



# A cost-effective, analytical method for measuring metabolic load of mitochondria

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## ABSTRACT

Analysis of cellular energetics is central to understanding metabolic diseases including diabetes and cancer. The two most commonly used methods to monitor cellular respiration are the Seahorse-XF system, and Glo™ assays, which are considered “gold standards”. These commercial methods measure energetics indirectly and require considerable financial investment. Here we describe an alternative assay that enables accurate quantification of NADH turnover and that is affordable. This method measures resazurin reduction to resorufin at rising concentrations in the presence of purified mitochondrial extracts until NADH becomes a rate-limiting factor. This indicates the maximal level of NADH turnover in each sample and therefore infers metabolic activity. Here we compare MRC5, MCF7 and MDA231 cell lines which have differing metabolic profiles.

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## 1. Introduction

Cellular metabolism is a balance between activities of the tricarboxylic acid (TCA) cycle, glutaminolysis and the electron transport chain (ETC), which are based in the mitochondria, and glycolysis and the pentose phosphate pathway (PPP), which are based in the cytosol. The ATP yield from glycolysis is highly inefficient: it generates 2 molecules of ATP per molecule of glucose, whereas up to 36 are produced by oxidative phosphorylation (OxPhos; [1]. However, the main advantage of glycolysis is that, unlike OxPhos, it does not produce reactive oxygen species (ROS), which can cause significant damage to the cell, particularly to the DNA and mitochondria (Reviewed by Ref. [2]. Moreover, glycolysis generates secondary metabolites that can be used as substrates to fuel flux through the PPP including the essential nucleic acid precursor ribose-5-phosphate (R5P), aromatic amino acid precursor erythrose-5-phosphate (E5P) [4]. During tumourigenesis, cellular metabolism is often skewed towards glycolysis, a phenomenon known as the “Warburg Effect” [5].

Examining highly complex metabolic pathways in cells is challenging. The gold standard assays rely on by-products or reduction of a substrate as means for indirect measurement of cellular glycolytic load. For example; the *Seahorse XF Glycolytic Rate Assay* by Agilent utilises the symport of lactate with protons out of the cell in combination with carbonic acid derived mitochondrial acidification to deduce cellular glycolytic load. The *Glucose-Glo™ Assay* (Promega) is an enzymatic assay that quantifies the concentration of remaining glucose in “spent” medium. This is done by *in vitro* glucose dehydrogenase activity, reducing NAD<sup>+</sup> to NADH supplying the necessary co-factor, NADH, for pro-luciferin reductase to generate a substrate for luciferase. Glucose concentration can then be inferred from the relative fluorescence between samples.

As the assays described above are both indirect and costly, we have developed a direct and cost-effective alternative method to analyse cellular respiration, which can be used for cells grown in 2D monolayer, suspension, or in 3D. This method does not measure cellular viability like many commercially available kits (Abcam ab129732 for example). It is instead, a refinement of one described in part by Ref. [6]; and by Ref. [7]; where resazurin in the presence of oxidative phosphorylation inhibitors, such as rotenone and antimycin A, is used as an indicator of mitochondrial health. The method described here also takes observations by Ref. [8] into

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account by reading initial resorufin fluorescence, reducing the effect of autofluorescent dihydroresorufin on the result.

## 2. Method

### 2.1. Preparation of mitochondrial extracts

Human embryonic lung fibroblasts (MRC5), and human breast cancer cells (MCF7 and MDA231) were grown in standard 2D monolayer, harvested by scraping and washed in ice-cold PBS (centrifuged at 300 g for 5 min at 4 °C). PBS was discarded and pellets resuspended in ice-cold extraction buffer (200 mmol<sup>-1</sup> Mannitol, 70 mmol<sup>-1</sup> Sucrose, 1 mmol<sup>-1</sup> EGTA, 10 mmol<sup>-1</sup> HEPES, pH 7.5) supplemented with protease and kinase inhibitors, and lysed with 30 strokes in a 2 cm<sup>3</sup> glass Dounce homogeniser. Unlysed cells were then removed by centrifugation (500 g for 5 min at 4 °C), the supernatant was retained, and centrifuged at 1000 g (10 min, 4 °C) to precipitate nuclei, which were also discarded. The supernatant was finally centrifuged at 10,000 g (20 min, 4 °C) to pellet mitochondria, which were resuspended in cold extraction buffer and spun again at 10,000 g (20 min, 4 °C) to wash mitochondria. At this point the supernatant was removed and the pellet resuspended in Locke's Buffer (154 mmol<sup>-1</sup> NaCl, 5.6 mmol<sup>-1</sup> KCl, 2.3 mmol<sup>-1</sup> CaCl<sub>2</sub>, 1 mmol<sup>-1</sup> MgCl<sub>2</sub>, 3.6 mmol<sup>-1</sup> NaHCO<sub>3</sub>, 5 mmol<sup>-1</sup> glucose and 5 mmol<sup>-1</sup> HEPES pH 7.5). Protein concentration was assessed by Bradford assay.

