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IN-VITRO MITOCHONDRIAL UNCOUPLER ASSAY PLUS WASHOUT ON THE CYRIS® ANALYSIS PLATFORM

ABSTRACT

The following exemplary protocol describes the preparation, run, and results of an in-vitro assay to investigate the reactions of L929 mouse fibroblasts to treatment and washout of the mitochondrial chain uncoupler carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP).
The CYRIS® platform is able to perform this automated assay with a deeper insight into the substance mode of action due to its ability to monitor the oxygen consumption rate (OCR), extracellular acidification rate (ECAR)and microscopic images of the cells label-free in parallel and in real time. The cells are measured for 12 hours without treatment for baseline, 12 hours under treatment with different FCCP concentrations (0 nM, 63 nM, 125 nM, 250 nM and 500 nM) and 10 hours after treatment for washout.

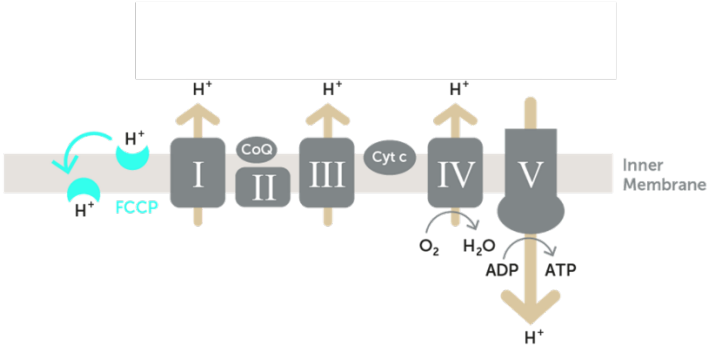
The results show a dose- and time-dependent reaction of the cells to FCCP. Correlations between different cell parameters are clearly visible.

It is possible and may be necessary to adapt the protocol to other cell lines, cell numbers, and culture times.

It is recommended to read the entire protocol once complete before

INTRODUCTION

An in-vitro assay with the uncoupler FCCP, as performed here, can be used as method to analyze the effects of drugs, which have an effect directly on the mitochondrial respiratory chain. FCCP transports protons from the membrane side with high proton density to the other side with low density. In this way, it decouples the proton gradient that the cell tries to maintain across the inner mitochondrial membrane through the respiratory chain. FCCP has a very strong effect. However, the effect is reversible and therefore can be washed out.



The site of action of FCCP in the mitochondrial respiratory chain.

The aim of this assay is to identify the effects of FCCP on L929 cells that result from certain combinations of drug concentrations and treatment times. To show these dose- and time-dependent effects, in this assay different high effective (500 nM) to low effective (63 nM) concentrations are tested and washed out over a complete experiment time of 34 hours.

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 needed deep-well plate (DWP) storage vessels for baseline and treatment measuring are prepared with measurement media and the appropriate concentrations of FCCP in measurement medium, followed by acclimatization 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. By starting the corresponding sequence program, the assay 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 where detached and counted. A sample of 150 µl containing 40,000 cells (266,666 cells/ml) was seeded out into the wells 2-6, 8-12, 14-18 and 20-24 of the sterile sensor plate. The wells 1, 7, 13 and 19 are filled only with 150 µl culture medium and work as media control wells without cells. The sensor plate should be left resting still in the workbench for 20 minutes to allow the cells to sediment evenly and after this it is transferred to the standard cell incubator (37° C, 5% CO2, 95% humidity) for at least 3 hours to attach properly.

Reagents

Prewarm sterile measuring medium with 5% FCS to 37° C in a water bath.

The active substance FCCP is normally stored as powder. Therefore, it is necessary to prepare a stock solution in organic solutions like DMSO and Ethanol. Prepare a 2 mM stock of FCCP in DMSO. Aliquot the stocks in small amounts (e.g. 50 µl) and freeze them at -20 °C. Like this they are stable for one month.

Preparation of DWP(1) for the 12-hour baseline and 10-hour washout measurement:
Fill every well of one sterile DWP with 7 ml warm measuring medium with 5% FCS and place it directly into the CYRIS® incubator at position 5 for acclimatization. Cover it for transport from the sterile workbench to the incubator.

Preparation of DWP(2) for the 12-hour treatment measurement:
For the preparation of the assay media, thaw the drug stock solution: Let one aliquot of FCCP stock solution completely warm up to room temperature and mix it by gently tapping.

Mix 16 ml prewarmed measuring medium with 5% FCS with 4 µl FCCP stock solution (2 mM) to create a final concentration of 500 nM FCCP in measuring medium. Fill wells 6, 12, 18 and 24 of the same DWP with 4 ml of this solution.

