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Measurement of Oxygen Consumption Rate (OCR) in iPSC-Derived Neurons and iPSCs Using Seahorse XFe96 Analyzer

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Ali Ghoochani[1,2,3,4], Monther Abu-Remaileh[1,2,3,4]

[1]Department of Chemical Engineering, Stanford University, Stanford, CA 94305, USA;
[2]Department of Genetics, Stanford University, Stanford, CA 94305, USA;
[3]The Institute for Chemistry, Engineering and Medicine for Human Health (Sarafan ChEM-H), Stanford University, Stanford, CA 94305, USA;
[4]Aligning Science Across Parkinson's (ASAP) Collaborative Research Network, Chevy Chase, MD, 20815, USA

asap

Ali Ghoochani
Stanford University

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We use this protocol and it's working

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Abstract

This protocol describes the procedure to measure mitochondrial oxygen consumption rate (OCR) in iPSC-derived neurons and undifferentiated iPSCs using the Seahorse XFe96 Analyzer.

Materials

-Seahorse XFe96 microplates (Agilent Technologies)
- Poly-D-Lysine (for neurons)
- rhLaminin (for iPSCs)
- Seahorse XF Base Medium (Agilent Technologies)
- DMEM/F12 (Thermo Fisher Scientific)
- Neurobasal-A (Thermo Fisher Scientific)
- MEM Non-Essential Amino Acids (Thermo Fisher Scientific)
- GlutaMAX (Thermo Fisher Scientific)
- N2 Supplement (Thermo Fisher Scientific)
- B27 Supplement with/without antioxidant (Thermo Fisher Scientific)
- NT-3 (10 ng/ml)
- BDNF (10 ng/ml)
- Mouse Laminin (1 µg/ml)
- Doxycycline hydrochloride (2 µg/ml)
- Pyruvate (1 mM)
- Glucose (20 mM)
- Oligomycin (1 µM final)
- 2,4-Dinitrophenol (DNP, 50 µM final)
- Rotenone (0.5 µM final)
- StemFlex™ Medium (Thermo Fisher Scientific)
- Seahorse XFe Report Generator
- Protein quantification reagents

Maintain iPSCs

1 Culture iPSCs in StemFlex™ Medium on rhLaminin-coated plates (2.5 µg/mL in 1x DPBS).

2 Replace medium every other day.

Initiate Pre-Differentiation (Day -3 to 0)

3 Detach iPSCs using ReLeSR™ and centrifuge to pellet.

4 Resuspend in N2 Pre-Differentiation Medium:

- Knockout DMEM/F12
- 1x MEM Non-Essential Amino Acids
- 1x N2 Supplement
- 10 ng/mL NT-3
- 10 ng/mL BDNF
- 1 µg/mL Mouse Laminin
- 10 µM Thiazovivin
- 2 µg/mL doxycycline

5 Seed 1.5 × 10^6 cells/well in rhLaminin-coated 6-well plates with 2 mL/well of medium.

Pre-Differentiation (Day -3 to 0)

6 Maintain cells without medium change.

7 On Day 0, dissociate with Accutase and centrifuge.

Neuronal Differentiation and Maturation (Days 0–21)

8 Plate 5 × 10^4 pre-differentiated cells per well into **Poly-D-Lysine-coated Seahorse XFe96 microplates.**

9 Culture in Classic Neuronal Medium (**1:1 DMEM/F12 and Neurobasal-A**) supplemented with:

- 1x MEM NEAA
- 0.5x GlutaMAX
- 0.5x N2
- 0.5x B27 with/without antioxidant (Based on the experiment)
- 10 ng/mL NT-3
- 10 ng/mL BDNF
- 1 µg/mL Mouse Laminin
- 2 µg/mL doxycycline

10 Day 7: Half-medium change, no doxycycline.

11 Day 14: Repeat half-medium change with same composition (no doxycycline).

12 Day 21: Cells ready for Seahorse assay.

Preparation for Seahorse Assay

13 Preparation of Sensor Cartridge (a day before experiment):

13.1 Hydrate the sensor cartridge:

The day prior to measuring OCR, hydrate the sensor cartridge with Seahorse XF Calibrant 200 µL /well and 400 µL PBS/ edge well and keep the sensor cartridge at 37 °C in a non-CO2 incubator overnight

Preparing the Assay Medium

14 On the day of the assay, replace the growth medium with pre-warmed assay medium (180 µL) containing Seahorse XF base medium, 1 mM pyruvate, and 20 mM glucose.

15 Incubate the microplate in a non-CO2 incubator at 37 °C for 01:00:00 to equilibrate the temperature and pH.

Preparing Modulating Agents

16 In the meantime, prepare following modulating agents (10X) in assay medium for injection:

	A	B	C	D
	Port Loading Design	**10X concentration**	**Add to Port volume**	**Final well concentration (µM)**
	Port A: Oligomycin	10 µM	20 µL	1 µM
	Port B：2,4-Dinitrophenol (DNP)	500 µM	25 µL	50 µM
	Port C：Rotenone	5 µM	27 µL	0.5 µM

16.1 Load 10X concentration of agents into port of the cartridge (From step 13)

Calibrating the Analyzer

17 Place the loaded sensor cartridge into analyzer and run the program. The calibration usually takes 00:15:00 .

Measuring the OCR

18 After calibration is done, transfer the Seahorse XFe96 cell microplate (from step 3.1) to the analyzer and press "continue"

Post-Assay Procedures

19 Protein Normalization:

19.1 After the assay, aspirate the assay medium and lyse the cells with a suitable lysis buffer and measure protein concentration using a BCA assay.

19.2 Use this data for normalizing the OCR results.

OCR Measurement in iPSCs (Undifferentiated)

20 Plate 5 × 10^4 iPSCs per well into rhLaminin-coated Seahorse XFe96 microplates in StemFlex™ Medium.

21 Incubate 48 h at 37°C, 5% CO_2.

22 **Seahorse Assay :**

22.1 follow same steps as in **Steps 13–19** above.