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PERFORMING A MITOCHONDRIAL STRESS TEST ON THE CYRIS® ANALYSIS PLATFORM

ABSTRACT

The following exemplary protocol describes the preparation, run, and results of a so-called “mitochondrial stress test” with adherent cells of the cell line L929 in the CYRIS analysis platform.

The mitochondrial stress test is utilized to create a profile of the key parameters of mitochondrial functions by measuring the oxygen consumption rate (OCR) of cells under the influence of different inhibitors and an uncoupler. Thereby the basal respiration, ATP production, proton leak, maximal respiration, reserve capacity, and non-mitochondrial respiration can be defined.

Here we show the procedure with the example of the cell line L929 and a fast preparation protocol (seeding and measuring the cells in one day). It is possible and may be necessary to adapt the protocol to other cell types, cell numbers, and culture times.

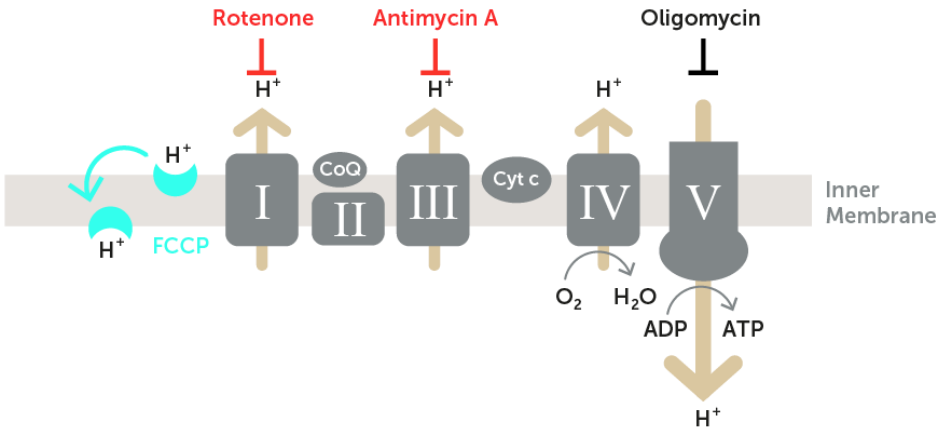
It is recommended to read the entire protocol once completely before the first test procedure to avoid problems with time management.

INTRODUCTION

The function of mitochondria, as the powerhouses of the cell, is of great interest in biological and medical research. The assessment of the current function or dysfunction of mitochondria helps to characterize cellular states and to better illustrate the effects and causes of diseases.

This is important because primary and secondary mitochondrial dysfunctions may play a key role in diseases like Alzheimer's disease, muscular dystrophy, Lou Gehrig's disease, diabetes, and cancer.

A common method of documenting the performance of mitochondria is to stress mitochondria in living cells in a targeted way so that they reveal their maximum ingestible states. This is comparable to a performance test of an athlete. By applying very specifically acting substances, different areas of the mitochondrial respiratory chain are modulated, which leads to measurable cellular reactions that can be used to assess mitochondrial states. The measured parameter is the time-resolved oxygen consumption (oxygen consumption rate, OCR) since oxygen is consumed directly in the respiratory chain.



This so-called "mitochondrial stress test" has a precise sequence in which different active ingredients are applied one after the other. After the monitoring of the baseline of cellular oxygen consumption under standard culture conditions, oligomycin is applied to the cells. It inhibits complex V (ATP-synthase) of the electron transport chain, which utilizes the proton gradient over the inner mitochondrial membrane to synthesize ATP. Since this proton gradient is now no longer degraded for ATP production, the oxygen-driven proton pumps which generate the gradient are blocked by the high gradient. As a result, the oxygen consumption drops by the amount that is normally used to produce ATP. What remains is the small amount of oxygen consumption that must be used to compensate for losses through the membrane (proton leakage) and non-mitochondrial processes in the cell which consume oxygen.

The next substance applied (carbonyl cyanide-p-trifluoromethoxyphenylhydrazone, FCCP) makes the inner mitochondrial membrane, which maintains the proton gradient, permeable. This causes the gradient to collapse. The cell now tries to rebuild the gradient using the full potential of the oxygen-driven proton pumps (maximum oxygen consumption) to produce ATP. The oxygen consumption that now occurs reflects the maximum possible respiration. The difference between basal respiration and maximum respiration is called spare capacity.

The third addition is a mixture of rotenone and antimycin A which blocks complexes I and III and fully stops the electron transport chain. As a result, no more oxygen is used for pumping protons and the rate of cellular oxygen consumption drops to a minimum, which indicates non-mitochondrial oxygen consumption. The recorded profile of the course of the oxygen consumption rates reflects the mitochondrial performance of the measured cells. It is a good basis to compare different cell types or cells in different states.