Mix 16 ml prewarmed measuring medium with 5% FCS with 2 µl FCCP stock solution (2 mM) to create a final concentration of 250 nM FCCP in measuring medium. Fill wells 5, 11, 17 and 23 of the same DWP with 4 ml of this solution.

Mix 16 ml prewarmed measuring medium with 5% FCS with 1 µl FCCP stock solution (2 mM) to create a final concentration of 125 nM FCCP in measuring medium. Fill wells 4, 10, 16 and 22 of the same DWP with 4 ml of this solution.

Mix 16 ml prewarmed measuring medium with 5% FCS with 0.5 µl FCCP stock solution (2 mM) to create a final concentration of 63 nM FCCP in measuring medium. Fill wells 3, 9, 15 and 21 of the same DWP with 4 ml of this solution.

Fill wells 1, 2, 7, 8, 13, 14, 19 and 20 (media / cell control groups) of the same DWP with 4 ml of measuring medium with 5% FCS only and place It directly into the CYRIS incubator at position 6. Cover it for transport from the sterile workbench to the incubator.

Prepare waste DWP:
Put an empty DWP into the CYRIS incubator at positions 2 and 3 for wasted media.

Deep-well filling in list form

Measured function	Substance	DWP position	Volume measuring medium for mix	Amount of 2 mM FCCP stock solution	Wells to be filled with mix	Volume of mix per well
Baseline / Washout	None	5	168 ml	/	1-24	7 ml
Treatment	FCCP 500 nM	6	16 ml	4 µl	6, 12, 18, 24	4 ml
	250 nM		16 ml	2 µl	5, 11, 17, 23	4 ml
	125 nM		16 ml	1 µl	4, 10, 16, 22	4 ml
	63 nM		16 ml	0.5 µl	3, 9, 15, 21	4 ml
	0 µM		32 ml	/	1, 2, 7, 8, 13, 14, 19, 20	4 ml
Waste	None	2, 3	/	/	/	/

