IN-VITRO MITOCHONDRIAL UNCOUPLER ASSAY PLUS WASHOUT WITH IMMOBILIZED SUSPENSION CELLS ON THE CYRIS® ANALYSIS PLATFORM

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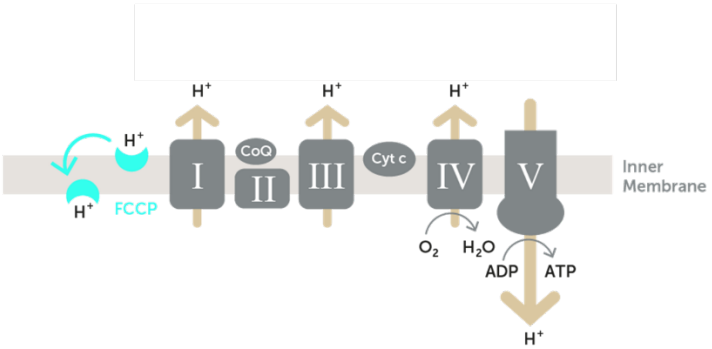
ABSTRACT

The following protocol describes the preparation, performance, and results of an in-vitro assay to investigate the response of the suspension cell line YAC-1 to treatment and washout of the mitochondrial chain uncoupler carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) using our automated cell analysis platform CYRIS®. In an effort to perform high quality measurements on suspension cells in our platform with automated fluidics and imaging, which could be a major challenge with suspension cells, we developed a protocol using *Cell-Tak Cell and Tissue Adhesive* from Corning® to immobilize the suspension cells to the surface of our sensor plates. For optimal immobilization of suspension cells, it is recommended to determine the minimum effective density of Cell-Tak by a preliminary dose-response experiment as previously described in the application note "Performing an experiment to determine the optimal amount of Cell-Tak to immobilize suspension cells for further analysis on the CYRIS platform".

The CYRIS® platform enables the automated examination of the effects of FCCP on suspension cells due to its ability to monitor oxygen consumption rate (OCR), extracellular acidification rate (ECAR) and microscopic images of the cells, label-free, in parallel and real-time. The cells are measured for 2 hours without treatment for a baseline, 6 hours under treatment with different FCCP concentrations (0 nM, 125 nM or 250 nM) and 2 hours after treatment for washout. The suspension cells were effectively immobilized to the surface of our sensor plate throughout the entire experiment. The uncoupling of the respiratory proton gradient by FCCP treatment of suspension cells was reflected by increased OCR and ECAR due to the associated additional effort of the cell to stabilize this gradient. The effect of FCCP was not permanent and could be washed

INTRODUCTION

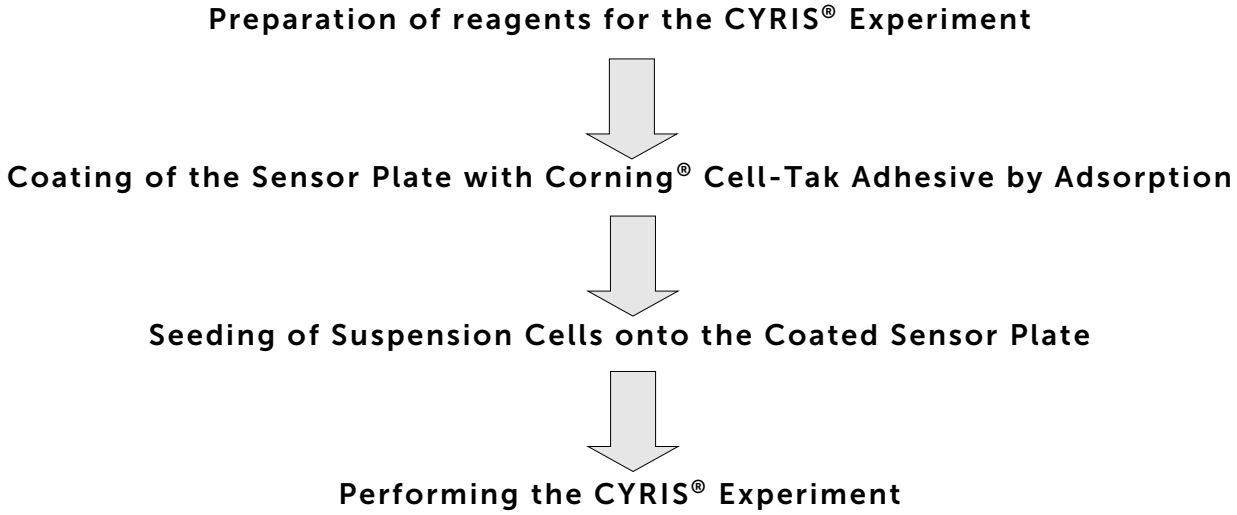
An in-vitro assay with the uncoupler FCCP can be used as a method to analyze the effects of drugs that act directly on the mitochondrial respiratory chain. FCCP transports protons from the membrane side with high proton density to the side with low density. In this way, it decouples the proton gradient that drives the ATP synthase and which cells therefore want to maintain across the inner mitochondrial membrane to produce ATP in 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.

