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PERFORMING A HYPOXIA ASSAY ON THE CYRIS® ANALYSIS PLATFORM

ABSTRACT

The following exemplary protocol describes the preparation, implementation, and results of a hypoxia assay with adherent cells of the cell lines L929, HepG2 and J774A.1 in the CYRIS analysis platform.

Oxygen (O2) is essential for the entire organism, but also at the cellular level. Cellular respiration can only function through the provision of sufficient oxygen. In the event of hypoxia, cells switch from aerobic energy production to fermentation to generate energy under anaerobic conditions.

The hypoxia assay is utilized to create hypoxic conditions and track the cellular adaption to the reduced oxygen supply by measuring the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of the cells.

Here we show the procedure with different cell lines (L929, HepG2 and J447A.1). 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

Oxygen (O_2) is essential for an organism in order to produce energy and maintain a stable pH value. Cellular respiration can only function through the provision of sufficient oxygen. In this process, oxygen serves as a terminal electron acceptor in the mitochondria for energy production. In the event of oxygen deficiency, the cells switch from aerobic energy production to fermentation to generate energy under anaerobic conditions (without oxygen). If there is a decreased O_2 supply in a tissue, this state is called hypoxia. Its causes include exposure to high altitudes, anemia, stroke, heart attack or tumor growth. Therefore, it is of particular interest to study the effects of hypoxic conditions on the cellular level.

A common method to study hypoxia in living cells is to culture them under hypoxic conditions. Here we investigate three different cell lines (L929, HepG2, J774A.1) for 24h under hypoxic conditions and for an additional 24h while re-establishing normoxia (no active control, oxygen is passively leaking back into the chamber). To induce hypoxia, we reduce the oxygen content of the chamber atmosphere to 5%, compared to the regular oxygen content in our laboratory of approx. 20.9%.

In this context, the time-resolved oxygen consumption rate (OCR) and the time-resolved extracellular acidification rate (ECAR) are optimal parameters to track the cellular adaption to the reduced oxygen supply.

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 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 oxygen content of the chamber atmosphere is set to 5%. After 24h it is set back to 20.9% (unregulated). The monitoring is performed automatically and the acquired data is displayed in real time.

MATERIAL AND METHODS

Cell culture

L929 (mouse fibroblasts), HepG2 (human hepatocarcinoma-derived cells) and J774A.1 (mouse macrophages) were maintained in DMEM high glucose supplemented with 5% and 10% FCS (culture medium), respectively. On the day of the assay itself, the cells were detached and counted. A sample of 150 µl containing 50,000 cells (333,333 cells/ml) was seeded from the L929 cells into the wells 1-2, 7-8, 13-14 and 19-20, from HepG2 cells into the wells 3-4, 9-10, 15-16 and 21-22 and from J774A.1 cell into the wells 5-6, 11-12, 17-18 and 23-24 of the sterile sensor plate. The sensor plate was left resting in the workbench for 20 minutes to allow the cells to sediment evenly, and

afterwards it was transferred to the standard cell incubator (37°C, 10% CO2, 95% humidity) for at least five hours to attach properly. It is possible and may be necessary to adapt the culture time to other cell types, e.g., for fibroblasts three hours are sufficient for the cells to attach.

Reagents

Prewarm sterile measuring medium with 5% and 10% FCS to 37°C in a water bath.

Preparation of DWP (1) for 24 hours baseline measurement during hypoxia: Fill every well of one sterile DWP with 8 ml warm measuring medium directly with 5% and 10% FCS and put it 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 24 hours baseline measurement during normoxia: Fill every well of one sterile DWP with 8 ml warm measuring medium directly with 5% and 10% FCS and put it into the CYRIS incubator at position 5 for acclimatization. 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 and 2 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
Baseline during hypoxia	None	5	/	/	1-24	8 ml
Baseline during normoxia	None	6	/	/	1-24	8 ml
Waste	/	3	/	/	/	/
Waste	/	2	/	/	/	/