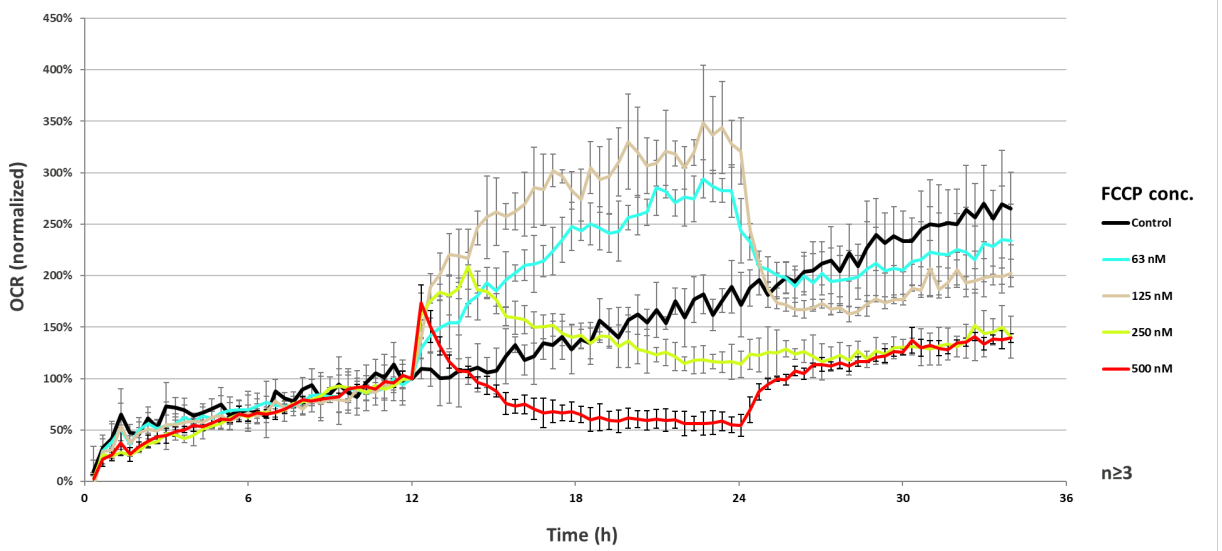
Fluidic

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

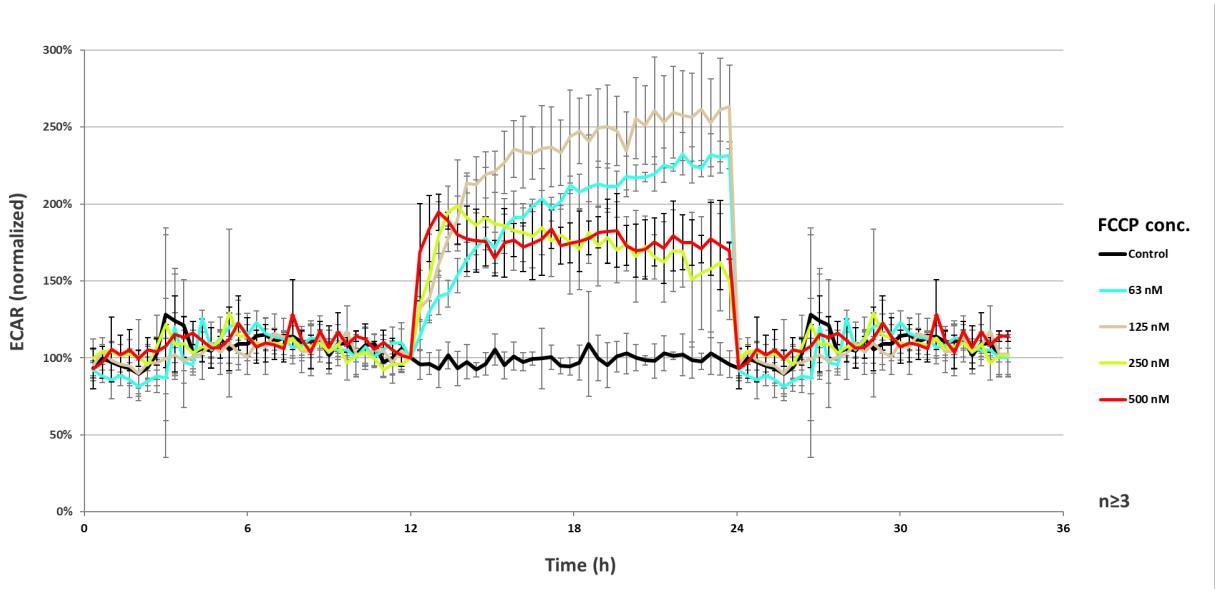
RESULTS AND DISCUSSION

The test settings included a preincubation of L929 cells without any active substance for 12 hours, a treatment of the cells with different concentrations of FCCP (0 nM, 63 nM, 125 nM, 250 nM, 500 nM) for the next 12 hours and a washout after treatment for 10 hours. After selecting and launching the appropriate program, the platform executed the test procedure autonomously. During the whole assay, the OCR and the ECAR, as well as morphology based on regular microscope images every 20 minutes, were recorded.

The result values recorded by the platform can be evaluated statistically. We normalized every well to the point just before treatment (12h). Then, we grouped wells treated with the same FCCP concentration and calculated the mean value and standard deviation. In this example, obvious outliers were dealt with in such a way that they were removed from the group formation. Such outliers can be, for example, an incorrectly treated well or incorrectly read out well. However, this means that the number of overall valid replicates falls from n=4 to n≥3.



OCR of L929 cells under the influence of different FCCP concentrations.



ECAR of L929 cells under the influence of different FCCP concentrations.