With this assay, we aimed to investigate the effect of different FCCP concentrations on the suspension cell line YAC-1 with our automated cell analysis platform CYRIS®. For this purpose, the suspension cells were immobilized with *Cell-Tak Cell and Tissue Adhesive from Corning®*, which is a special adhesive based on proteins isolated from the marine mussel *Mytilus edulis*. We have previously developed a protocol to immobilize suspension cells on the surface of our sensor plates, which is necessary because otherwise the cells would be flushed out of the sensor well by the periodic liquid flow generated by the device and would therefore no longer be measurable. To guarantee optimal immobilization of suspension cells to the sensor plate and in order to save costs, it is recommended to determine the minimum effective density of Cell-Tak for your particular cell type by a preliminary dose-response experiment (see application note "Performing an experiment to determine the optimal amount of Cell-Tak to immobilize suspension cells for further analysis on the CYRIS platform").
Here we describe a protocol to study the effect of FCCP on the suspension cell line YAC-1. Therefore, immobilized YAC-1 cells were treated with different FCCP concentrations. The assay is set up with a 2-hour baseline measurement without any active substance, a 6-hour treatment phase, and a 2-hour washout measurement.

ASSAY WORKFLOW



MATERIAL AND METHODS

Materials Required

Reagents

- Cell-Tak Adhesive (Corning®)
- DMEM high glucose medium
- Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (Sigma-Aldrich)
- Fetal calf serum (FCS)
- INCYTON measuring medium
- Phosphate buffered saline (PBS) (pH 7.4)
- Sodium bicarbonate buffer ($NaHCO_3$) (0.1 M, pH 8)
- Sodium hydroxide solution (NaOH) (1 M)

Devices and disposable materials

- Centrifuge
- INCYTON CYRIS® flox
- INCYTON metabolic sensor plate
- Pipettes and pipette tips (1-10 µl, 2-200 µl, 50-1000 µl)
- Standard cell incubator
- Vacuum pump, flexible tube and glass Pasteur pipettes
- 15 ml sterile conical tube
- 1.5 ml micro reaction tube

Method

Reagents preparation

The data from only using the wells of half a sensor plate (12 wells) is sufficient to assess the necessary results. For this reason, all of the following information is based on only 12 wells. The other half can be used later in a different experiment.

The active substance FCCP is normally stored as powder. Therefore, it is necessary to prepare a stock solution in organic solutions like DMSO or Ethanol. Prepare a stock solution of 2 mM FCCP in DMSO, make aliquots of 50 µl and store the aliquots at -20 °C. In this state, they are stable for one month.

Prepare sterile measuring medium supplemented with 10 % FCS and prewarm it to 37 °C in a water bath.

