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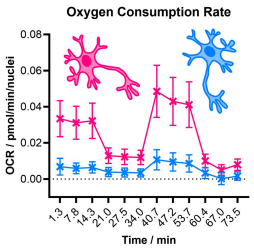
Seahorse OCR/ECAR, MitoStress Test and ATP-Rate Test for iNeurons V.1



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We use this protocol and it's working

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Abstract

Protocol for measurements of oxygen consumption rate (OCR) and extracellular acidifcation rate (ECAR) of differentiated iNeurons using the Seahorse XF96 bioanalyser. Protocol describes simultaneous conduction of Seahorse XF Cell Mito Stress Test and Seahorse XF Real-Time ATP Rate Assay through measurements made following automated sequential mitochondrial toxin additions. This protocol assumes each half of a plate has same samples/conditions with Columns1-6 being used for the Mito Stress Test and Columns7-12 for the Real-Time ATP Rate Assay.

Materials

- iNeuron hPSC Line (iNeuron hPSC Line established on WTC11 hPSC background with TET-ON system at the AAVS1 safe-harbour locus for overexpression of murine Ngn2 and dCas9-KRAB at the CLYBL safe harbour locus was a kind gift from the lab of Michael Ward)
- mTeSR Plus (STEMCELL Technologies, 100-0276)
- Ca^{2+}/Mg^{2+}-free Dulbecco's Phosphate Buffered Saline (DPBS) (Gibco, 14190169)
- 0.5M EDTA (Invitrogen, 15575020)
- Geltrex (Gibco, A1413302)
- TrypLE Express (Gibco, 12604021)
- Dulbecco's Modified Eagle Medium (DMEM) (Gibco, 11995-065)
- Heat-inactivated foetal bovine serum (FBS) (Gibco, A5256801)
- 5mM Y27632 ROCKi (MedChem, HY-10071)
- DMEM/F12 - HEPES (Gibco, 11330032)
- Neurobasal (Gibco, 21103049)
- Glutamax Supplement (Gibco, 35050061)
- Non-essential amino acids (NEAA) (Gibco, 35050061)
- N2 supplement (Gibco, 17502048)
- B27 Supplement (Gibco, 17504044)
- Insulin (~10mg/ml, Sigma, I9278)
- B-mercaptoethanol (50mM, Gibco, 31350010)
- Accutase (Sigma, A6964)
- BrainPhys (STEMCELL Technologies, 5790)
- BDNF (Peprotech, 450-02)
- NT-3 (Peprotech, 450-03)
- Doxycycline (Dox) (Sigma, D5207)
- Agilent Seahorse XFe96
- Seahorse XF96 Cell Culture Microplates (Included in Agilent, #103793-100)
- Seahorse XFe96/XF Pro Extracellular Flux Assay Kits (Included in Agilent, #103793-100)
- Seahorse XF Calibrant Solution (Included in Agilent, #103793-100)
- Seahorse XF DMEM Assay Medium without phenol red (Included in Agilent, #103680-100)
- Seahorse XF 1.0 M Glucose Solution (Included in Agilent, #103680-100)
- Seahorse XF 100 mM Pyruvate Solution (Included in Agilent, #103680-100)
- Seahorse XF 200 mM Glutamine Solution (Included in Agilent, #103680-100)
- Seahorse XF Cell Mito Stress Test Kit (Agilent, #103015-100) >> Note see below reagents for "home-made" toxin stock alternatives
- Oligomycin (Sigma O4876)
- FCCP (Sigma C2920)
- Rotenone (Sigma R8875)
- Antimycin A (Sigma A8674)
- Hoechst 33342 (Thermo Scientific, 62249)
- Opera Phenix High-Content Confocal Microscope System

Buffers/Stocks:

- Geltrex is diluted 1:100 in serum free DMEM for coating of culture surfaces
- see methods for mitochondrial toxin stock preparations.

Safety warnings

Oligomycin, Antimycin and Rotenone are mitochondrial poisons which should be handled with appropriate PPE and disposed of through safe disposal routes

Preparation of mitochondrial toxin stocks

1 Agilent recommend that mitochondrial toxins used for the mitostress test and ATP-Rate test are specifically the ones purchased from them. We have not noted any negative impacts on assay quality using "home-made" toxin stocks prepared from Sigma products with DMSO resuspension and -20 °C storage.

Appropriate dilutions when using the Agilent Seahorse XF Cell Mito Stress Test Kit and "home-made" alternatives will be described.

