Fixing hippo neurons to assess endogenous NEMO during oxidative stress

Forked from Investigation_of_mitophagy_in_Hippo_neurons

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We use this protocol and it's working

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ABSTRACT

We altered a previously developed method (dx.doi.org/10.17504/protocols.io.bxpfpmjn) in order to investigate the interaction of endogenous NEMO with damaged mitochondria in Hippocampal rat neurons. Mitochondrial damage was induced by the administration of 35 nM Antimycin A, a complex III inhibitor, over 1 hr. Here, we visualized neuronal mitochondria with a mito-targeted construct, Mito-SNAP, and a fluorescent SNAP ligand, JaneliaFluor 646, though other mitochondrial markers may be suitable. Endogenous NEMO was visualized with the commercially available anti-NEMO primary antibody (abcam). AntA-treated neurons exhibited fragmented mitochondria. We did not observe a change in the appearance of NEMO labeling in AntA treated cells versus non-treated cells.

MATERIALS

BSA block: 0.2% Triton-X/3% BSA in PBS.

BEFORE START INSTRUCTIONS

Prepare poly-L-lysine (PLL)-coated surfaces on which to plate hippocampal neurons by pipetting 700-1000 uL 0.5 mg/mL PLL onto glass-bottomed dishes, prioritizing coverage of the glass imaging window. Neurons may also be fixed on glass coverslips.

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Plating and maintenance of hippocampal neurons

6d

1 Plate primary hippocampal Sprague Dawley rat neurons (embryonic day 18) on 35 mm glass-bottomed, PLL-coated dishes at 250,00 cells per dish in MEM supplemented with 10% horse serum, 33 mM D-glucose, and 1 mM sodium pyruvate. This is day 0.

Note

Information for dissection and separation of hippocampal neurons is not included because we outsource these steps to an on-campus core facility.

2 Incubate neurons for at least 2 but no more than 5 hours at 37 degrees C, 5% CO2. 2h

3 Replace media with Neurobasal (Gibco) supplemented with 33 mM D-glucose, 2 mM GlutaMAX (Invitrogen), 100 units/ml penicillin, 100 ug/ml streptomycin, and 2% B-27 (ThermoFisher) (Maintenance Media; MM) and continue to maintain at 37 degrees C, 5% CO2. 1d

4 Add AraC (5 uM) one day post-plating to prevent glial proliferation

5 Allow neurons to grow until Day 6.

Transfection of primary neurons

2d

6 On day 6, transfect neurons. 0.3 ug Mito-SNAP per 35-mm dish. Use 3.8 uL Lipofectamine 2000 2h

Transfection Reagent (ThermoFisher) per dish.

6.1 Equilibrate a volume of fresh MM equal to 2 mL per dish by incubating MM in a flask in the cell culture incubator. 45m

6.2 Replace conditioned MM with 1 mL fresh MM for each dish. Save conditioned MM in a fresh flask in the incubator.

6.3 Prepare transfection solutions A and B by adding 0.3 ug Mito-SNAP to 150 uL Neurobasal (A) and 3.8 uL Lipofectamine 2000 to 150 uL Neurobasal (B) for each dish. Rotate by hand 8 times and incubate at room temperature for 5 min. 5m

6.4 Briefly centrifuge A and B with a tabletop centrifuge. Then combine A and B and rotate by hand 8 times to mix. Incubate at room temperature for 20 min. 20m

6.5 Add A+B (300 uL) to each dish of primary neurons dropwise.

6.6 Incubate at 37 degrees 45 min. 45m

6.7 Replace transfection media with 50%/50% fresh and conditioned media.

7 Incubate transfected neurons 36-48 hours. 2d

Fluorescent labeling in neurons

1h

8 Incubate neurons in 2 uM SNAP ligand (Janelia-Fluor 646) for 30 min. 30m

9 Wash 2X, then rest 30 min in conditioned media. 30m

Mitophagy induction in neurons

1h

10 Replace neuron media with MM containing 35 nM Antimycin A (or, for control conditions, media containing no small molecule inhibitor) and incubate for 1 hr. 1h

10.1 During this period, prepare fixation reagent (see next step)

Fix, permeabilize, and block neurons

35m

11 Prepare 4% paraformaldehyde (PFA)/4% sucrose in PBS

11.1 Thaw a 10 mL aliquot of 4% PFA in PBS using a 37 degree water bath 7m

11.2 Add 0.4 g sucrose to aliquot

11.3 Rotate at R.T. for 10 min to dissolve 10m

11.4 Warm 4% PFA/4% sucrose to 37 degrees 10m

12 Gently add at least 0.5 mL fixative to each dish and incubate 10 min at 37 degrees 10m

13 Aspirate PFA and wash samples with PBS 4X 5 min 20m

14 Permeabilize cells by adding at least 300 uL 0.2% Triton-X in PBS for 5 min 5m

15 Block cells with at least 300 uL BSA block (see Materials) for 50 min

15.1 Prepare primary antibody solution during this step.

Perform immunolabeling and Hoechst stain 2d

16 Prepare primary solution with a volume of 200 uL * number of dishes. Use anti-NEMO (abcam, raised in rabbit) at a 1:500 dilution in BSA block.

16.1 Centrifuge primary antibody at 4 degrees, max speed, for 4 min before use. After centrifugation, pipet from top of antibody aliquot.

17 Aspirate blocking solution from each sample and gently add 180 uL primary solution.

18 Incubate samples in primary solution overnight in a dark environment. 1d

19 The following day, aspirate primary solution and add 100-200 uL PBS to wash for 5 min. Carry out PBS wash for a total of 4 times. 20m

20 Prepare secondary solution with a volume of 110 uL * number of dishes. Use anti-rabbit 488 secondary antibody at 1:200 dilution in BSA block.

20.1 Centrifuge primary antibody at 4 degrees at max speed for 4 min before use. After centrifugation, pipet from top of antibody aliquot.

21 Aspirate PBS from final wash and add 100 uL secondary solution to each sample, making sure to submerge all areas of the glass window. Incubate 1 hr at room temperature. 1h

22 Aspirate secondary solution and add 100-200 uL PBS to wash for 5 min. Carry out PBS wash for a total of 2 times. 10m

23 Prepare Hoechst solution with a volume of 110 uL * number of dishes. Use Hoechst dye at 1:1000 dilution in PBS.

24 Aspirate PBS from second wash and add Hoechst solution. Incubate 5 min at room temperature. 5m

25 Repeat step 22. 10m

26 Aspirate final PBS wash and gently add enough volume of Vectashield to cover surface of glass window.

Imaging

27 Image samples, for example, with a Nikon Eclipse Ti Microscope with a 100X objective (Apochromat, 1.49-N.A. oil immersion) and an UltraView Vox spinning disk confocal system (PerkinElmer). Collect Z-stacks at 0.15 nm/step with Volocity acquisition software (PerkinElmer).