VALIDATION NEURONS

Which NeuN?

* Storks et al. 2023 made homogenates and then resuspended the pellet in a 1:100 dilution of **NeuN (ABN78C3, Millipore Sigma)** and incubated under agitation overnight in the dark at 4°C. We then centrifuged the nuclei, rinsed with PBS, and then resuspended in 200–400 uL for counting. Everything was fixed with 4% paraformaldehyde for at least three days. Check if fixing with our method may work.

What was the dissolvent in the NeuN dilution?

* Farrow et al. 2021 dissected brains and post-fixed for 1 h in 4 % paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4 before being placed in 1% NaN3 in 0.1 M PBS for a minimum of three days. Later, they resuspended the pellet in 100 μL of homogenization buffer containing a 1:100 dilution of mouse anti-NeuN monoclonal antibody (EMD Millipore, USA) for 30 min on ice. The brain tissue lysate was washed by adding 1 mL of ice-cold PBS (pH 7.4) and centrifuged at 300 g for 3 minutes and the supernatant discarded. The pellets were resuspended in 100 μl of homogenization buffer containing a 1:200 dilution of goat anti-mouse IgG-FITC antibody (EMD Millipore, USA) for 30 minutes on ice in the dark. Brain tissue lysate was washed by adding 1 ml of PBS (pH7.4) and centrifuged at 300 g for 3 minutes. The supernatant was discarded, and the cells resuspended in 100 μl of PBS (pH 7.4) and were stored on ice in the dark. Why this last steps? Is it related to the fluorophore conjugate for NeuN?

We may need to **fix the tissue 4% PFA** once the homogenates are done.

We may need to permeabilize the tissue. Are we doing that for DNA damage? Check our protocols with Dalton

We need a fluorophore conjugate for NeuN for the flow cytometer. Ask Mick and tell him about our plan.

ACKNOWLEDGEMENTS:

The authors acknowledge Microscopy Australia (ROR: 042mm0k03) at the Centre for

Advanced Microscopy, The Australian National University, a facility enabled by

NCRIS and university support.

Staining brain homogenates with NeuN+ in *Lampropholis delicata*

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1 March 2025

# 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):
   1. Transfer the tissue to a 1.5mL centrifuge tube containing 100µL of 125U/mL of pre-warmed collagenase (type II) in 1X PBS.
   2. Incubate for 30-min at 32°C
2. For those regions that do not need collagenase (e.g.: Olfactory bulbs) / after digesting with collagenase:
   1. Mash the tissue through a 100µm mesh filter using the rubber stopper of an insulin syringe.
   2. Rinse through the mesh filter with 1mL of cold 1X PBS.
   3. 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 |