### 2.2. Mitochondrial respiration assay

20 µg of mitochondrial protein extract was diluted in 25 µl of

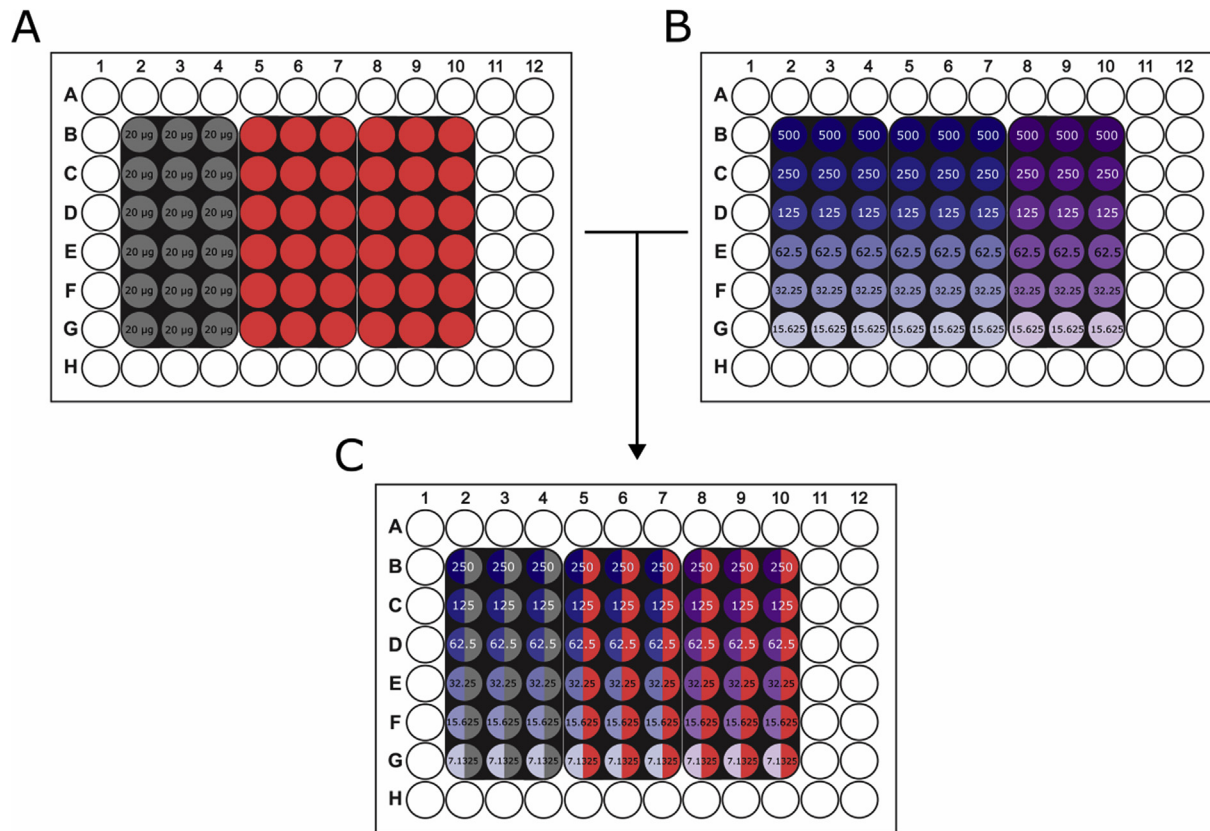
complete medium (DMEM, 10% FCS, 1 mmol<sup>-1</sup> glutamine) to a final concentration 0.8 µg µl<sup>-1</sup> and samples transferred to a glass-bottom black-walled 96-well plate (Eppendorf, CLS3904) in duplicate, see Fig. 1A. In a second, normal 96-well plate, serial dilutions of resazurin and resorufin solutions were prepared at double the final working concentration, as shown in Fig. 1B. Finally, the contents of plates A (mitochondrial extracts/medium diluent) and B (resazurin/resorufin solutions) were combined in equal volumes (25 µl) into a black 96-well plate, (see Fig. 1C), and the plate incubated at 37 °C, 5% CO<sub>2</sub> in a humidified atmosphere. After 1 h sample absorbance was measured using a spectrophotometer (BMG Labtech, FLUOstar Galaxy) with excitation set at 530 nm and emission at 590 nm. For rotenone treatment, 1 µM rotenone was added to isolated mitochondria at timepoint 0 h and left to act during the course of the assay.

