Extracellular Flux Analysis to investigate the impact of NF-κB on mitochondrial respiration in colorectal carcinoma (CRC).

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ABSTRACT

The reprogramming of cell metabolism is a hallmark of cancer. NF-κB transcription factors coordinate the host defence responses to stress, injury and infection. They also play a central role in oncogenesis, at least in part by regulating cell metabolism and the adaptation to energy stress conditions in various types of cancer, such as colorectal carcinoma (CRC). Here, we describe the XF Cell Mito Stress Test methodology aimed at characterising the metabolic and bioenergetic profile of CRC cells following the silencing of the essential NF-κB subunit, RelA. This methodology may also be applied to other cancers to reveal novel core vulnerabilities of malignant cells.

Keywords: Extracellular Flux Analysis; NF-κB; Colorectal carcinoma; cancer; metabolism

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1. INTRODUCTION

Metabolic reprogramming and energy stress adaptation play an important role in tumour progression and metastatic spread *(1-4)*. As such, the metabolic profiling of cancer cells is an increasingly more valuable approach to reveal novel core vulnerabilities of cancer cells, which could lead to better anticancer treatments. The advent of high-throughput respirometry and the availability of specific mitochondrial inhibitors have recently invigorated the development of methodologies that allow the measurement of bioenergetic parameters in intact cells *(5-7)*. Among these, extracellular flux (XF) analysis has become a mainstream method to measure the bioenergetic functions of cells and tissues. In particular, the Seahorse XFe96 respirometer can measure parameters such as the flux of oxygen (O_2), the oxygen consumption rate (OCR), the flux of protons (H^+), and the extracellular acidification rate (ECAR) in the medium immediately surrounding living cells, in real time and without the need to lyse cells or isolate mitochondria. This is significant because the OCR is a key readout of oxidative phosphorylation (OXPHOS), while ECAR reflects the rate of glycolysis [Figure 1].

The Seahorse XFe96 system comprises a 96-well plate coupled with a sensor cartridge containing an equivalent number of individual sensor probes [Figure 2]. The tip of each probe is equipped with two separate polymer-embedded fluorophores that are sensitive to either O_2 or H^+. The quenching chemistry of the fluorophores in response to variations in O_2 and H^+ concentrations in the medium is responsible for the sensitivity of the probes. Both oxygen and protons fluxes are directly linked to the intensity of the fluorescence signals relative to the signals of a standard solution, and any changes in signal intensity can therefore be inferred to be proportional to changes in O_2 or H^+ concentrations, respectively. The rates of change in O_2 or H^+ concentration can be calculated by measuring the fluorescent signals over time *(8)*.

During each measurement cycle, the sensor cartridge is lowered into the wells to create a transient microchamber of defined volume [Figure 2]. Drug delivery ports located on the cartridge itself then allow the automatic injection via air pressure of specific compounds. Fiber optic bundles are inserted into the probes. Light is forced into the sensor to excite the fluorophores. The resulting emitted light is then transmitted back through the fiber optic bundle and measured by the detector. The fluorescent emission is measured for a specified period of time (measurement time). Next, the slope of the linear decline (for O_2 concentration over time) or incline (for H^+ concentration over time) provides a basis for calculating the OCR (in pmol/min) or ECAR (mpH/min) value, respectively [Figure 2] *(9)*. Gerencser et al have reported the algorithm for calculating the OCR using XF respirometers *(10)*.

The XF Cell Mito Stress Test is a comprehensive standard assay to assess key parameters of respiratory function by the measurement of the OCR *(9)*. The assay uses the following modulators of respiration to selectively target specific components of the electron transport chain (ECT): a) Oligomycin, an inhibitor of ETC complex V, also known as ATP synthase; b) a mitochondrial uncoupler, such as carbonyl cyanide-p-trifluoromethoxyphenyl-hydrazone (FCCP) or 2,4-dinitrophenol (2,4-DNP); and a combination of rotenone (an inhibitor of ETC complex I) and antimycin A (an inhibitor of ETC complex III). In brief, the first measurement is “basal OCR”, which is measured before injection of the mitochondrial inhibitors. Once the basal measurements have been recorded, the inhibitors are serially injected to measure the following bioenergetic parameters: 1] “ATP-linked respiration”; 2] “maximal respiration”; and 3] “non-mitochondrial respiration”. Specifically, by blocking ATP synthase, oligomycin allows the measurement of “ATP-linked respiration” *(11)*. An increase in ATP-linked OCR indicates a surge in ATP demand, while a decrease in this parameter indicates a reduced ATP demand, a lack of substrate availability, and/or an impairment in OXPHOS *(11)*. The mitochondrial uncoupler (FCCP or 2,4-DNP) is then used to collapse the inner membrane gradient and so allow the ETC to function at maximal capacity. Finally, rotenone and antimycin are added together to shut down the ETC and thereby unveil the residual, non-