Home-Made Stocks
Molecular weights:
Oligomycin (Sigma O4876) = 790 MW (note is a mixture of Oligomycin A, B and C)
FCCP (Sigma C2920) = 254.17 MW
Rotenone (Sigma R8875) = 394.4 MW
Antimycin A = 531.6 MW (Sigma A8674)

Resuspension:
[M] 15 millimolar (mM) Oligomycin = 5 mg in 422 µL DMSO
[M] 50 millimolar (mM) FCCP = 10 mg in 787 µL DMSO
[M] 25 millimolar (mM) Antimycin A = 25 mg in 1881 µL DMSO
[M] 50 millimolar (mM) Rotenone = 20 mg in 1014 µL DMSO

Dilutions:
[M] 11 millimolar (mM) FCCP = 220 µL [M] 50 millimolar (mM) + 780 µL DMSO
[M] 6 millimolar (mM) Antimycin + Rotenone = 240 µL [M] 25 millimolar (mM) Antimycin + 120 µL [M] 50 millimolar (mM) Rotenone + 640 µL DMSO

Mito Stress Kit Stocks (to be made on the day of assay):
[M] 100 micromolar (µM) Oligomycin = 63nmol + 630 µL XF DMEM
[M] 100 micromolar (µM) FCCP = 72nmol + 720 µL XF DMEM
[M] 50 micromolar (µM) Rotenone+Antimycin-A = 27nmol of both + 540 µL XF DMEM

!!NOTE: MITOCHONDRIAL TOXINS SHOULD BE HANDLED WITH CARE AND ANY WASTE DISPOSED THROUGH APPROPRIATE DISPOSAL ROUTES!!

iNeuron hPSC Maintenance

2 **Maintenance of iNeuron hPSCs and iNeuron Differentiation**
Conducted as described in protocol hPSC Culture, "iNeuron Differentiation and Culture in N2B27 vs BrainPhys for Immunofluorescence and Biochemistry Assessments of Mitophagy" (https://www.protocols.io/private/E5851436F8F111EF86990A58A9FEAC02). Specifics of the seeding and culture of iNeurons for the Seahorse assay are described below in go to step #3

iNeuron Culture

3h 4m

3 **Seeding of d3 iNeurons on Seahorse XF96 plate**
3h
Before beginning make sure you have Geltrex coated all 96-wells of the Seahorse XF96 plate plates (Generally aim to do Overnight but > 03:00:00 will be fine).

3.1 Harvest d3 iNeurons by accutase as already described in "iNeuron Differentiation and Culture in N2B27 vs BrainPhys for Immunofluorescence and Biochemistry Assessments of Mitophagy".

Prepare 2×10^5 cells per ml suspension in N2B27 or BrainPhys media. 100ul of this suspension (=2×10^4 cells) will be required for every XF96-well seeded.

3.2 Remove geltrex from plates, add cells as prepared above in to all wells except the perimeter wells (only use central 6 rows x 10 columns).

Note: I have found edge effects associated with seeding neurons in XF96-well Seahorse plate format to be particularly bad. Therefore tend to only seed the internal 60-wells of a 96-well, and instead fill the perimeter wells with the same volume of media as done for the experimental wells with cells.

Note: the 4x corner wells must not have cells as these will be used as background correction wells. As with the other empty perimeter wells, just add an equal volume of media.

3.3 **d4 Onwards: Half media change**

Perform a half media change with N2B27 or BrainPhys twice a week.
I usually perform the first media change after seeding on d6 but in this case just top each XF-96-well up with additional 100 µL of culture media (200 µL final volume)

I then perform the next half media change on d9, removing 100 µL of culture media and replacing with 100 µL of fresh culture media.

Seahorse Assay

1h 45m

4 **Day Prior to Seahorse Assay: Hydrate XFe Analyser Cartridge**

As described in protocol by agilent: DAY OF LOADING CARTRIDGE XFe...

Notes on Differentiation Stage for Assay:
Collection of cells on a variety of differentiation stages might be interesting.
For assessing differences in OCRs between iNeurons cultured in N2B27 and BrainPhys, we typically assay cells on d24. Therefore this cartridge hydration step is performed on d23.

