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REAL TIME ACTIVATION AND SUBSEQUENT MITOCHONDRIAL STRESS TEST OF MACROPHAGES ON THE CYRIS® ANALYSIS PLATFORM

ABSTRACT

The following exemplary protocol describes the preparation, run, and results of a real time activation and subsequent mitochondrial stress test of adherent cells of the mouse macrophage cell lines J774A.1 in the CYRIS analysis platform.

Macrophages, as an important part of the cell-mediated immunity, are activated by external stimuli. Through this, they change their physiological state and also their morphology. By targeted activation, they can be polarized into pro-inflammatory (M1) and immunoregulatory (M2) macrophages. These groups also differ in their metabolic activity.

Typical stimuli are pathogen fragments (M1) or cytokines (M2). These can be applied to a macrophage culture and thus force polarization for subsequent investigations.

Here we show the polarization of a non-activated (M0) J447A.1 mouse macrophage cell line to M1 and M2 followed by a mitochondrial stress test to determine mitochondrial activity. The complete procedure (polarization and stress test) is performed and analyzed in real time by our CYRIS analysis platform.

INTRODUCTION

Macrophages are key player of the cell-mediated immunity. Activated by external stimuli, they are able to change their physiological status and become effector cells highly adapted to the needs of the organism. These include pro-inflammatory (M1) macrophages, which are activated by pathogen fragments and phagocytize harmful biological material, and immunoregulatory (M2) macrophages, which are activated by cytokines and suppress inflammatory processes in wound healing (Galván-Peña S and O'Neill LAJ 2014; Viola A et al. 2019).

To study the physiology of macrophages it is possible to trigger polarization by targeted activation. This change to a different phenotype takes about 6-12 hours in a monolayer culture and is characterized by various genetic, metabolic and, in the case of M1 macrophages, morphological changes. As long as they are polarized, M1 and M2 can now be studied for their specific properties.

Here we show the polarization of a non-activated (M0) J447A.1 mouse macrophage cell line to M1 and M2 followed by a mitochondrial stress test to determine mitochondrial activity. The complete procedure (polarization and stress test) is performed and analyzed in real time by our CYRIS analysis platform. Thereby we record the time-resolved oxygen consumption rate (OCR) and the time-resolved extracellular acidification rate (ECAR) as well as microscopic imaging.

The application of 100 ng/ml bacterial Lipopolysacharide (LPS) for M1 polarization and 20 ng/ml recombinant murine Interleukine 4 (rmIL-4) for M2 polarization over 24h performed by the fluidic robot serves hereby as activation. Immediately afterwards, a standard mitochondrial stress test is performed automatically by the platform to evaluate the mitochondrial capacity of the polarized classes. For this, 0.5 µM FCCP was previously titrated out as the optimal amount of uncoupler.

In order to obtain an independent assessment for successful polarization, in a parallel independent experiment, cells were polarized according to the same scheme, but then not subjected to an mitochondrial stress test, but the mRNA was isolated directly from the sensor plate and analyzed by qPCR for the gene expression of the two polarization markers nitric oxide synthase (M1) and arginase 1 (M2) (Speidel JD et al. 2021).

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

ASSAY WORKFLOW

In preparation for the assay, the cells of a vital cellular culture of J447A.1 macrophages are seeded into a sterile sensor plate and cultured for 5 hours 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 supplemented with substances for polarization and the mitochondrial stress test. They are 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 polarization procedure and the mitochondrial stress test is performed and monitored automatically, and the acquired data is displayed in real time. Thereby, the measuring media supplemented with LPS and IL-4 for polarization are pipetted in a closed loop (storage deepwell=waste deepwell) in order to keep the consumption of expensive media supplements low. In the relatively short period of 24 hours and with half the usual amount of medium as buffer, this procedure has no negative effect on the cells. Used supplemented measuring media from the mitochondrial stress test are discarded as normal into a waste deepwell, since only very small amounts are required due to the short measurement time per substance (36 min).

MATERIAL AND METHODS

Cell culture

Maintain J774A.1 (mouse macrophages) in DMEM high glucose supplemented with 10% FCS (culture medium). On the day of assay performance, detach and count the cells. Seed out a sample in 150 µl culture medium containing 40,000 cells (266,666 cells/ml) into the wells (7-24) of a sterile sensor plate. Well 1-6 can be used to culture a different control cell line if necessary. Let the sensor plate rest in the workbench for 20 minutes to allow the cells to sediment evenly and then transfer the cells in the sensor plate into a standard cell incubator (37° C, 5% CO_2, 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.

