100
65	35	100