Assaying Microtubule Stability in Primary Neurons

<u>Protocol for immunofluorescence (IF) and immunoblot (IB)</u>

This protocol is developed for primary cultures of dorsal root ganglia (DRG) neurons (12 DIV) dissected from adult mice *(Pero ME et al., 2021)* and hippocampal and cortical neurons (14-21 DIV) dissected from rat embryos *(Qu X et al., 2017).*

1. Plate cells on coated coverslip in 12 well dishes (100 µg/ml of poly-D-lysine (PDL) 1X and 10 µg/ml of laminin for DRG neurons; PDL 1X for hippocampal and cortical neurons).

2. Treat neurons for the established times. As a positive control, use taxol (5 uM for 3 hr).

3. 1 h before the end of the treatment, add nocodazole (1 µg/mL for DRG; 0.2 µg/mL for hippocampal and cortical neurons).

Move the plate onto a slide warmer incubator kept at 37° C

4. At the end of the incubation time, gently wash each well with 1 ml of warm PHEM 1X buffer once before adding the extraction buffer.

5. Gently, add 80 µl of extraction buffer (PHEM buffer supplemented with 0.05% triton-X 100, protease inhibitor cocktail, and 10 µM of taxol) in the center of the well→leave 5 minutes for DRG neurons or 1.5 minutes for hippocampal and cortical neurons.

For IB follow the next steps

6. Gently and thoroughly collect the supernatant (*soluble fraction*) from each well into an eppendorf tube containing 20 µl of Laemmli Buffer 5X in PHEM.

7. Gently add 100 µl of Laemmli Buffer 1X in PHEM on top of the extracted cell layer, scrape the neurons and collect the pellet (*microtubule fraction)*.

8. Boil the samples (soluble and microtubule fraction) at 90°C for 5 min.

9. Run equal volumes of microtubule and soluble fraction on a 10% polyacrylamide gel for IB analysis of total levels of α-tubulin (DM1A).

For IF follow the next steps

6. Add dropwise 2X volume of fixative buffer (8% PFA and 0.2% glutaraldehyde in 1x PHEM) and incubate for another 30 min at 37 °C.

7. Wash with PBS 1X and process for immunofluorescence.

8. Stain for βIII-tubulin

9. Analyze images using ImageJ software by measuring the average intensity of proximal neurites (within 100 μm from the cell body).

<u>Pre extraction buffer 1ml</u>:

PHEM 2X	500 ul
Prot Inhibitor stock 100X-	10 ul
Triton 100X -	0.5 ul
Taxol 10uM-	1 ul
H20	488.5 ul

<u>1X Laemmli buffer in PHEM 1ml</u>:

PHEM 2X	500 ul
Laemmli buffer 5X	200 ul
H_2O	300 ul

<u>PHEM 2X (500 ml)</u>:

Weigh out the following:

a. 18.14 g PIPES
b. 5.96 g HEPES
c. 3.80 g EGTA
d. 0.41 g $MgCl_2$

Bring up to volume in H_2O