ASSAY WORKFLOW

In preparation for the assay, the cells to be analyzed are seeded into the sterile sensor plate and cultured for 2–6 hours (depending on the cell type) under standard cell culture conditions. During this time, the CYRIS® analysis platform is set up for the assay. For this purpose, the various deep-well plate (DWP) storage vessels are prepared with measurement media and appropriate concentrations of active agents and acclimatized in the CYRIS® climate chamber. The pipetting robot is equipped with sterile tips. After the cells have adhered sufficiently, the culture medium on the cells is replaced by fresh measurement medium, the special fluidic lid is closed, and the sensor plate is inserted into the platform. Once the corresponding sequence program starts, the mitochondrial stress test is performed automatically and the acquired data is displayed in real time.

MATERIAL AND METHODS

Cell culture

L929 mouse fibroblasts were maintained in DMEM high glucose supplemented with 5% FCS (culture medium). On the day of assay performance, the cells were detached and counted. A sample of 150 µl containing 120,000 cells (800,000 cells/ml) was seeded out into every well of the sterile sensor plate, except media control wells. Control wells were only filled with 150 µl of culture media. The sensor plate was left resting in the workbench for 20 minutes to allow the cells to sediment evenly, and afterward it was transferred to the standard cell incubator (37°C, 10% CO_2, 95% humidity).

Reagents

The active substances oligomycin A, FCCP, antimycin A, and rotenone are normally stored as powders. Therefore, it is necessary to prepare stock solutions in organic solutions like DMSO and ethanol. Prepare a 1 mM stock of oligomycin in DMSO, a 1 mM stock of FCCP in DMSO, a 10 mM stock of antimycin A in ethanol, and a 10 mM stock of rotenone in DMSO. Aliquot the stocks in small amounts (100 µl) and freeze them at -20 °C. In this state, they are stable for one month.

For the preparation of the assay media, thaw the drug stock solutions: let one aliquot consisting of oligomycin A, FCCP, antimycin A, and rotenone stocks completely warm up to room temperature and mix it by gently tapping.

Preparation of DWP for 1-hour baseline measurement: Fill every well of one sterile DWP with 3 ml warm measuring medium and place directly into the CYRIS incubator at position 5 for acclimatization. Cover it for transport from the sterile workbench to the incubator.

Preparation of DWP for 40-minute “ATP-production” measurement: Mix 25 ml prewarmed measuring medium with 25 µl oligomycin A stock (1 mM) to get a final concentration of 1 µM in measuring medium. Fill wells 1, 24, and 4–21 of a sterile DWP with 1 ml of this solution. Fill wells 2, 3, 22, and 23 (control wells) with 1 ml of the pure measuring medium only and place DWP directly into the CYRIS incubator at position 6. Cover it for transport from the sterile workbench to the incubator.

Preparation of DWP for 40-minute “maximal respiration” measurement (important: different cell types vary in their response to FCCP. At higher doses, a reduced response can be observed. The FCCP dose for the maximal OCR for a particular cell type must be determined empirically in separate experiments): Mix 10 ml prewarmed measuring medium with 5 µl FCCP stock (1 mM) to get a final concentration of 0.5 µM in measuring medium. Fill wells 1, 24, and 4–9 of a sterile DWP with 1 ml of this solution. Mix 10 ml prewarmed measuring medium with 2.5 µl FCCP stock (1 mM) to get a final concentration of 0.25 µM in measuring medium. Fill wells 10–15 of the DWP with 1 ml of this solution. Mix 10 ml prewarmed measuring medium with 1.3 µl FCCP stock (1 mM) to get a final concentration of 0.13 µM in measuring medium. Fill wells 16–21 of the sterile DWP with 1 ml of this solution. Fill wells 2, 3, 22, and 23 (control wells) with 1 ml of the pure measuring medium only and place DWP directly DWP into the CYRIS® incubator at position 7. Cover it for transport from the sterile workbench to the incubator.

Preparation of DWP for 40-minute “non-mitochondrial respiration” measurement: mix 25 ml prewarmed measuring medium with 2.5 µl antimycin A stock (10 mM) and 2.5 µl rotenone stock (10 mM) to get a final concentration of 1 µM each in measuring medium. Fill wells 1, 24, and 4–21 of a sterile DWP with 1 ml of this solution. Fill wells 2, 3, 22, and 23 (control wells) with 1 ml of the pure measuring medium only and directly put the DWP into the CYRIS incubator at position 2. Cover it for transport from the sterile workbench to the incubator.

Prepare waste DWP: put an empty DWP in the CYRIS® incubator on position 3 for wasted media.