4.1 Aliquot 25 mL of Seahorse XF Calibrant in a 50ml falcon tube and place in non-CO2 37 °C incubator overnight.

4.2 Open XFe analyser cartridge in a TC hood and place the sensor cartridge upside down next to the utility plate

4.3 Add 200 µL of sterile H2O to each well of the utility plate using a multichannel

4.4 Lower the sensor cartridge onto the utility plate, submerging the sensors in the water.

4.5 Place assembled sensor cartridge and utility plate in a non-CO2 37 °C incubator overnight

Note: Verify that the incubator is properly humidified

4.6 Switch on the Seahorse XFE96 Analyser machine and set the incubator on to 37 °C overnight

4.7 Also perform a full media change for the neurons, to give 200 µL final volume.
This must be done extremely gently as the neurons are quite prone to detachment upon full media changes.

5 **Day of Seahorse Assay**

Prepare XF Assay Media:
Will need ~ 100 mL total
In a 50ml falcon (x2)
48.5 mL XF DMEM Medium (w/out Phenol Red)
500 µL Seahorse XF Glucose ([M] 1.0 Mass Percent solution) fridge
500 µL Seahorse XF Pyruvate ([M] 100 millimolar (mM) solution) fridge
500 µL Seahorse XF L-Glutamine ([M] 200 millimolar (mM) solution) freezer aliquots

5.1 Place prepared XF Assay Media in 37 °C water bath.

5.2 Remove the assembled sensor cartridge with utility plate and calibrant from the incubator non-CO2 37 °C incubator

5.3 In a TC hood place the sensor cartridge upside down next to the utility plate

5.4 Remove and discard the water from the utility plate using a multichannel

5.5 Fill each well of the utility plate with 200 µL of the pre-warmed XF Calibrant
Lower the sensor cartridge onto the utility plate, submerging the sensors in calibrant

5.6 Place assembled sensor cartridge with XF Calibrant in utility plate in a non-CO2 37 °C incubator for 01:00:00 prior to loading the injection ports of the sensor cartridge. 1h

5.7 Around 00:45:00 later remove 180 µL of cell culture media from each of the wells (including background correction. (this should leave 20 µL remaining). 45m

5.8 Add 200 µL of XF Assay media per well

Note: be very careful as neurons are very delicate at this stage.

5.9 Remove 200 µL of XF assay media from cells (again should leave 20 µL) and add 200 µL of fresh XF assay media again

5.10 Remove 200 µL of XF assay media from the first 6 columns and add 160 µL XF assay media per well (this should give 180 µL total)
These first 6 columns (1-6) will be used for the Mito Stress Test and the end 6 columns (7-12) for the Real-Time ATP Rate Assay.

5.11 Incubate the cell plate at 37 °C without CO2 for 60 minutes prior to the assay.

5.12 It should now be time to add compounds to the sensor cartridge plate injector ports (1hr after incubation with XF calibrant).

Use the pipette guides:
For A put A/D with A at top left.
For B put B/C with B at top left.
For C put B/C with C at top left.
Use multichannel with non-filtered p200 (they have slightly narrower tip end).
Place vertically until get little pressure (don't force).
Reverse pipette 20 µL and try to angle off the droplet prior to removing.
Do in order A>D so don't get any contamination from latter injections during early injections.

5.13 Into the first 6x Columns (1-6 for mito stress test) add 20ul of each of appropriate toxin:
A = [M] 10 micromolar (µM) Oligomycin
B = [M] 11 micromolar (µM) FCCP
C = [M] 6 micromolar (µM) Rotenone/Antimycin (and [M] 24 micromolar (µM) hoechst for downstream imaging of nuclei for normalisation)

See tables below for working stock preparation.

	Compound	Injection Port	Desired Final Concentration / uM	Well Volume upon addition / ul	Concentration of working stock for 20ul addition to ports / uM	Volume of Working solution required with plenty of excess / ul	Volume Stock required / ul	Volume XF media / ul	Note

	Oligomycin	A	1	200	10	1500	150	1350	
	FCCP	B	1	220	11	1500	165	1335	
	Rotenon+AntimycinA	C	0.5	240	6	1500	180	1320	also add 1.8ul of 20mM Hoechst (final dilution of 1:10000 in well)

Preparation of Injection Port Working Dilutions for MitoStress Test using MitoStress Test Kit Reagents

"Home-Made" Toxin Stocks prepared in go to step #1 :

- [M] 15 millimolar (mM) Oligomycin
- [M] 11 millimolar (mM) FCCP
- [M] 6 millimolar (mM) Rotenone+AntimycinA