All reagents were from Sigma unless otherwise stated.

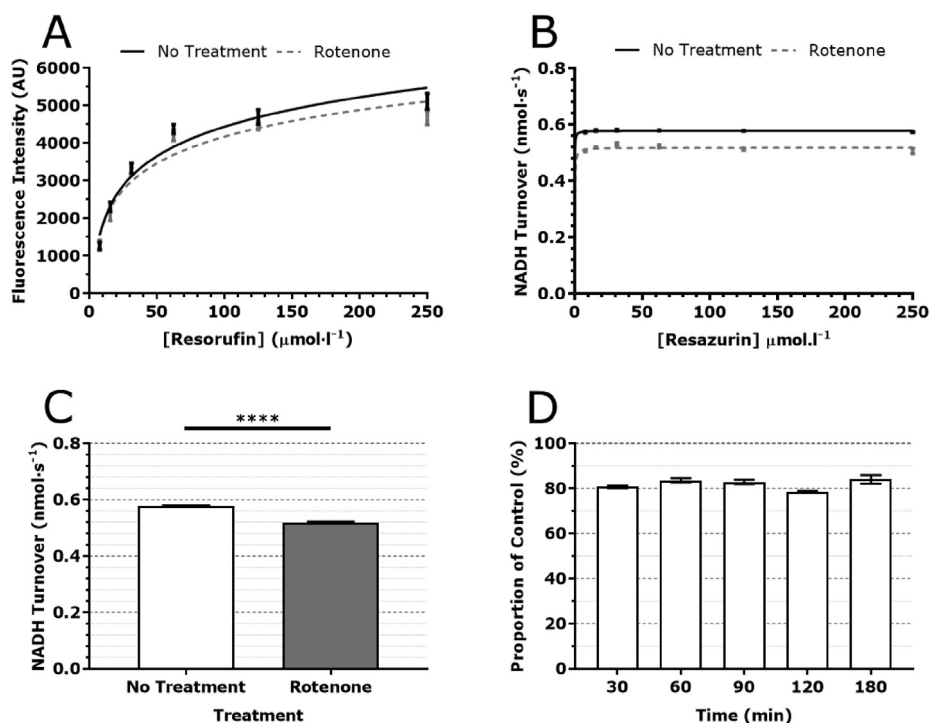
## 3. Results

To determine NADH turnover, and thus the extent of OxPhos in each cell line, a series of calculations were made. First the 590 nm emission values from the resorufin standards (Fig. 1C, columns 8–9, rows B–G), were used to plot a standard curve, which was expressed in arbitrary units (AU), (Fig. 2A). Next, any background 590 nm signal from the resazurin blanks was subtracted from the corresponding 590 nm mitochondrial values. Values less the background were then transformed through the standard curve in Fig. 2A to calculate the resorufin concentration in each mitochondrial sample.

The resorufin concentrations obtained were then plotted against the original resazurin concentration to produce the Michaelis-



**Fig. 1. Plate layout for mitochondrial activity assay.** A, layout of black walled 96-well plate with mitochondrial extracts and wells containing 25 µl of medium as a diluent for blanks and standards. B, 2x concentrated resazurin and resorufin, twofold serially diluted from 500 µmol<sup>-1</sup> to 15.625 µmol<sup>-1</sup>. C, a twofold dilution of resazurin and resorufin from plate B, into plate A.