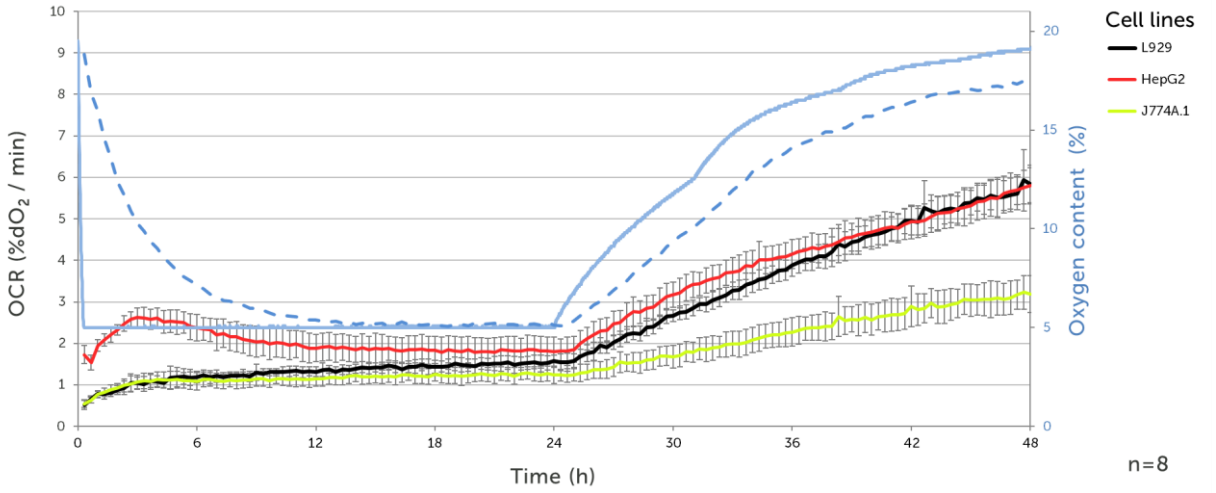
Fluidic

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

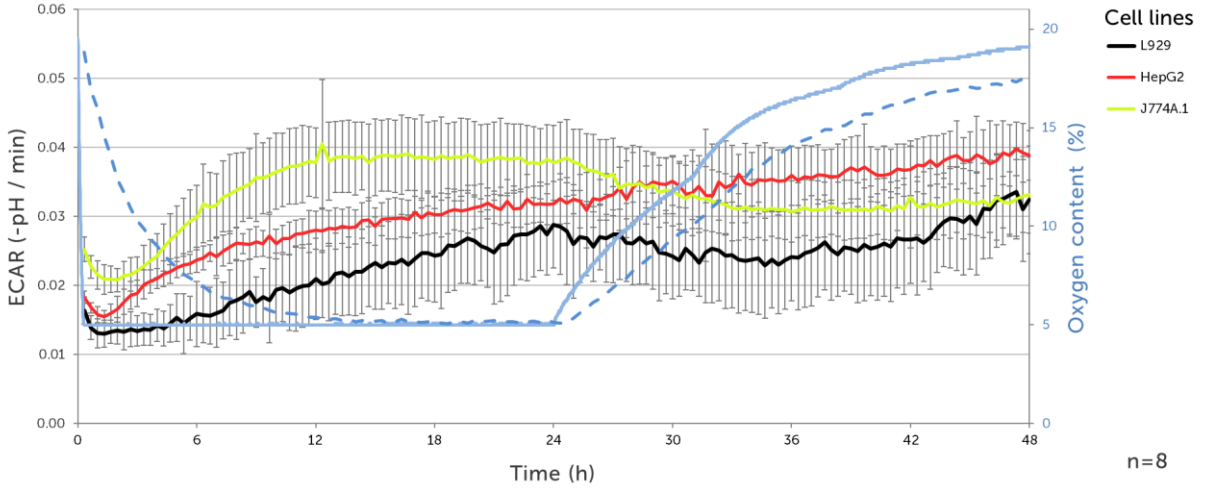
RESULTS AND DISCUSSION

Oxygen consumption and extracellular acidification

Raw OCR and ECAR 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 combined all OC or ECA rates of the individual cell lines into a separate group (L929, HepG2, J774A.1). The mean rates and standard deviations of these groups are plotted against time. Additionally, we plotted the measured oxygen content of the chamber atmosphere (blue full line) and the approximated oxygen content of the medium directly at the cells, derived from the raw data (blue dashed line). It should be noted that, due to the slow physical transfer of oxygen between a liquid (medium) and the gas phase (chamber atmosphere), deoxygenation and reoxygenation takes significantly longer in the medium than in the chamber atmosphere. Furthermore, the passive re-establishment of normoxia is not fully completed within the hours 24 to 48, which is due to the good gas tightness of the chamber and the associated low reflux of oxygen.



OCR of different cell lines under the influence of hypoxia and re-oxygenation.



ECAR of different cell lines under the influence of hypoxia and re-oxygenation.

Within the first 15 hours under hypoxia (atmosphere oxygen level of 5%), the oxygen concentration in the culture media also constantly dropped to 5% and the oxygen consumption of all cell lines stagnated at a low level.

