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β -Oxidation measurements in primary neurons V.1

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Beta Oxidation



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Protocol status: Working

We use this protocol and it's working

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Abstract

In this protocol, we assess β -oxidation in primary hippocampal neurons derived from mice using Etomoxir, an irreversible inhibitor of carnitine palmitoyltransferase 1a (CPT-1a). Etomoxir blocks fatty acid oxidation (FAO) by targeting CPT-1a, thereby inhibiting palmitate-driven β -oxidation. This is followed by a mitochondrial stress test to evaluate metabolic changes.

Guidelines

The protocol needs prior approval by the users' Institutional Animal Care and Use Committee (IACUC) or equivalent ethics committee.

Materials

Equipment

- 1) Seahorse XFe96 Analyzer.
- 2) Incubator without CO₂.
- 3) High-Content Imaging Logos Bio CELENA X High-Content Imaging system

Reagents

- 1) Seahorse XFe96 cell culture plates and corresponding sensor cartridges (Agilent Technologies, Cat. no. 103798-100).
- 2) XF Calibrant (Agilent Technologies, Cat. No. 100840-000). Store at room temperature.
- 4) Seahorse XF Cell Mito Stress Test Kit (Agilent Technologies, Cat. No. 103010-100).
- 5) XF assay media DMEM without phenol red, sodium bicarbonate, and sodium pyruvate (Agilent Technologies, Cat. No. 103575-100).
- 6) Seahorse XF 1.0 M glucose solution (Agilent Technologies, Cat. No. 103577-100). Store at 4°C.
- 7) Seahorse XF 100 mM pyruvate solution (Agilent Technologies, Cat. No. 103578-100). Store at 4°C.
- 8) Seahorse XF 200 mM glutamine solution (Agilent Technologies, Cat. No. 103579-100). Store at -20°C.
- 9) Paraformaldehyde 16% Aqueous Solution EM Grade (Electron Microscopy Science, Cat. No. 5710)
- 9) DAPI Nucleic Acid Stains (Thermo Fisher Scientific, Cat. No. D21490).
- 10) Poly-L-lysine (Sigma-Aldrich, Cat. No. P2636).
- 11) Neurobasal medium (Gibco-Thermo Fisher, Cat. No. 21103-049).
- 12) penicillin (100 U ml⁻¹)–streptomycin (100 µg ml⁻¹) (penicillin and streptomycin 10,000 U ml⁻¹ Invitrogen-Thermo Fisher, Cat. No. 15140-122).
- 13) GlutaMAX supplement (GlutaMax 100× Gibco-Thermo Fisher, Cat. No. 35050-061).
- 14) Fetal Bovine Serum (Gibco, Cat. No. 26140-079).
- 15) B27 supplement, serum-free, 50× (Gibco-Thermo Fisher, Cat. No. 17504-044).
- 16) Cytosine β-D-arabinofuranoside (Ara-C) (Sigma-Aldrich, Cat. No. C1768).
- 17) Etomxier (MedChemExpress, Cat. No.: HY-50202)

Software

- 1) Seahorse Wave Desktop software v.2.6.1.56
- 2) Seahorseanalytics.
- 3) ImageJ

β-Oxidation measurements in primary neurons

1 Primary Neuron Culture Protocol

- 1.1 Sacrifice pregnant wild-type (C57BL/6J) mice at embryonic day 16 (E16) via CO₂ asphyxiation followed by cervical dislocation.
- 1.2 Perform hysterectomy to isolate E16 embryos.
- 1.3 Dissect hippocampi (or cortices) from embryonic brains under a dissection microscope.
- 1.4 Transfer dissected tissues into a 1.5 mL Eppendorf tube containing HBSS without Ca²⁺ and Mg²⁺.
- 1.5 Add 2.5% trypsin and incubate for 10 minutes at 37°C.
- 1.6 Stop digestion by adding horse serum and 1% DNase I, incubating for an additional 10 minutes at 37°C.
- 1.7 Gently triturate the digested tissue using a P1000 pipette until no visible clumps remain.
- 1.8 Centrifuge the cell suspension at 120 × g for 7 minutes at room temperature.
- 1.9 Resuspend the pellet in Neurobasal medium supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin, 1× GlutaMAX, and 10% FBS.
- 1.10 Plate neurons at a density of 25,000 cells per well in XFe96 cell culture microplates pre-coated overnight with 1 mg/mL poly-L-lysine (PLL) to ensure optimal attachment.
- 1.11 Replace culture media 4–6 hours post-plating with Neurobasal medium supplemented with: 100 U/mL penicillin, 100 µg/mL streptomycin, 1× GlutaMAX, 1× B27 supplement.
- 1.12 Change media every 2–3 days thereafter.