	A	B	C	D	E	F	G	H	I
	Compound	Injection Port	Desired Final Concentration / uM	Well Volume upon addition / ul	Concentration of working stock for 20ul addition to ports / uM	Volume of Working solution required with plenty of excess / ul	Volume Stock required / ul	Volume XF media / ul	Note
	Oligomycin	A	1	200	10	1500	1	1499	
	FCCP	B	1	220	11	1500	1.5	1498.5	
	Rotenon+Antimycin A	C	0.5	240	6	1500	1.5	1496.7	also add 1.8ul of 20mM Hoechst

	A	B	C	D	E	F	G	H	I
									(final dilution of 1:10000 = 2uM in well)

Preparation of Injection Port Working Dilutions for MitoStress Test using "home-made" stocks

5.14 Into the last 6x Columns (7-12 for Real-Time ATP Rate Assay) add 20ul of each of appropriate toxin:

A = [M] 15 micromolar (µM) Oligomycin

B = [M] 5.5 micromolar (µM) Rotenone/AntimycinA

C = [M] 24 micromolar (µM) hoechst (for downstream imaging of nuclei for normalisation)

See tables below for working stock preparation.

	Compound	Injection Port	Desired Final Concentration / uM	Well Volume upon addition / ul	Concentration of working stock for 20ul addition to ports / uM	Volume of working solution required for 48+6 wells / ul	Volume Stock required / ul	Volume XF media / ul	Note
	Oligomycin	A	1.5	200	15	1500	225	1275	
	Rotenon+AntimycinA	B	0.5	220	5.5	1500	165	1335	
	Media	C		240		1500	0	1500	also add 1.8ul of 20mM Hoechst (final dilution of

									1:10000 in well)

Preparation of Injection Port Working Dilutions for Real-Time ATP Rate Assay using MitoStress Test Kit Reagents

"Home-Made" Toxin Stocks prepared in go to step #1 :

- [M] 15 millimolar (mM) Oligomycin
- [M] 11 millimolar (mM) FCCP
- [M] 6 millimolar (mM) Rotenone+AntimycinA

	Compound	Injection Port	Desired Final Concentration / uM	Well Volume upon addition / ul	Concentration of working stock for 20ul addition to ports / uM	Volume of working solution required for 48+6 wells / ul	Volume Stock required / ul	Volume XF media / ul	Note
	Oligomycin	A	1.5	200	15	1500	1.5	1498.5	
	Rotenon+AntimycinA	B	0.5	220	5.5	1500	1.375	1498.625	
	Media	C		240		1500	0	1498,2	also add 1.8ul of 20mM Hoechst (final dilution of 1:10000 in well)

Preparation of Injection Port Working Dilutions for Real-Time ATP Rate Assay using "home-made" stocks

5.15 Set up computer and calibrate sensors:
Open the Wave software on the Seahorse computer and select Templates.
Select the Mito Stress Test assay (not the acute injection one).
Add how many groups you have.

Check the protocol section (it should be already set up). Normally it does 3 mixes and then 3 min measure for 3 times per each step = 3 data points per drug.
Click on "Start Run".
Save file when promoted to do so.
Follow the prompts on screen
Remove lid from calibration plate + loaded cartridge and place in machine (A1 = TOP LEFT)
Click "I'm ready" when done.
The system will now start calibration of the cartridge for both ECAR and OCR sensors. (about 20min)

5.16 When prompted by the software (i.e. calibration has finished), remove 200 µL of XF assay media from the last 6 columns of cell plate (this should leave 20 µL) and add 160 µL of fresh XF assay media per well (this should give 180 µL total).

5.17 Then **remove lid** from cell plate and replace the calibration plate with the cell plate (A1 top left)

Note >> keep lid to put back on for imaging

5.18 Click "Load Cell Plate" to start equilibration of the plate at 37 °C .
When ready, the system will automatically start acquiring the baseline measurements.
When all is done, eject the plates and discard the sensor cartridge but keep the cell plate >> replace lid

Nuclei Imaging and Normalisation

6 There are a number of different strategies for normalisation. In our hands we found a good linear correlation between nuclei cell count and basal OCR.

6.1 Load the seahorse plate into an Opera Phenix high content screening microscope

6.2 Using the 20x water objective image hoechst (Ex=375nm, Em=435-515nm) across all fields of the wells.

6.3 Analyse the number of nuclei using Columbus software.
Use the nuclei count per well as factor to normalise OCR data in the Seahorse Wave software.

Protocol references

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