Here we seed out an identical sensor plate with the same number of cells and polarize it in the same way. Subsequently, the mRNA of the cells of all wells is isolated separately and examined by qPCR for increased gene expression of the associated marker genes.

Reagents for macrophage polarization

Prepare a stock solution of 0.1% bovine serum albumin (BSA) in PBS. Dissolve 10 mg BSA (Sigma-Aldrich) in 10 ml PBS, make aliquots of 1 ml and store the aliquots at -20°C.
Prepare a rmIL-4 stock solution with a concentration of 20 ng/µl. Dissolve 10 µg rmIL-4 (Immunotools) in 500 µl PBS with 0.1% BSA and make aliquots of 10 µl. Store the aliquots at -20°C.

Prepare a LPS stock solution with a concentration of 1 mg/ml. Dissolve 1 mg LPS (Sigma-Aldrich) in 1 ml PBS, make aliquots of 50 µl and store the aliquots at -20°C.

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

Preparation of DWP (1) for 24 hours closed loop macrophage polarization: Prepare measuring medium with 10% FCS containing 100 ng/ml LPS for M1 polarization. Thaw one aliquot of the LPS stock solution and prepare a predilution containing 100 ng/µl LPS by diluting 3 µl LPS stock solution in 27 µl PBS. Dilute 25 µl of the LPS predilution in 25 ml measuring medium with 10% FCS. Fill well 13-18 of one sterile DWP with 4 ml of this LPS working solution. Prepare measuring medium with 10% FCS containing 20 ng/ml rmIL-4 for M2 polarization. Thaw three aliquots of the rmIL-4 stock solution and dilute 25 µl of the rmIL-4 stock solution in 25 ml measuring medium with 10% FCS. Fill well 19-24 of one sterile DWP with 4 ml of this rmIL-4 working solution. Fill well 7-12 of a sterile DWP with 4 ml warm measuring medium with 10% FCS for the M0 macrophages and put it into the CYRIS incubator at position 5 for acclimatization. Cover it for the transport from the sterile workbench to the incubator.

Regents for subsequent mitochondrial stress test

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 2 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 drug stock solutions: let one aliquot of oligomycin A, FCCP, antimycin A and rotenone stocks completely warm up to room temperature and mix it by gently tapping.

Preparation of DWP (2) for 36 minutes "ATP-production" measurement: mix 19 ml prewarmed measuring medium with 10% FCS with 19 µl oligomycin A stock (1 mM) to get a final concentration of 1 µM in measuring medium. Fill wells 7-24 of a sterile DWP with 1 ml of this solution and directly put the DWP into the CYRIS® incubator at position 6. Cover it for transport from the sterile workbench to the incubator.

Preparation of DWP (3) for 36 minutes "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 separated experiments). Mix 19 ml prewarmed measuring medium with 10% FCS with 4.75 µl FCCP stock (2 mM) to get a final concentration of 0.5 µM in measuring medium. Fill wells 7-24 of a sterile DWP with 1 ml of this solution and directly put the DWP into the CYRIS incubator at position 7. Cover it for transport from the sterile workbench to the incubator.

Preparation of DWP (4) for 36 minutes "non-mitochondrial respiration" measurement: mix 19 ml prewarmed measuring medium with 10% FCS with 1.9 µl antimycin A stock (10 mM) and 1.9 µl rotenone

stock (10 mM) to get a final concentration of 1 µM each in measuring medium. Fill wells 7-24 of a sterile DWP with 1 ml of this solution 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/ predilution for mix	Wells to be filled with mix	Volume of mix per well
M-Polarization	LPS & rmIL-4	5	24 ml	/	19-24	4 ml
			25 ml	25 µl	13–18	4 ml
			25 ml	25 µl	7–12	4 ml
ATP production	Oligomycin	6	19 ml	19 µl	7-24	1 ml
Maximal respiration	FCCP	7	19 ml	4.75 µl	7-24	1 ml
Non-mitochondrial respiration	Antimycin & Rotenone	2	19 ml	1.9 µl + 1.9 µl	7-24	1 ml
Waste	/	3	/	/	/	/

Fluidic

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

RNA isolation

In order to verify the macrophage polarization, here we polarize in a parallel independent experiment the cells in the sensor plate in the same manner, isolate the mRNA of the cells and analyze it by qPCR for expression of the marker genes nitric oxide synthase (M1) and arginase 1 (M2). For RNA isolation the NucleoSpin RNA Plus KIT (Macherey-Nagel) is used.