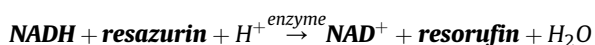
**Fig. 2.** Mitochondrial metabolic load MRC5 cells with and without Rotenone treatment. **A**, resorufin standard curves, no treatment:  $y = 1132.8\ln(x) - 775.85$ ;  $R^2 = 0.9576$ ; rotenone:  $y = 1025.1\ln(x) - 544.47$ ;  $R^2 = 0.9335$ . **B**, Michaelis-Menten curves for treated and untreated mitochondrial extracts. **C**,  $V_{\max}$  calculated in **B**, representing maximal NADH turnover analysed by two-tailed unpaired *t*-test with Welch's correction (\*\*\*\*,  $P < 0.0001$ ). **D**,  $V_{\max}$  of rotenone treated MRC5 cells over time, as a percentage of the relevant control.

Menten graph shown in Fig. 2B. As the reactions were carried out with resazurin in excess, in this experiment NADH is the only limiting factor, thus the resorufin concentration can be used to calculate the rate of “NADH turnover” ( $V_{\max}$ ) of the isolated mitochondria directly. Fig. 2B and C clearly show that mitochondria isolated from MRC5 cells and treated with rotenone have a reduced  $V_{\max}$  value compared to the untreated controls. This is also true of  $V_{\max}$  values calculated at 30 min intervals (Fig. 2D). The  $V_{\max}$  for NADH for each condition obtained is expressed as  $\text{nmol} \cdot \text{s}^{-1}$  as this is more physiologically relevant to subcellular enzyme kinetics. Note that a Michaelis-Menten curve could not fit data generated at 15 min or 180 min, as the signal-noise was too high at 15 min and variation too large at 180 min. These limitations led us to conclude that the optimum time to take readings was between 30 min and 120 min.

To demonstrate the applicability of this method we compared the metabolic load (NADH turnover) of two malignant breast cancer cell lines: MCF7 and MDA231. As shown in Fig. 3B and C, the data obtained clearly indicate that MCF7 cells reduce resazurin twice as quickly as MDA231 cells ( $V_{\max} = 2.5 \text{ nmol} \cdot \text{s}^{-1}$  versus  $1.2 \text{ nmol} \cdot \text{s}^{-1}$ ). We then calculated the Michaelis constants ( $k_M$ ) for each, i.e. the concentration at which half the NADH-dependent mitochondrial enzymes were oxidised from NADH to  $\text{NAD}^+$ . As shown in Fig. 3D, these were also significantly different for the two lines:  $16.25 \mu\text{mol} \cdot \text{l}^{-1}$  for MCF7, versus  $2.6 \mu\text{mol} \cdot \text{l}^{-1}$  for MDA231.

#### 4. Discussion

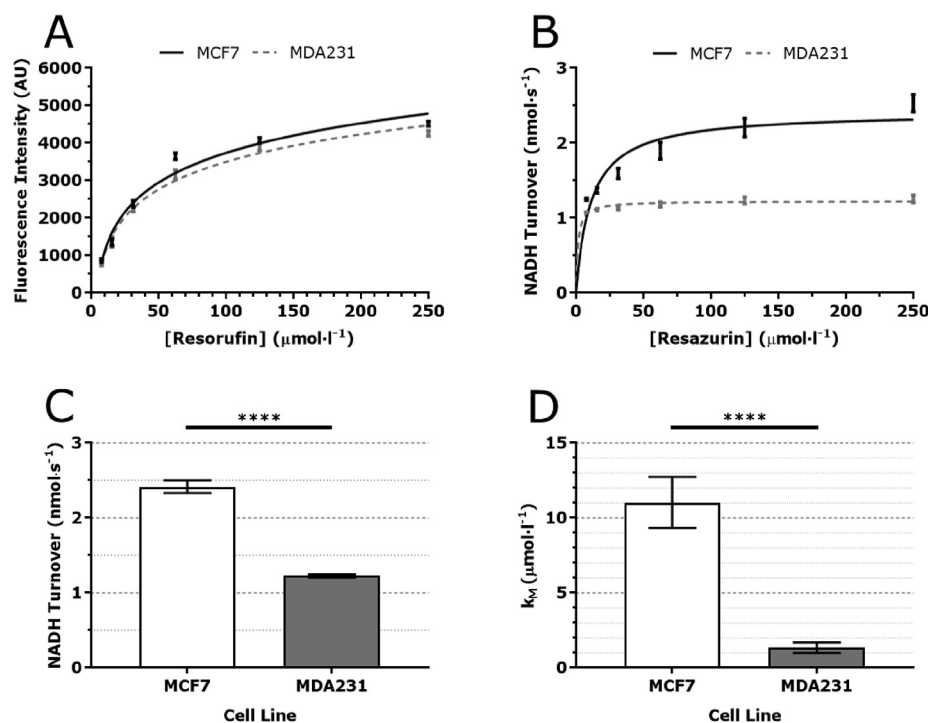
This NADH-turnover assay is simple way to measure the metabolic load of a cell population. Based on oxidation of NADH to  $\text{NAD}^+$ , it measures the reduction of resazurin to resorufin as follows:



In this reaction, assuming there is a steady flux of NADH and  $\text{NAD}^+$ , assaying the same mass of mitochondria in the presence of increasing concentrations of resazurin until the resazurin is present in excess ( $>32.5 \mu\text{mol} \cdot \text{l}^{-1}$  for MCF7, and  $>5.2 \mu\text{mol} \cdot \text{l}^{-1}$  for MDA231, as assessed from  $2xk_M$ ), mitochondrial NADH turnover can be quantified directly from the conversion reaction. This is because, when resazurin is in excess, NADH becomes the principle limiting factor in the reaction and thus  $V_{\max}$  on a Michaelis-Menten curve indicates at which concentration NADH limits the reaction. Higher  $V_{\max}$  values infer a higher rate of oxidative phosphorylation.

In this example we compared the metabolic load of mitochondria isolated from two breast cancer cell lines, grown in 2D monolayer culture. The data report that MCF7 cells are much more efficient at OxPhos than MDA231 cells, which is consistent with the metabolic landscape in non-migratory and metastatic cancers represented by MCF7 and MDA231 cells respectively [9–12]. Furthermore, the dependence of MDA231 cells on oxygen-independent metabolism suggests that these cells derive from a population which experienced sustained hypoxia during tumourigenesis [3,13–15]. A  $V_{\max}$  value as low as that observed for MDA231 cells suggests minimal NADH production via oxidation of NADH to  $\text{NAD}^+$  by NADH dehydrogenase, and thus low OxPhos. Minimal NADH production means there must also be minimal glutaminolysis as entry into the TCA cycle by glutamate via  $\alpha$ -ketoglutarate would cause  $\text{NAD}^+$  reduction.

In conclusion we have described a simple, quick and economical method to measure OxPhos in isolated mitochondria that can be used to report mitochondrial metabolic load. There is a trade-off between accuracy and cost, but this assay provides a good “*first port of call*” for research interested in metabolic dysregulation. If one is only monitoring metabolism, earlier time points can be used (15 and 30 min), however, if a treatment is incorporated into the experimental design, 1 h is more suitable as this allows time for the treatment to take effect. Note that beyond 1 h the assay becomes



**Fig. 3. Mitochondrial metabolic load of consecutive passages of MCF7 and MDA231 cells.** A, resorufin standard curves, MCF7:  $y = 1142.2\ln(x) - 1522.9$ ;  $R^2 = 0.9692$ ; MDA231:  $y = 1061.3\ln(x) - 1390.4$ ;  $R^2 = 0.9869$ . B, Michaelis-Menten curves for MCF7 and MDA231 mitochondrial extracts. C,  $V_{max}$  calculated in B, representing maximal NADH turnover analysed by two-tailed unpaired *t*-test with Welch's correction (\*\*\*\*,  $P < 0.0001$ ). D,  $K_M$  calculated in B, analysed by two-tailed unpaired *t*-test with Welch's correction (\*\*\*\*,  $P < 0.0001$ ).

less reliable, likely due to a build-up of dihydroresorufin. Dihydroresorufin is a colourless and afluorescent compound formed from resorufin oxidation that has recently been reported to affect the fluorometric “Amplex Red” assay [8]. Another useful parameter in this assay, with respect to mitochondrial homeostasis is the mass of mitochondria as a function of the number of cells or mass of tissue from which they are prepared. Identical calculated  $V_{max}$  between two samples may still inform on altered mitochondrial dynamics and behaviour if the mitochondrial mass of the relative samples is different.

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### Declaration of competing interest

The authors declare that they have no competing interests.

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