Preparation of DWP (Pos. 5) for the 2-hour baseline measurement and 2-hour washout measurement: Fill wells 1-12 of a sterile DWP with 2 ml warm measuring medium with 10 % 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 (Pos. 6) for the 6-hour treatment measurement:
Completely thaw an aliquot of the FCCP stock solution.
Mix 12 ml of pre-warmed measuring medium containing 10 % FCS with 0.75 µl FCCP stock solution (2 mM) to create a final concentration of 125 nM FCCP in the measuring medium. Fill wells 5-8 of a sterile DWP with 3 ml of this solution.
Mix 12 ml of pre-warmed measuring medium containing 10 % FCS with 1.5 µl FCCP stock solution (2 mM) to create a final concentration of 250 nM FCCP in the measuring medium. Fill wells 9-12 of a sterile DWP with 3 ml of this solution.
Fill the wells 1-4 of a sterile DWP with 3 ml of warm measuring medium containing 10 % FCS for the untreated control group and place it directly into the CYRIS® incubator at position 6 for acclimatization. Cover it for transport from the sterile workbench to the incubator.

Prepare waste DWPs: Put empty DWPs in 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	24 ml	/	1-12	2 ml
Treatment	FCCP 250 nM	6	12 ml	1.5 µl	9-12	3 ml
	125 nM		12 ml	0.75 µl	5-8	3 ml
	0 µM		12 ml	/	1-4	3 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.

Adsorption coating procedure

As a high density of Cell-Tak will not necessarily improve the performance of your experiment but will increase the costs of an experiment, it is recommended to determine the minimum effective density of Cell-Tak by a preliminary dose-response experiment (see application note "Performing an experiment to determine the optimal amount of Cell-Tak to immobilize suspension cells for further analysis on the CYRIS platform"). In this experiment we used a Cell-Tak concentration of 0.062 µg/µl. Prepare the Cell-Tak solution as indicated below in list form. Use a 1.5 ml micro reaction tube to dilute the indicated amount of Cell-Tak into the $NaHCO_3$ buffer. Then add the indicated volume of NaOH, mix thoroughly and dispense immediately 88 µl Cell-Tak solution to the wells 1-12 of the sensor plate. Incubate the sensor plate at 37 °C for 1 h. In the meantime prepare the cell suspension as describe in the cell culture section.

Preparation of Cell-Tak solution in list form:

$NaHCO_3$ (0.1 M, pH 8)	1.026 µl
Cell-Tak stock solution (1.85 µg/µl)	Add 36 µl
NaOH (1 M)	Add 18 µl
Total volume	1080 µl
Final Cell-Tak concentration	0.062 µg/µl

After incubation aspirate the Cell-Tak solution via the side channels of the sensor plate using a glass pipette connected to a vacuum pump and wash the wells once with 100 µl PBS each. Aspirate the PBS via the side channels of the sensor plate and add immediately 150 µl of prepared cell suspension into the wells 1-12 of the sensor plate as described in the cell culture section.

Cell culture

The data from only using the wells of half a sensor plate (12 wells) is sufficient to assess the necessary results. For this reason, all of the following information is based on only 12 wells. The other half can be used later in a different experiment.

YAC-1 cells (a mouse lymphoma cell line) are maintained in DMEM high glucose supplemented with 10 % FCS (culture medium). On the day of the experiment, count the cells and dilute the cell suspension in a 15 ml sterile conical tube in culture medium to a concentration of 4 x 10^6 cells/ml. Incubate the cell suspension in a standard cell incubator (37 °C, 5 % CO_2, 95 % humidity) until further use.