Metabolic results

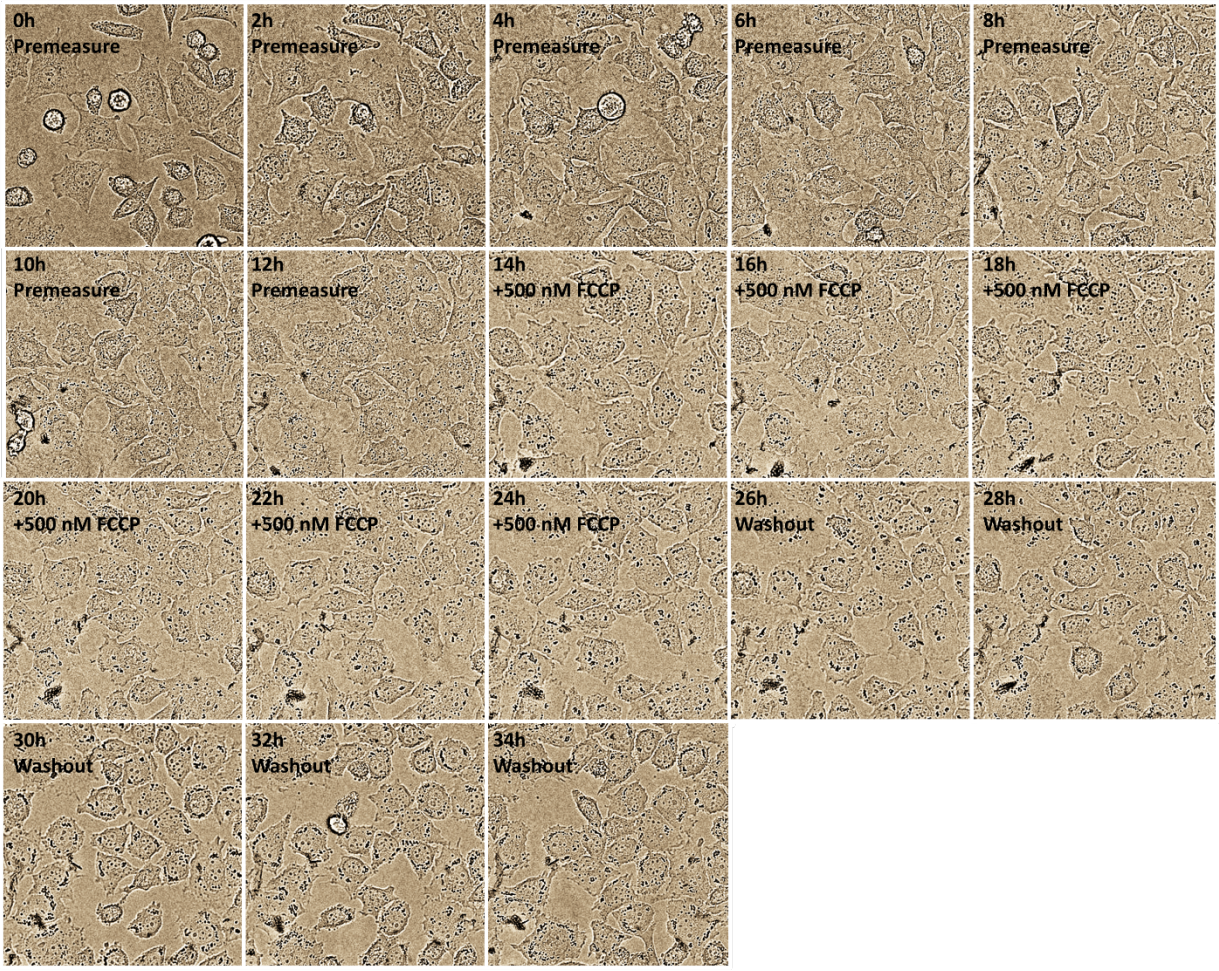
The results of the metabolic monitoring clearly show the time- and dose-dependent changes of OCR and ECAR in a quantitative and temporal profile. The OCR of the control group is growing steadily. Low FCCP concentrations (63 nM and 125 nM) are leading to an increased OCR due to the slight uncoupling of the respiratory proton gradient and the associated additional effort for the cell to stabilize this gradient. Nevertheless, proliferation continues apparently unaffected. The higher turnover in the respiratory chain and proliferation is also reflected in the ECAR, which increase steadily at low FCCP concentrations to provide reduction equivalents.

At higher FCCP concentrations (250 nM and 500 nM), the uncoupling is so strong that the respiratory chain can no longer be operated. After a short time, the cells strongly decrease their consumption of oxygen and their proliferation. ATP production over the respiratory chain is therefore decreased, too. As a compensatory behavior, the cell generates more ATP via glycolysis, which is characterized by the persistently increased acidification.

Through washout, cells return to a level of oxygen consumption equal to their density. This also shows that cells treated with higher concentrations of FCCP lag behind the control in their ability to proliferate. They continue at the OCR level, which they had directly before the start of the treatment.

Imaging results

Because of the saved time point corresponding to each image, the results of the constant microscopic imaging can be investigated in a timeline. Here we have enlarged a region of the images from well 22 and placed it one after the other in such a way, that an image every 2 hours Is shown over the entire experiment time.



Magnified imaging of the same L929 cells over 36 hours under pretreatment, 500 nM FCCP treatment and washout.

In the first 12 hours (pretreatment) a steady increase of density is shown (compare 0h to 12h). After the beginning of FCCP treatment, the cells became more flat and intracellular precipitations appear (compare 12h with 14h). Over the treatment period, the cell density does not increase anymore (compare 14h to 24h). With the washout, the amount of intracellular precipitations is slightly decreasing and the cells lose their flatness (compare 14h to 34h).

With the movie function in the ACE data viewer (not presentable here) you can see that the migration of the cells is heavily impacted by FCCP and restored when the drug is washed out.

CONCLUSIONS

This application note shows the exemplary performance of an in-vitro assay to investigate the reactions of L929 mouse fibroblasts to treatment and washout of the mitochondrial chain uncoupler FCCP. With this protocol, it is possible to show a clear dose- and time-dependent effect across multiple parameters. OCR, ECAR and microscope images are continuously recorded and show different responses to lower and higher concentrations of FCCP. These effects are related to the mitochondrial respiratory chain and to glycolysis. On the morphologic level, an increasing FCCP concentration leads to a higher suppression of migration and proliferation. Intracellular precipitations appear.

The effects of FCCP are not permanent and can be washed out. Thereby the cells return to a normal behavior very quick.

The performed assay could demonstrate the advantages of automated execution and multiparametric real-time detection on the CYRIS platform. In particular, the comparability of all values recorded in parallel from the same cells should be emphasized.

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