When the macrophage polarization is complete, remove the lid of the plate and aspirate the medium via the side channels using a glass pipette connected to a vacuum pump. Lysate the cells by adding 100 µl lysis buffer directly to the cells in the sensor plate and pipetting up and down. Transfer the lysate into a 0.5 ml micro tube. Repeat this step two more times. The lysates can be stored at -20°C until further use or proceed directly with RNA isolation according to the manufacturer's instructions.

To determine the RNA concentration and quality the NanoDrop 1000 spectrophotometer (Thermo Scientific) is used to measure the absorbance of the RNA at the wavelengths of 260 nm and 280 nm. The purity of the RNA solution is determined by the ratio OD260 / OD280 and should be about 2.0.

RT-PCR

The High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) is used for reverse transcription polymerase chain reaction (RT-PCR) according to the manufacturer's instructions. 100 ng of RNA were transcribed into cDNA.

RT-PCR preparation per sample:

Buffer	2 µl
dNTPs	0.8 µl
Primer	2 µl
RT	1 µl
Nuclease-free water	4.2 µ
RNA sample	100 ng in 10 µl

qPCR

In order to analyze the expression of the marker genes nitric oxide synthase (M1) and arginase 1 (M2) specific RNA transcripts is detected by qPCR using the iTaq Universal SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions and transcript-specific primers. To evaluate the relative gene expression, β-Actin is used as a reference gene.

qPCR preparation per sample and gene:

SYBR Green	12.5 µl
Forward-Primer	1 µl
Reverse-Primer	1 µl
Nuclease-free water	4.2 µ
cDNA sample	2 µl

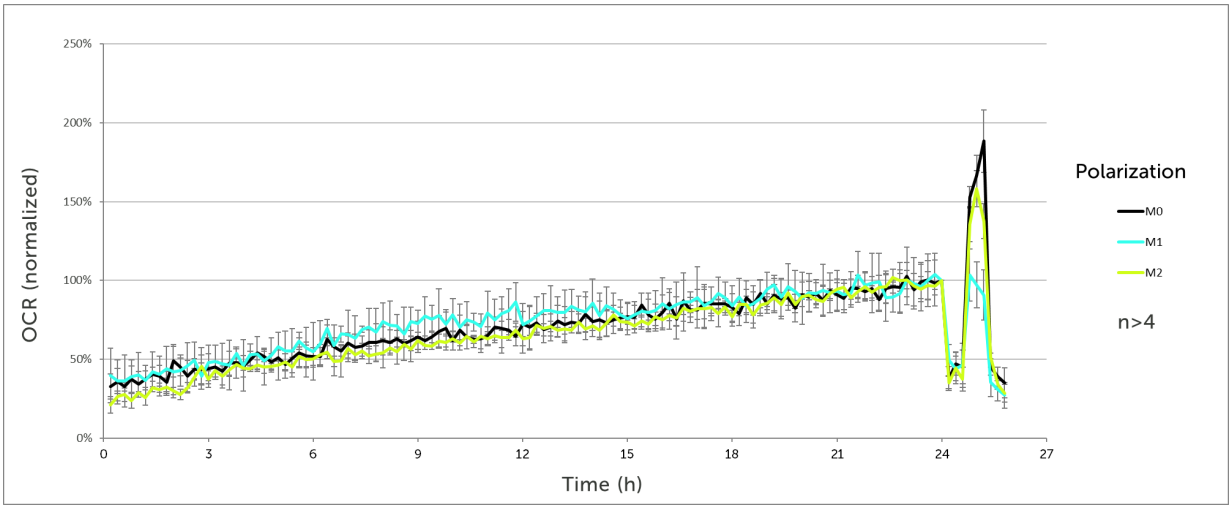
Sequence of primer:

Nitric oxide synthase (Nos2)	Forward primer (5'-3')	CCTGTGAGACCTTTGATG
	Reverse primer (5'-3')	CCTATATTGCTGTGGCTC
Arginase 1 (Arg1)	Forward primer (5'-3')	GGAACCCAGAGAGAGCATGA
	Reverse primer (5'-3')	TTTTTCCAGCAGACCAGCTT
β-Actin	Forward primer (5'-3')	TCCATCATGAAGTGTGACGT
	Reverse primer (5'-3')	GAGCAATGATCTTGATCTTCAT