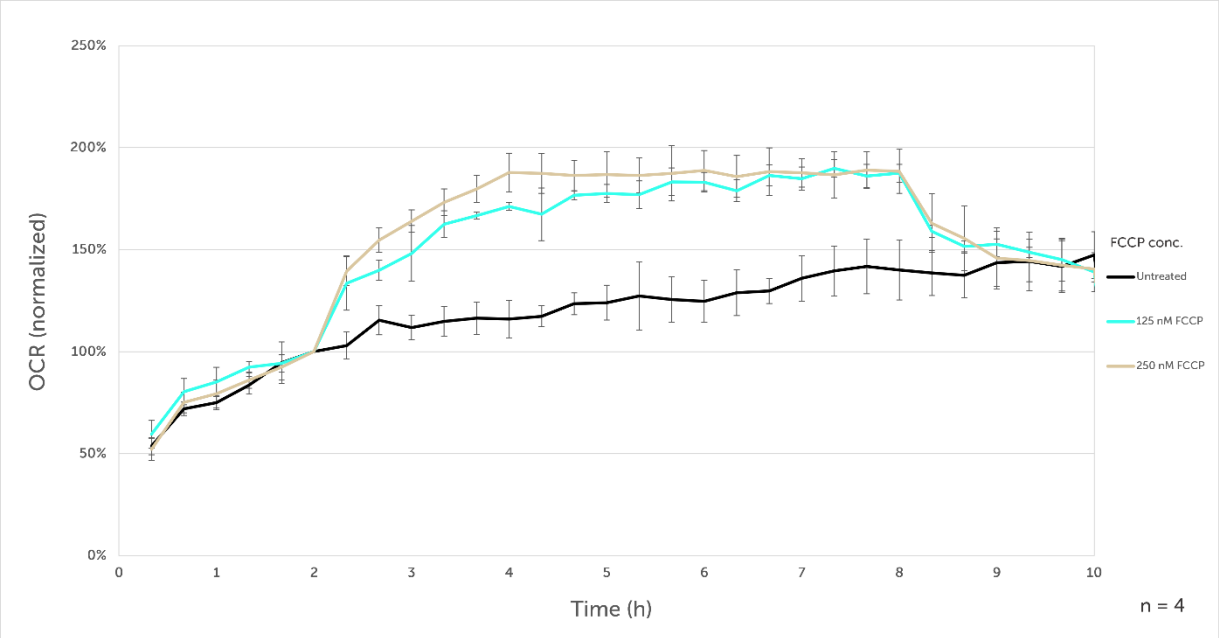
Immediately before dispensing the cell suspension into the precoated sensor plate, centrifuge the cell suspension at 300 x g for 5 min, discard the medium supernatant and resuspend the cell pallet in the necessary amount of warm measuring medium supplemented with 10 % FCS to recreate a concentration of 4 x 10^6 cells/ml. Carefully add 150 µl cell suspension containing 600,000 cells into the wells 1-12 of the precoated sensor plate. Cover the sensor plate with a standard multiwell plate lid and leave it in the sterile workbench for 20 minutes to allow the cells to distribute evenly in the wells. Transfer the sensor plate into a standard cell incubator (37 °C, 5 % CO_2, 95 % humidity) and incubate for 60 min to allow the cells to attach properly. After incubation aspirate the medium via the side channels of the sensor plate using a glass pipette connected to a vacuum pump and distribute 500 µl fresh measuring medium supplemented with 10 % FCS between the two side chambers of the culture wells 1-12. Make sure that the medium levels in each of the three chambers are the same. Cover the plate with the special fluidic lid and insert the sensor plate into the platform. Start an ACE experiment process with 2 hours pretreatment from position 5, 6 hours treatment from position 6, and 2 hours washout from position 5. Every measuring cycle should have a 100 µl medium exchange and 1 image per well every 20 minutes.