mitochondrial respiration, which generally increases in the presence of mitochondrial stressors such reactive oxygen species (ROS) *(11)*. In addition these bioenergetic parameters, the following other functions can be extrapolated: 4] “proton leak”, which is calculated by subtracting non-mitochondrial respiration from the value of ATP-linked respiration; and 6] “spare respiratory capacity” (SRC), which is calculated by subtracting basal respiration from the value of maximal respiration. An increase in proton leak could be due to for instance to an elevation of uncoupling proteins (UCP) activity or damage to the inner mitochondrial membrane or ETC complexes *(11)*. Conversely, the SPR reflects the respiratory reserve available for ATP production to support cell survival during energy stress conditions or increased metabolic demand *(11, 12)*. A typical OCR graph obtained by using a Cell Mito Stress Test is depicted in [Figure 3].

NF-κB transcription factors are central coordinating regulators of immunity and inflammation. They also play a key role in oncogenesis *(13, 14)*. In mammals, the NF-κB transcription factor family consists of five highly conserved protein subunits, termed RelA/p65, which is generally the dominant NF-κB subunit *(13, 15, 16)*, RelB, c-Rel, NF-κB1(p105/p50) and NF-κB2 (p100/p52) (*17*). Several studies have shown that NF-κB signalling is involved in the regulation of energy metabolism and metabolic stress adaptations in various types of cancer, including colorectal carcinoma (CRC) *(13, 18-21)*.

Here, we describe the XF Cell Mito Stress Test methodology for the purpose of investigating how NF-κB affects cell metabolism in the mouse colon cancer cell line, CT-26, upon expression of non-specific (ns) or RelA-specific (RelA) small-hairpin (sh)RNAs *(20)*, using a Seahorse XFe96 respirometer.

2. EQUIPMENT & MATERIALS

2.1 Equipment:

1. Seahorse XFe96 Extracellular Flux analyser.

2. Non-CO_2 incubator.

3. Seahorse XFe96 FluxPak, including XF96 Cell Culture Microplate and XFe96 sensor cartridge.

4. Cell Counter.

5. Water Bath.

2.2 Reagents:

1. CT26 colon cancer cell line expressing ns-shRNAs or RelA-shRNAs. [*see* **Note 1**].

2. Cell culture growth medium: RPMI-1640 medium without glucose, 10% FBS, 150 U/mL penicillin, 200 U/mL streptomycin, 10 mM HEPES buffer, 1 mM sodium pyruvate.

3. 1x Phosphate Buffer Saline (PBS).

4. Seahorse XF calibrant solution (Agilent).

5. XF RPMI assay medium: RPMI medium, pH 7.4, 1 mM pyruvate, 2 mM glutamine, and 10 mM D-glucose.

6. 10 mM D-Glucose.

7. 2 mM Glutamine.

8. 1 mM Sodium Pyruvate.

9. 1 mM Oligomycin A stock solution.

10. 5 mM 2,4-Dinitrophenol stock solution.

11. 1 mM Rotenone stock solution.

12. 1 mM Antimycin A stock solution.

3. METHODS

The XF Cell Mito Stress Test is carried out over a two-day period. Accordingly, the experimental workflow has been divided into two sections: 1] The first describes the steps to be undertaken on the day before the assay (day 0) (**3.1**); 2] while the second describes the steps to be undertaken on the day of the assay (day 1) (**3.2**) [Figure 4].

