

# Real-time monitoring of mitochondrial and cellular bioenergetics in cultured neurons in a microplate

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## Introduction

Mitochondrial dysfunction is central to many neurodegenerative disorders and is known to be involved in psychiatric disorders as well. In neurons, mitochondria are located in axons, dendrites, growth cones and pre- and post-synaptic terminals where their functions are regulated by local signals. Until the advent of the Seahorse XF (extracellular flux) analyzer it was difficult or impossible to monitor the respiration of intact neural cells in culture without using vast quantities of material and detaching them from the plates, this in turn has hampered research aimed at detecting subtle changes in mitochondrial function. The XF24 allows respiration rate (oxygen consumption rate, OCR) and glycolysis to lactic acid (extracellular acidification rate, ECAR) to be quantified simultaneously in 20 wells in parallel using small numbers of cell in microplate. In the present study, we evaluated bioenergetics of cultured cortical neurons using XF24 Analyzer. Primary neurons isolated from E16 murine embryonic were cultured for 1 day and 7 days respectively. Bioenergetics of the cultured neurons under various conditions was monitored.

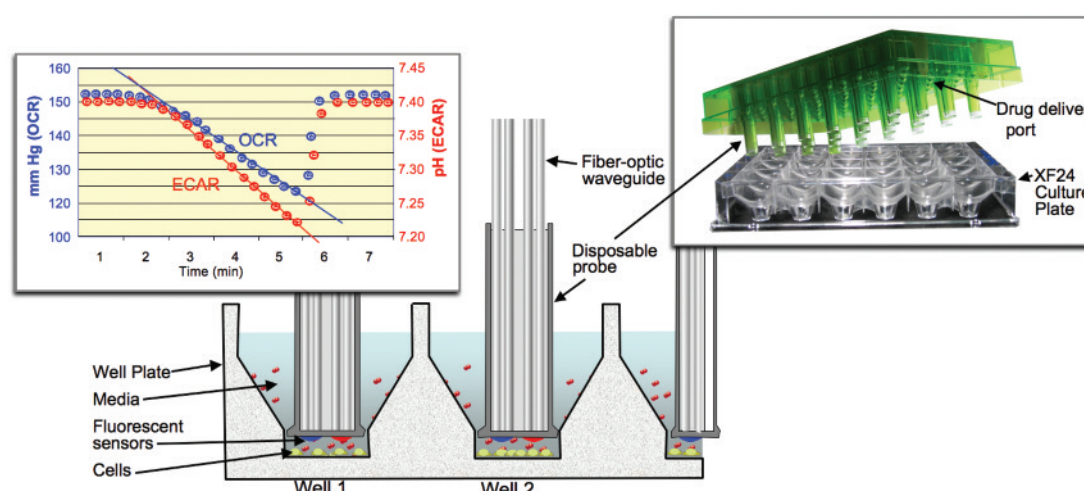
An important criterion of neuronal survivability is the ability of the cells to increase its respiration (and hence ATP production) to cope with an increased ATP demand (1, 2). By performing a single experiment with sequential addition of ATP synthase inhibitor oligomycin, mitochondrial uncoupler FCCP and respiration inhibitor cocktail myxothiazol and rotenone, we were able to answer the fundamental questions of mitochondrial function in cells, that is, the rate of mitochondrial ATP synthesis, the magnitude of the inner mitochondrial proton leak and the capacity of the mitochondrion's electron transport chain to respond to an increased cellular ATP demand. Taken together, XF24 can be a powerful tool to measure bioenergetics in cultured neurons and may aid the investigation of pathogenesis of neurological diseases as well as to discover intervening therapeutics.

## XF Bioenergetic Assay

OCR and ECAR measurements were made using the XF24 Analyzer (Seahorse Bioscience, Billerica, MA01862) as described by Wu et al.(3). XF assay medium was low buffered bicarbonate free DMEM unless specified.

Briefly, assay protocols including compound injection is preprogrammed through an excel template. Compounds of interest were loaded in drug delivery system of sensor cartridge before calibration. Culture medium was replaced with XF assay medium ~ 1 hour prior to assay.

### 1. How XF24 works



At the heart of the XF system is a method that generates a small, temporary, volume of media around the cells in a well of microplate. This small volume of approximately 7  $\mu$ l is created by lowering the disposable probe to within 200  $\mu$ m of the well plate bottom. This small measurement volume amplifies changes in analyte concentrations, i.e., oxygen and protons, allowing highly sensitive and time resolved data to be collected from a set of fluorescent sensors. Once the measurement is achieved in this small volume, the probe is lifted and the media around the cells is restored to its original condition. This non-destructive method allows multiple measurements under basal and stimulated conditions to be made with a common cell population.