RESULTS AND DISCUSSION

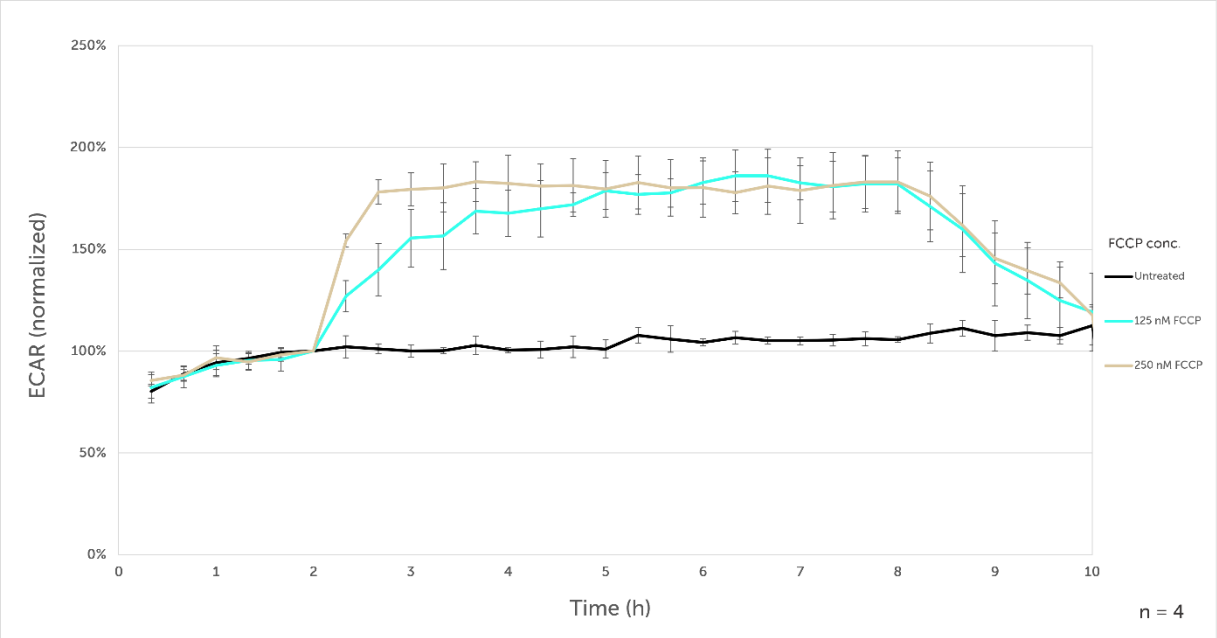
The test settings included a preincubation of YAC-1 cells without any active substance for 2 hours, a treatment period with different FCCP concentrations for the next 6 hours and a washout phase of 2 hours. After selecting and launching the appropriate program, the platform executed the test procedure autonomously. Throughout the experiment, the oxygen consumption rate and extracellular acidification rate of YAC-1 cells, as well as morphology based on microscope images, was recorded every 20 minutes.

Metabolic results

Raw measuring of OCR and ECAR data can be exported directly to spreadsheet applications. This makes it possible to analyze the data according to one's own preferences. In this example, we normalized the data of every well with YAC-1 cells (1-12) to the timepoint of treatment start (2 hours), created groups according to the different treatments and calculated the mean value and standard deviation of OCR and ECAR for each group plotted against time.



OCR of YAC-1 cells immobilized to our sensor plate and under the influence of different FCCP concentrations.