Deep-well filling in list form:

Measured function	Substance	DWP position	Volume measuring medium for mix	Volume stock solution for mix	Wells to be filled with mix	Volume of mix per well	Wells with measuring medium only
Baseline	None	5	/	/	/	3 ml	1–24
ATP production	Oligomycin	6	25 ml	25 µl	1, 24, 4–21	1 ml	2, 3, 22, 23
Maximal respiration	FCCP (3 different conc.)	7	10 ml	5 µl	1, 24, 4–9	1 ml	2, 3, 22, 23
			10 ml	2.5 µl	10–15	1 ml	/
			10 ml	1.25 µl	16–21	1 ml	/
Non-mitochondrial respiration	Antimycin & Rotenone	2	25 ml	2.5 µl + 2.5 µl	1, 24, 4–21	1 ml	2, 3, 22, 23
Waste	/	3	/	/	/	/	/

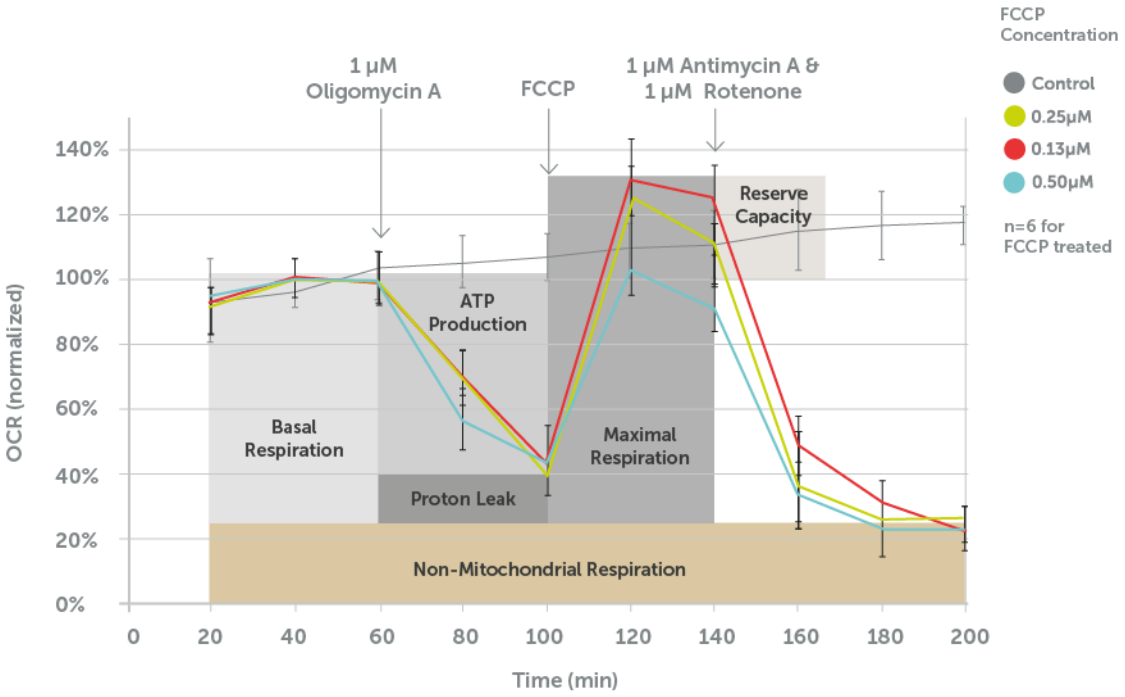
Fluidic

Equip the robot head with 24 sterile pipette tips and attach it to the robot arm in the CYRIS climate chamber.

RESULTS AND DISCUSSION

Raw measuring of OCR data can be exported directly to spreadsheet applications. This makes it possible to evaluate the data according to your own preferences. In this example, we first normalized the individual rates of each well by setting the mean of the second and third baseline rates (40 min and 60 min) as 100%.

Subsequently, we combined all rates, which were treated the same way, into a separate group (control, 0.50 µM FCCP, 0.25 µM FCCP, and 0.13 µM FCCP). The mean rates and standard deviations of these groups are plotted against time.



The experiment results show an undisturbed, correct course. All measured OCRs were evaluable and consistent. The controls show a normal course.

The different treatment groups differ in their kinetics only at the periods where different FCCP concentrations act on them. In addition, they show only small standard deviations.

The usable oxygen for ATP production is 44% of the basal respiration.

Maximum respiration appears under the influence of 0.13 µM FCCP. This shows that the optimal FCCP concentration is in the range of this value. The OCR is 131% of the basal respiration. Therefore, the cells have a reserve capacity of 31% in this case.

The non-mitochondrial oxygen consumption of the cells is 22%.

Continuous cell imaging reveals vital, sub-confluent cells that show virtually no significant changes over the measurement period. However, this was not to be expected, because during the measurement, only brief and very localized influence on the respiratory chain was taken.

CONCLUSION

This application note shows the exemplary performance of a mitochondrial stress test on the automated cell analysis platform CYRIS. We created a profile of the key parameters of mitochondrial functions by measuring the oxygen consumption rate (OCR) of L929 mouse fibroblasts under the influence of different inhibitors and uncouplers.

Different FCCP concentrations for titration show a clear dose-depend effect on maximum respiration, with a maximum OCR at 0.13 µM FCCP.

The ratio of the oxygen consumption for this case is 100% for basal respiration, 44% for ATP production, 131% for maximum respiration, and 22% for non-mitochondrial respiration.

The performed assay could demonstrate the advantages of automated execution in combination with real-time detection on the CYRIS platform as a tool for mitochondrial profiling.

With the protocol shown, it is possible to automatically generate cell-specific mitochondrial profiles of any adherent cell type. Only the FCCP concentration used has to be adjusted to the specific reaction of the different cell types.

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