The fluorescent sensors are coupled to a fiber-optic waveguide which mediates conversion of optical signals to digital data resulting in a quantitative measurement of oxygen partial pressure (a measure of mitochondrial respiration) and proton concentration (an indicator of glycolysis). The sensors reside on the ends of 24 plastic probes that comprise the XF sensor cartridge. The sensor cartridge also contains an integrated drug delivery system that stores and injects up to 4 compounds per well during an experiment. The XF assay is assembled by fitting the cartridge over the 24-well cell culture microplate and placing it into the analyzer that has been preprogrammed through an excel template to execute a user-defined protocol.



## Materials and Methods

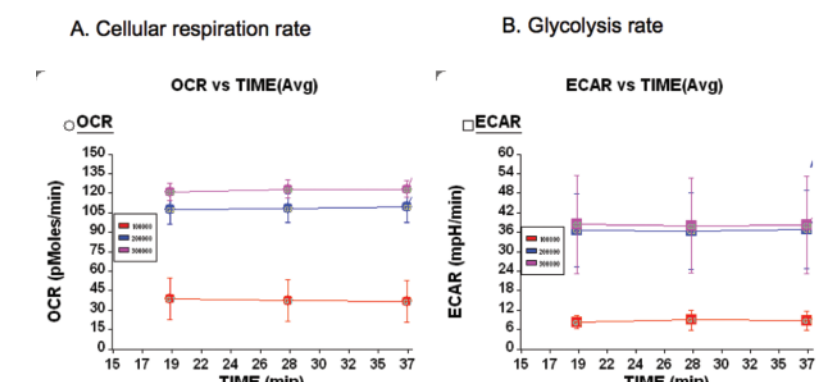
### Embryonic cortical neurons and Chemical Reagents

Primary cortical neurons were isolated from cortices of E16 murine embryos. They were seeded in Neurobasal medium (Invitrogen, Carlsbad, CA) containing 10% FBS and 100  $\mu$ g/ml penicillin-streptomycin on poly-D-lysine coated XF24 plates. After 2 hours attachment, medium was replaced with Neurobasal medium supplemented with B27 (Invitrogen) supplemented. Neurons were cultured for 1 or 7 days in 37°C/10% CO<sub>2</sub> incubators before XF assay.

Bicarbonate free DMEM, sodium pyruvate, oligomycin, FCCP, myxothiazol, rotenone and araC were obtained from Sigma (St. Louis, MO). All compounds were prepared according to the manufacturers' instructions.

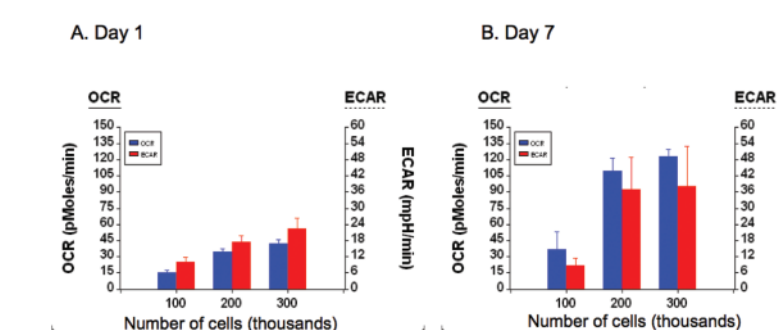
## Results

### 2. Real time monitoring of cellular respiration rate and glycolysis rate of cultured neurons.



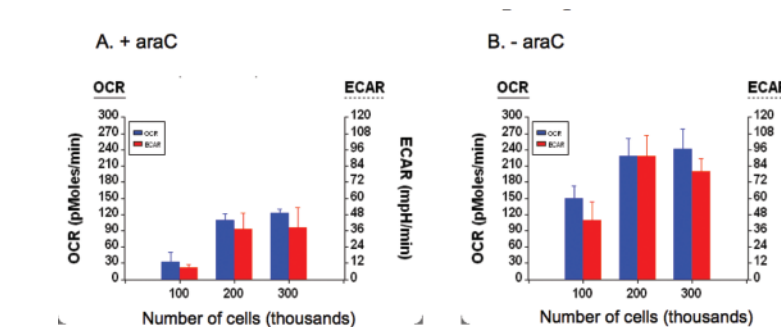
Neurons were seeded at 100, 000, 200,000 and 300,000 cell/well were cultured for 7 days before XF assay. Both OCR (A) and ECAR (B) increased as the number of cells increased per well.