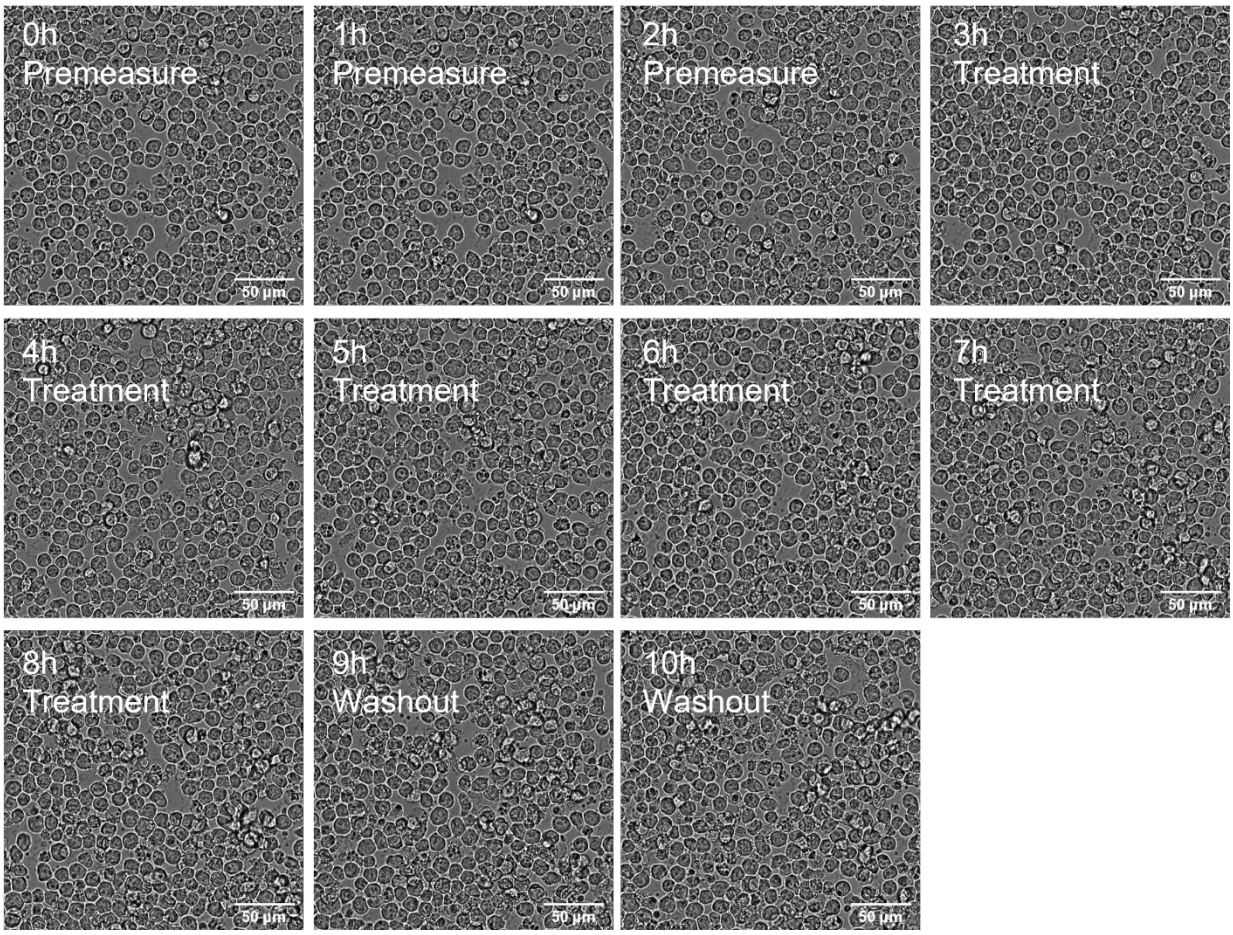


ECAR of YAC-1 cells immobilized to our sensor plate and under the influence of different FCCP concentrations.