- 1.13 Add 4 μM Cytosine β -D-arabinofuranoside at DIV 3–4 to inhibit glial proliferation for neuronal-only cultures.
- 2 Open the Wave software.
- 3 Choose the Acute Mito stress configuration.
- 4 Design a new template for your experiment.
- 5 Define the injection strategy as follows: Port A: Etomoxir, final concentration 40 μM , Port B: Oligomycin, final concentration 2 μM , Port C: FCCP, final concentration 2.5 μM , and Port D: Rotenone/Antimycin A (Rot/Anti), final concentration 1 μM each.
- 6 Select the appropriate assay medium: Mito Stress Assay Medium: DMEM without phenol red, sodium bicarbonate, or sodium pyruvate, supplemented with 10 mM glucose, 2 mM glutamine, and 1 mM pyruvate.
- 7 Specify the cell type as primary neurons and complete all fields following the software wizard prompts to finalize the group definitions.
- 8 Generate a group list according to the assay.
- 9 In the Plate Map tab of the Seahorse Wave software, assign each experimental group to the appropriate wells based on experimental design, and designate background (blank) wells according to the cell map to ensure accurate normalization and background correction.
- 10 In the instrument protocol tab, define the injection sequence to be A, B, C, and D and the cycles (three cycles each measurement, each cycle consisted of a 1-minute mixing phase (to ensure uniform exposure of cells to reagents), a 2-minute wait phase (to allow the system to stabilize), and a 3-minute measurement phase (to monitor pH changes in real time).
- 11 Take the cartridge from the incubator and remove the purple support and place the cartridge back in the utility plate.
- 12 Distribute each intermediate solution for the glycolysis assay as follows: Etomxier (injection port A, 20 μL , 240 μM , 6X dilution), Oligomycin (injection port B, 20 μL , 14 μM ,

7X dilution), FCCP (injection port B, 20 μ L, 20 μ M, 8X dilution), and Rot/Anti (injection port C, 20 μ L, 9 μ M, 9X dilution) in each of 96 wells, each well has 100 μ L of assay media.

- 13 Equilibration by running the assay button on the screen.
- 14 Place the sensor cartridge with the utility plate on the machine tray, taking care of the cartridge orientation (the notch needs to be placed on the bottom left).
- 15 Confirm the presence of the cartridge to the software by clicking the confirmation button displayed on the screen.
- 16 Let the calibration and equilibration step run.
- 17 Wash the cells with PBS 3 times.
- 18 Permeabilize the cells with 0.1% TritonX100 for 5 minutes.
- 19 Wash the cells with PBS 3 times.
- 20 Stain cells with DAPI (1 μ g/mL) for 10 minutes at room temperature.
- 21 Wash the cells with PBS 3 times.
- 22 Imaging of the 96-well plate using the CELENA® X High Content Imaging System at 10 $\times$ magnification.
- 23 Quantify the number of cells per well using an ImageJ script for automated cell counting.
- 24 Save the assay results and export them in Excel format. Analyse the data using Seahorse Report Generators provided by Agilent for automated calculation of metabolic parameters.



Protocol references

Angelini, A., Pi, X., & Xie, L. (2022). Evaluation of long-chain fatty acid respiration in neonatal mouse cardiomyocytes using SeaHorse instrument. *STAR protocols*, 3(2), 101392.