### 3. Cellular respiration and glycolysis rate of day 1 and day 7 neuron culture.



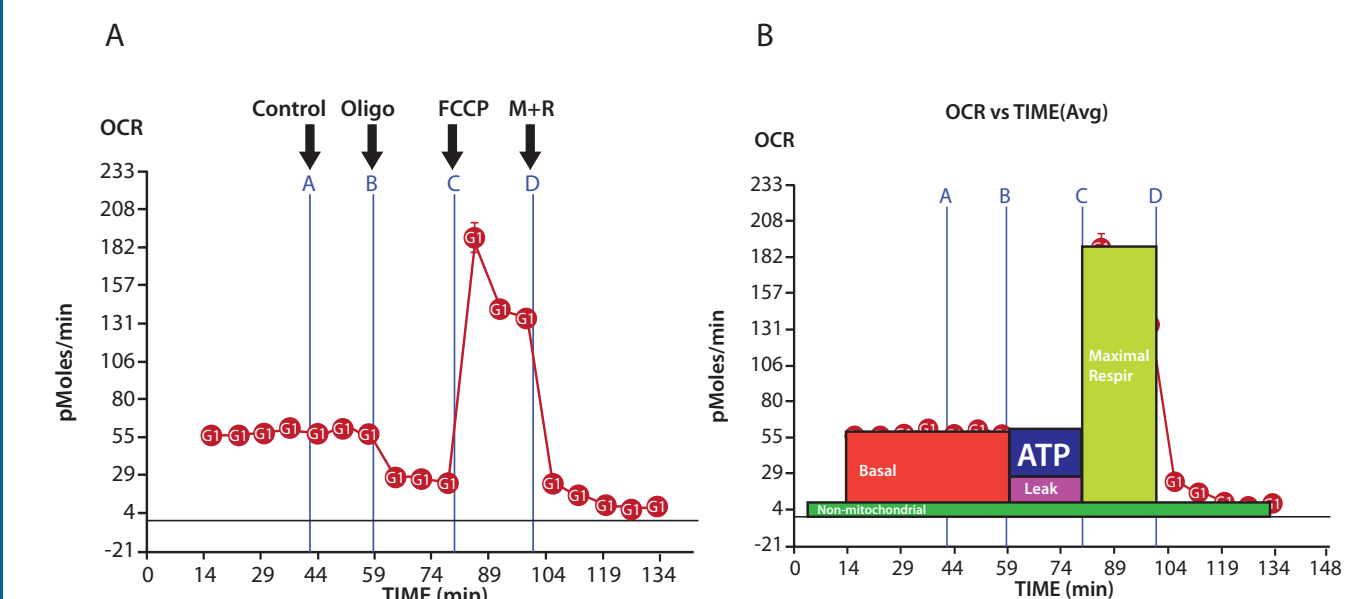
Neurons were seeded at 100, 000, 200,000 and 300,000 cell/well were cultured for 1 or 7 days before XF assay. Both OCR and ECAR were higher in day7 cultured neurons than day1 culture. The higher respiration rate correlated with significant neurite outgrowth of day7 neurons observed under microscope suggesting mitochondria biogenesis.

### 4. Metabolic rate of cultured neurons in the presence and absence of ara C



Neurons were cultured for 7 days in the presence (A) or absence (B) of 5  $\mu$ M ara C which is commonly used to control the proliferation of glial cells in cultures. The lower OCR and ECAR indicated more pure neuron population in 7 day neuronal culture.

### 5. Cellular respiration of cortical neurons: maximum respiration capacity, ATP turnover, proton leak and non-mitochondrial respiration.



After measuring basal respiration rate, compounds were added sequentially (preprogrammed in the instrument) into neurons during the assay. The effect on cellular respiration rates were measured after each compound addition (A). Additions 1, control medium; 2, 1.2  $\mu$ M oligomycin (Oligo) to inhibit the ATP synthase; 3, 4  $\mu$ M FCCP, an uncoupler to short-circuit the proton circuit and allow maximal respiration; 4, a cocktail of 1  $\mu$ M myxothiazol and 2  $\mu$ M rotenone (M+R) to inhibit electron transfer. 15 mM glucose and 10 mM sodium pyruvate is included in the assay medium. The right figure (B) shows the fundamental information that can be obtained from this single experiment.

## Summary

1. By monitoring oxygen consumption rate and extracellular acidification rate in real time, mitochondrial and cellular bioenergetics of small numbers of cultured neurons can readily be measured using XF24 Analyzer.
2. In a single experiment, fundamental functions of mitochondrial in cells have been measured by monitoring respiration rate alterations by mitochondrial perturbation agents. These include the rate of ATP synthesis, the magnitude of mitochondrial leak, and the capacity of the mitochondrion's electron transport chain to respond to an increased cellular ATP demand.

## References

1. Nicholls DG, Johnson-Cadwell L, Vesce S, Jakabsons M, Yadava N (2007) Bioenergetics of mitochondria in cultured neurons and their role in glutamate excitotoxicity. *J Neurosci Res.* 85:3206-12.
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