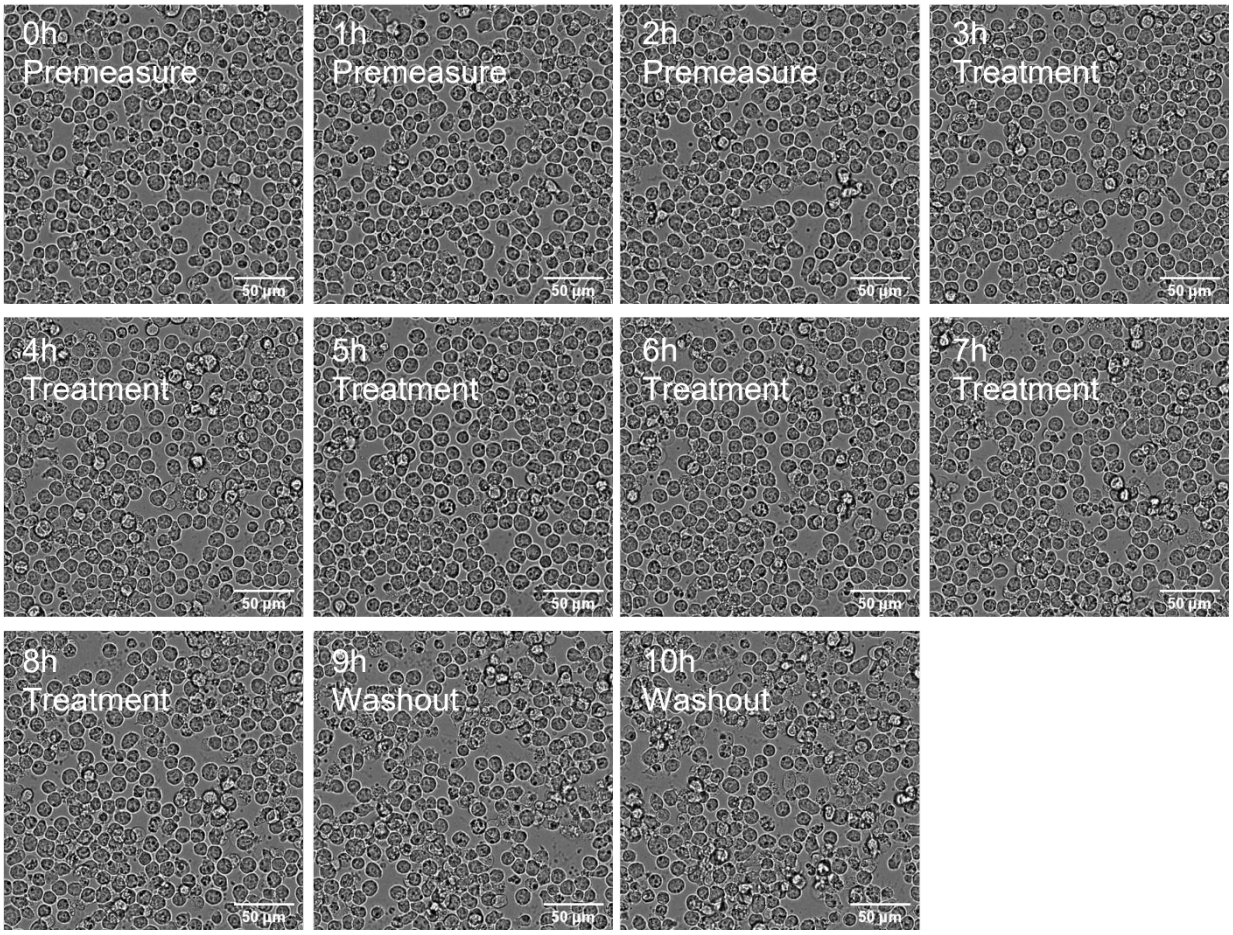
Based on the oxygen consumption rates, it could be observed that YAC-1 cells that were left untreated proliferated continuously during the entire experiment and the OCRs increased steadily. The treatment of suspension cells with 125 nM or 250 nM FCCP resulted in elevated OCR due to the slight uncoupling of the respiratory proton gradient and the associated additional effort for the cell to stabilize this gradient. The higher turnover in the respiratory chain and proliferation was also reflected in the ECAR, which increased during FCCP treatment to provide reduction equivalents. Through washout, cells return to a level of oxygen consumption equal to their density and to the untreated control group.

Imaging

Microscope images are taken periodically of all wells. This parallel imaging makes it possible to create time series and complements the metabolic data. In this case, FCCP did not cause any morphological changes of YAC-1 cells. However, the microscopic images revealed that the suspension cells were effectively immobilized to the surface of the sensor plate throughout the entire experiment. Here we have enlarged a region of the images of well 2 (untreated) and well 10 (treated with 250 nM FCCP) at different time points of the experiment.



Magnified microscopic images of untreated YAC-1 cells at different time points of the experiment.



Magnified microscopic images of YAC-1 cells treated with 250 nM FCCP at different time points of the experiment.

CONCLUSION

This application note shows the exemplary performance of an in-vitro assay to investigate the response of the suspension cell line YAC-1 to treatment and washout of the mitochondrial chain uncoupler FCCP. With this protocol, it is possible to show that suspension cells were effectively immobilized to the surface of our sensor plate throughout the entire experiment. The uncoupling of the respiratory proton gradient by FCCP treatment of suspension cells was reflected in increased OCR and ECAR due to the associated additional effort of the cell to stabilize this gradient. The effect of FCCP was not permanent and could be washed out. Thereby, the cells returned to a normal behavior very quickly. The performed assay could demonstrate that the CYRIS® platform enables the automated investigation of suspension cells under treatment and washout of an active substance such as FCCP. The effects of FCCP on suspension cells can be studied by the real-time and multiparametric detection of the platform.

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