Especially the HepG2 cell line is forced to a lower-than-normal level of oxygen consumption with the decreasing oxygen level in the medium.

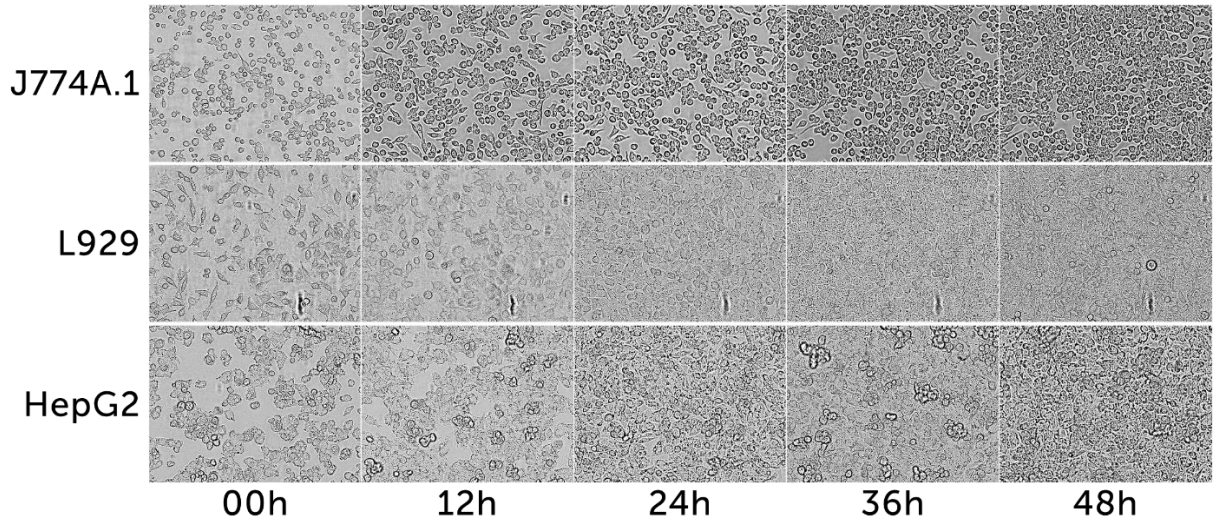
At the same time, the acidification of all cell lines increased, which indicates that the cells were forced to switch their metabolism partly from aerobic energy production to anaerobic glycolysis. Interestingly, the macrophage culture J774A.1 shows, compared to the other cell lines (L929 and HepG2), the highest acidification in combination with the lowest oxygen consumption. This may be related to the fact that macrophages are exposed to hypoxic conditions even under normal circumstances, such as skin injury, and therefore they can adjust their metabolism quickly to a reduced oxygen supply.

After 24h the oxygen level started as planned to passively re-oxygenate towards a state of normoxia.

The cells react immediately to the higher oxygen supply with a constant increase in oxygen consumption. This increase is driven by a higher consumption of the individual cell and by an increase in the number of cells. The acidification of the cell lines J774A.1 and L929 was significantly reduced with the oversupply of oxygen and only stabilized again with increasing cell numbers. In the cell line HepG2, on the other hand, acidification continued to increase at a constant rate.

Imaging

Since a microscope image was taken of all cultures at 20-minute intervals, it is possible to create time series and follow the development of the cells.



Continuous microscopic monitoring of all 3 cell lines over 48h.

The cells showed healthy growth that was not negatively affected to any noticeable degree by the hypoxic conditions (0-24h). Over the entire 48h experiment period (hypoxia and re-oxygenation), the cell numbers increased steadily.

CONCLUSION

This application note shows the exemplary performance of an automated 48h in-vitro experiment with 3 different cell lines under hypoxic (5% oxygen in atmosphere) and re-oxygenating conditions. All cell lines (L929, HepG2, J774A.1) exhibited differing behavior while dealing with the limited supply of oxygen. Especially the mouse macrophage cell line J774A.1 switched its metabolism to strongly increased acidification (Glycolysis driven) during hypoxia and reduced their acidification when the supply of oxygen was no longer limited. In contrast, HepG2 cells displayed a forced limitation of their oxygen consumption and a high amount of acidification during hypoxia, but acidification did not decrease when the oxygen limitation was removed.

Microscopic images showed a good health and steady growth of all cell lines during the 48h experimental period.

With this experiment we were able to demonstrate utilization of the CYRIS flox analysis platform for automated and multiparametric experiments under hypoxic conditions.

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