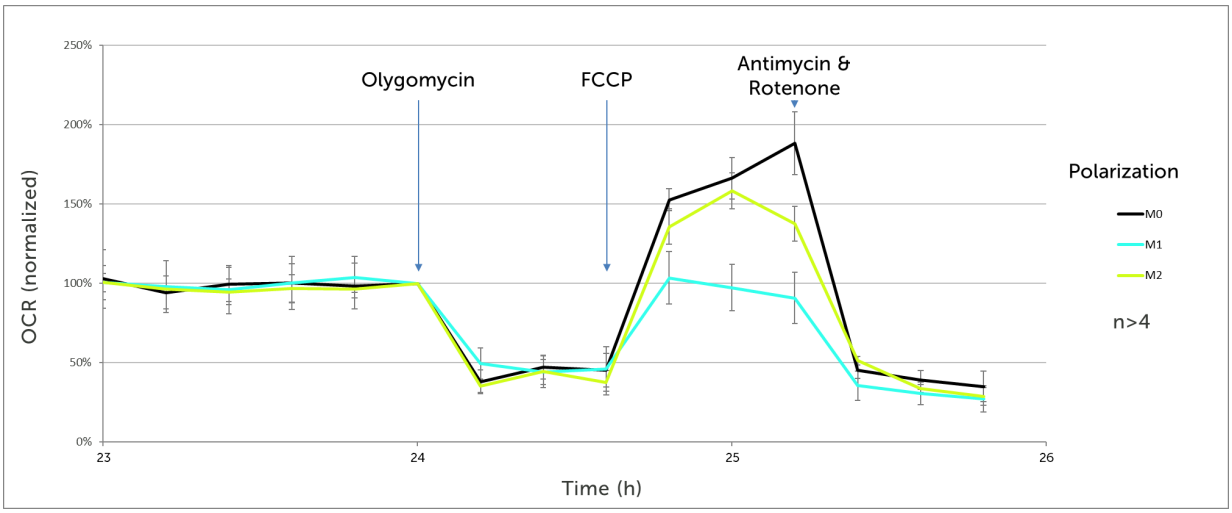
RESULTS AND DISCUSSION

Oxygen consumption rates

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 normalized all OCR rates to the timepoint directly before the mitochondrial stress test (24h) and combined the wells of different polarization types into groups with mean value and standard deviation plotted against time.



Normalized OCR of the three different polarization groups (M0, M1, M2) during polarization (0-24h) and mitochondrial stress test (24-26h).



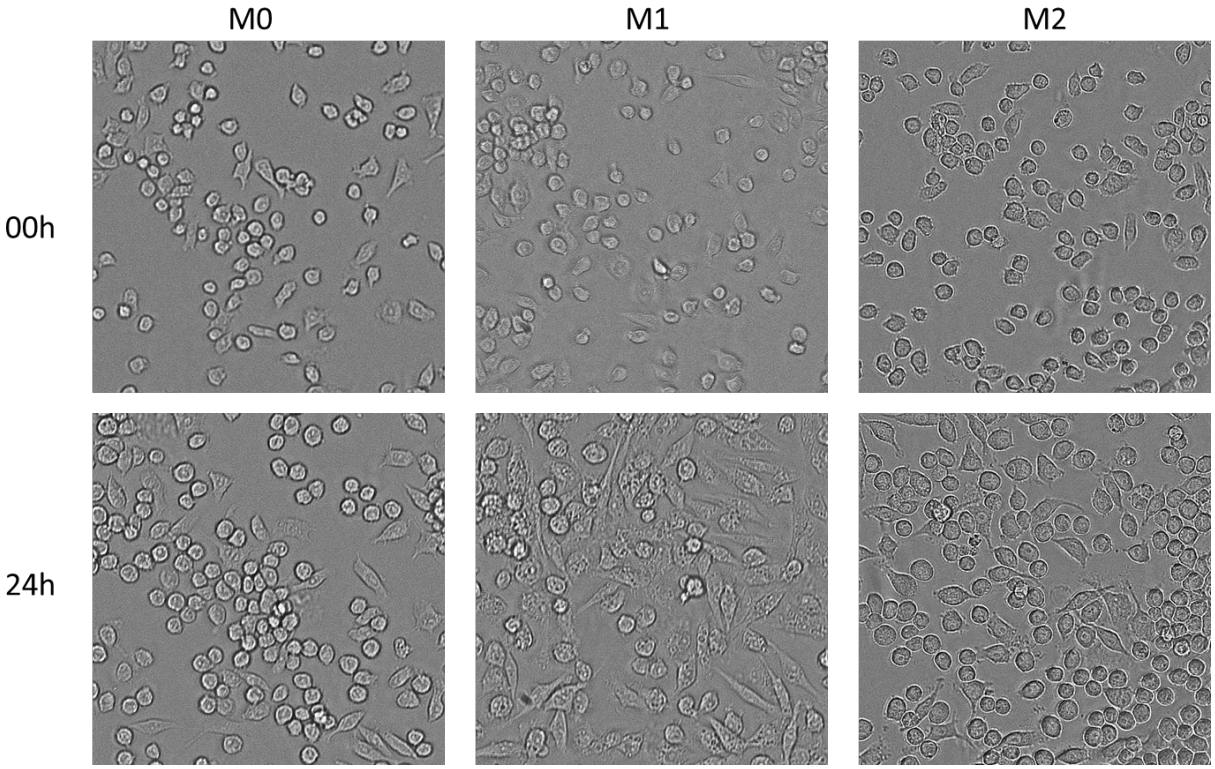
Normalized OCR of the three different polarization groups (M0, M1, M2) during mitochondrial stress test (enlarged illustration for better overview).