3.1 Seahorse XFe96 assay – day 0

1. Turn on the Agilent Seahorse XFe96 Extracellular Flux analyser and let it warm up overnight. [*see* **Note 2**].

3.1.1 Seeding the cells

1. Seed CT26 cells (80 µl/well, 25,000 cells) expressing ns-shRNAs (sh-ns) or RelA-shRNAs (sh-RelA) onto a Seahorse XF96 Cell Culture Microplate using the cell culture growth medium. [*see* **Note 3**].

2. Incubate the Microplate at 37°C, 5% CO_2 overnight. Monitor the growth and health of the cells using an inverted microscope.

3.1.2 Hydrating the XFe96 sensor cartridge

1. Aliquot at least 20 mL of XF Calibrant into a 50 mL conical tube and place the tube in a non-CO_2 37°C incubator overnight.

2. Open the XFe96 Extracellular Flux Assay Kit and remove the green sensor cartridge from the utility plate. Fill each well of the utility plate with 200 µL of sterile, tissue culture-grade water and place the sensor cartridge back into the utility plate, submerging the sensors into water.

3. Place the cartridge at 37°C in a non-CO_2 incubator overnight. [*see* **Note 4**].

3.1.3 Designing the experiments

1. Launch the Wave 2.6 software e open the assay template listed as “XF Mito Stress Assay”.

2. Prepopulated information relating to the injection strategy, cell pre-treatments, assay media, and cell types are provided in the “Group Definitions” menu. Modify the available experimental parameters as follow: a) In ”Injection strategy”, add the concentration of each drug used in the assay (*i.e.*, Port A: 1.8 μM Oligomycin A; Port B: 60 μM 2,4-DNP; Port C: 0.6 μM Rotenone/Antimycin A). b) In “Pre-treatments”, select “Control” and add “sh-ns”; then select “Experimental” and add “sh-RelA”. In “Assay Media”, add “XF RPMI assay medium, pH 7.4”. In “Cell Type”, delete the default entry and add “CT26”, specifying the cell seeding density for your own records.

3. Once the experimental groups have been named, select “Generate Groups”; the software will then automatically create two unique assay groups: CT26 sh-ns and CT26 sh-RelA.

4. Select the “Plate Map” menu to assign experimental groups to the microplate map by first choosing the group name in the list provided and then clicking individual experimental points on the microplate map. [*see* **Note 5**].

5. Select the “Instrument Protocol” menu to view the default instrument protocol [*see* **Note 6**].

6. Click “Run Assay” in the “Functions” ribbon to save the template file and start the assay.

3.2 Seahorse XFe96 assay – day 1

3.2.1 Preparing the assay medium and the compound working solutions.

1. Warm up the Seahorse XF RPMI medium to 37°C in a water bath.

2. Remove the Seahorse XF Cell Culture Microplates from the 37°C CO_2 incubator and examine the cells using an inverted microscope to confirm their confluence.

3. Remove the cell growth medium from the cell culture microplate and add 180 µL of pre-warmed assay medium.

4. Place the microplate into a 37°C non-CO_2 incubator for 1 hour prior to the assay.

5. Prepare 3 mL of working solutions in the assay medium for each compound by using the volumes specified in **Table 1.** [*see* **Note 7**].

6. Load the solutions onto the injection ports of the sensor cartridge by using the guides provided.

3.2.2 Running the assay

1. Browse the saved design file and then open it. Select the “Review and Run” tab and then choose “Start Run”.

2. Place the loaded sensor cartridge with the calibrant microplate into the instrument, and then select “I'm ready”. [*see* **Note 8**].

3. Following calibration and equilibration of the cell culture microplate, when prompted select “I'm ready”.

4. Remove the calibration utility plate and load the cell culture microplate and select “I'm ready” to run the assay.

3.3 Data analysis

1. The Seahorse Mito Stress Test assay can be analysed by using the Seahorse XF Mito Stress Test Report Generator, which automatically calculates the main Seahorse XF Cell Mito Stress Test parameters from the Wave data that were exported to Excel.

2. The Seahorse XF Report Generator can be installed together with the Wave 2.6 software or directly from the Agilent Cell Analysis website. [*see* **Note 9**].