Within the 24 hours of polarization all polarization groups show a steady increase of OCR over time. After application of Oligomycin all groups show a similar decrease in OCR, representing the amount of oxygen used for ATP-linked respiration. Under the influence of FCCP, M1 macrophages cannot increase their oxygen consumption above basal levels, suggesting that cells were already using their maximum possible respiration before the stress test. M0 and M2 macrophages, on the other hand,

significantly increase their oxygen consumption, although M2 macrophages cannot quite maintain the level of M0 macrophages. The different behavior under FCCP influence reflects the metabolic differences of the individual polarization groups. After addition of antimycin and rotenone, the oxygen consumption of all groups drops equally to the level of non-mitochondrial oxygen consumption.

Imaging

Since a microscope image was taken periodically of all cultures, it is possible to create time series and follow the polarization of the cells.

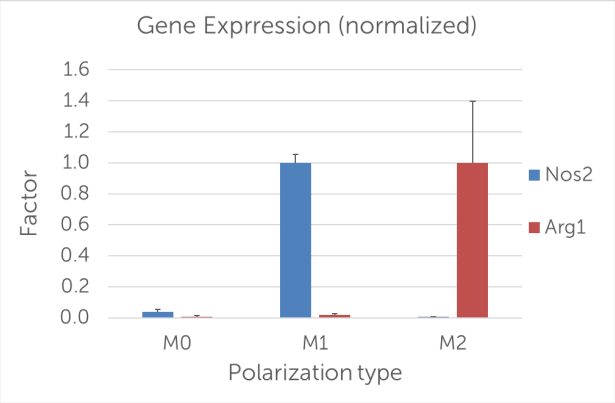


Microscopic images of the three different polarization groups (M0, M1, M2) before (0h) and after (24h) polarization in the analysis platform.

Non-polarized cells (0h) consistently show a rather small, rough, and cobblestone-like morphology with only a few elongated cells. After 24h polarization (24h), all groups increased in cell density due to proliferation. M0 macrophages show no change in morphology, while M1 macrophages show a distinctly elongated appearance and M2 macrophages appear larger and rounder in majority.
This shows that the polarization is accompanied by a traceable change in morphology.

qPCR

The results of qPCR show whether a particular marker gene is expressed more by polarized cells compared to non-polarized cells. The corresponding marker genes are nitric oxide synthase (Nos2) for M1 and arginase 1 (Arg1) for M2. To evaluate this, the respective individual measurements are grouped and normalized to the respective highest value. This reveals the ratio of the expression of the individual groups.



Normalized results of the expression of specific marker genes for M1 (Nos2) and M2 (Arg1).

The specific marker genes are clearly overexpressed in the polarized groups. This is a strong indicator that the polarization was successful.

CONCLUSION

This application note shows the polarization of a non-activated (M0) J447A.1 mouse macrophage cell line to M1 and M2 macrophages and a subsequent mitochondrial stress test, both completely performed and monitored in a single automated assay on the CYRIS analysis platform.

The assay was performed with the application of 100 ng/ml LPS for M1 polarization and 20 ng/ml rmIL-4 for M2 polarization over 24h and a standard mitochondrial stress test with 0.5 µM FCCP.

The results show a detectable polarization of the treated macrophages which is indicated by different oxygen consumptions in the mitochondrial stress test and by morphological changes of the cells after 24h.

A parallel evaluation of the polarization by qPCR shows a clear overexpression of the specific marker genes Nos2 (for M1) and Arg1 (for M2) compared to M0 macrophages.

Thus, it could be shown that the complete, complex process of polarization and subsequent testing can be performed and observed automatically on the platform.

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