4. NOTES

1. Production of high-titre lentiviral preparations in HEK-293T cells and lentiviral infections of CT-26 cells may be carried out as described in *(20)*.

2. The Agilent Seahorse XFe96 instrument needs to warm up for at least 5 hours. This step is required to allow the instrument and its program to stabilise at 37°C, since a stable 37°C temperature of the XF instrument is required for accurate Seahorse readings.

3. It is important to predetermine the optimal seeding density for each cell line used. The optimal cell seeding density may vary widely depending on the cell line, although this is typically in a range between 5×10^3 and 4×10^4 cells per well. The seeding

density must be determined empirically for each cell line. After seeding the cells, allow the plate to rest in the tissue culture hood at room temperature for at least one hour. This step facilitates an even distribution of the cells and reduces edge effects, which can be particularly pronounced for certain cell types. In all cases, empirically monitor the cell adherence using an inverted microscope.

4. The maximum time of incubation of the cartridge in the non-CO_2 incubator is 72 h. If for any reason the cartridge is hydrated for more than 24 h, then wrap it up in parafilm to avoid evaporation. In any case, the hydration period should be no less than 4 h.

5. The number of experimental replicates should be no less than 5.

6. The default instrument protocol does not require modifications . However, the name of the protocol command, the number of measurements recorded before or after injection, and the length of time for each measurement can be changed. Modifying the instrument protocol settings may affect how the data are acquired during the assay. For the CT-26 cell line, the default instrument protocol is recommended for the analyses.

7. In our system, 2,4-DNP was used instead of FCCP because it demonstrated less toxicity.

8. The calibration takes approximately 15 to 30 minutes. Remove the cartridge lid and verify the correct microplate orientation.

9. https://www.agilent.com/en/products/cell-analysis/cell-analysis-software/data-analysis/seahorse-xf-cell-mito-stress-test-report-generators.

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TABLES

Table 1 – Compound preparation instructions for loading onto the XFe/XF96 sensor cartridges*

	Stock solution (mM)	Final well (µM)	Stock solution volume (µL)	Media volume (µL)	10X (Port) (µM)	Volume added to port (µL)
Port A: Oligomycin	1	1.8	54	2946	18	20
Port B: 2,4 DNP	5	60	360	2640	600	22
Port C: Rot/AA	1	0.5	15	2985	5	25

*The starting volume of assay medium for the cell microplates is 180 µL per well.

FIGURE LEGENDS

Figure 1. Extracellular Flux (XF) analysis is the mainstream method for characterising the bioenergetic and metabolic profile of a cell. XF analysis measures the flux of oxygen – e.g. the oxygen consumption rate (OCR) – as well as the flux of protons – e.g., the extracellular acidification rate (ECAR). The OCR is largely the results of OXPHOS, while ECAR reflects the rate of glycolysis.

Figure 2. Schematic representation of a typical Seahorse XFe96 respirometer measurement cycle. During each measurement cycle, the sensor cartridge is lowered onto the wells to create a transient microchamber. Drug delivery ports located on the cartridge itself allow the automatic injection of specific compounds. Fiber optic bundles are inserted into the probes, and light is forced into the sensor to excite the fluorophores. Changes in fluorescence signals are proportional to changes in O_2 or H^+ concentrations. The slope of the linear decline (for O_2 concentration over time) or incline (for H^+ concentration over time) provides a basis for extrapolating the OCR value (expressed in pmol/min) or ECAR value (expressed in mpH/min), respectively.

Figure 3. Typical XF Cell Mito Stress Test profile, showing the key parameters of mitochondrial function. Basal OCR is measured before the injection of the mitochondrial inhibitors. Once the basal measurements are recorded, specific drugs are serially injected onto the microplate to measure the following bioenergetic parameters: 1] ATP-linked respiration; 2] maximal respiration; and 3] non-mitochondrial respiration. Additionally, 4] the proton leak value can be calculated by subtracting non-mitochondrial respiration from the ATP-linked respiration value, whereas 6] the spare respiratory capacity (SPR) can be calculated by subtracting basal respiration from the maximal respiration value.

Figure 4. Schematic representation of the XF Mito Stress Test workflow.