

## Summary

The XF24 non-invasively measures physiological changes in cellular energetics and metabolic pathways *in vitro* using optical biosensors in a 24 well format.

Shifts between aerobic respiration and glycolysis as a result of genetic, pharmacologic or physiologic manipulation can be measured in a single assay.

XF24 assays provide increased throughput in a drug discovery format that is superior to its single parameter predecessors.

## Measure Aerobic Respiration and Glycolysis Simultaneously

Determination of the contribution of aerobic respiration and glycolysis to cellular metabolism is not possible in most assay formats. However, by employing two sensors, one that measures oxygen consumption rate (OCR, an indicator of mitochondrial respiration) and another that measures extracellular acidification rate (ECAR, an indicator of lactic acid production or glycolysis), the XF24 can simultaneously determine both the aerobic and anaerobic components of cellular bioenergetics.

In Figure 1, A549 cells are treated with a variety of metabolic pathway inhibitors to alter the relative mix of aerobic and anaerobic metabolism. Untreated cells (●) show a normal basal mixture of both aerobic and anaerobic metabolism as indicated by an average rate of 125 pMoles/min OCR and 70 mpH/min ECAR, respectively. When exposed for 60 minutes to 100 mM of the glycolysis inhibitor 2-deoxyglucose (2-DG) (●), the cells shift to an almost exclusive aerobic phenotype as indicated by a low ECAR (approximately 20 mpH/min) and a high OCR (approximately 100 pMoles/min). Conversely, when incubated for 30 minutes in 1 μM of the Complex I inhibitor, Rotenone (●), the cells shift to an anaerobic phenotype with an average OCR of 20 pMoles/min and an average ECAR of 75 mpH/min. Lastly, incubation with both 2-DG and Rotenone (●) results in a precipitous drop in both energy yielding pathways that will lead eventually to cell death.

Using the same metabolic inhibitors as above and the mitochondrial respiration uncoupler, 2,4-DNP, we can sequentially add them to the same well to demonstrate how one can detect shifts in metabolic pathway utilization in real-time.

As shown in Figure 2, 2,4-DNP administration invoked a concomitant increase in OCR and ECAR. Subsequent addition of 2-DG to the same wells

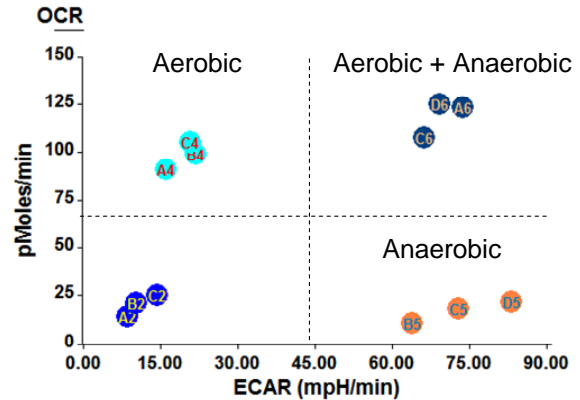


Fig.1. Pathway Determination in A549 cells. A549 cells, in triplicate wells, were treated with various metabolic inhibitors: vehicle only (●), 100 mM of glycolysis inhibitor 2-DG (●), 1 μM of the mitochondria complex I inhibitor, rotenone, (●) and 100 mM 2-DG plus 1 μM Rotenone (●), 10,000 cells per well were seeded 24 hours prior to the assay.

elicited a rapid decrease of the 2,4-DNP-stimulated ECAR to well below the baseline rate while OCR remained unaffected. Finally, addition of the mitochondrial respiration inhibitor Rotenone, after exposure of the same cells to 2,4-DNP and 2-DG, completely diminished OCR and further reduced ECAR.

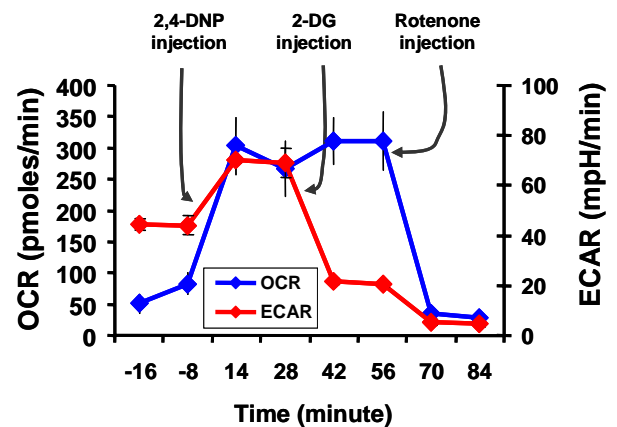


Fig.2 Real-time analysis of bioenergetic pathways in A549 cells. Mitochondria uncoupler 2,4-DNP (100 μM), glycolysis inhibitor 2-DG (100 mM) and mitochondria complex I inhibitor, rotenone (1 μM) were injected sequentially at the indicated time points into each well containing A549 cells after baseline rate measurement; Cell viability of the treated cells remained similar to control cells as determined by Calcein AM stain (data not shown). 10,000 cells per well were seeded 24 hours prior to the assay.

These data demonstrated that dynamic changes in the metabolic pathways that contribute to overall ATP turnover can be concurrently measured in a single assay using the XF24.

## Materials and Methods

**Cell Culture.** Human non-small cell carcinoma cell line A549 was obtained from the DCTD Tumor/Cell Line Repository at the NCI. Cells were cultured in growth medium consisting of RPMI 1640 (Invitrogen, CA), 10% FBS (Hyclone Logan, UT) and 100 units of penicillin and 100  $\mu\text{g/ml}$  streptomycin (Invitrogen). The cells were maintained in 75-cm<sup>2</sup> T flasks (BD Biosciences, San Jose, CA) in an incubator (Forma Scientific, Marietta, OH) controlled at 37°C, 95% humidity and 5% CO<sub>2</sub>. Cell culture medium was refreshed every other day. Every 2 to 3 days, A549 cultures were detached from the flasks using a 0.25% solution of trypsin (Invitrogen), counted by trypan blue exclusion using a ViCell (Beckman-Coulter, Fullerton, CA), and subcultured at a initial seeding density of 1.0 x 10<sup>5</sup>/ml (for a 2 day culture) or 5 x 10<sup>4</sup>/ml (for a 3-day culture) in 20 ml growth medium per T75 flask. All cultures were maintained at 80% to 90% confluence at the time of subculture.

**Test compounds.** 2-deoxyglucose (2-DG), 2,4-dinitrophenol (2,4-DNP) and rotenone were obtained from Sigma (St. Louis, MO). Concentrated stocks of 1000 mM 2-DG was prepared in assay medium. Concentrated stocks of 200 mM 2,4-DNP and 10 mM rotenone were prepared in DMSO. 2,4-DNP was further diluted to 2 mM followed by adjusting to pH 7.4. Rotenone was diluted to 0.01 mM. Calcein AM was purchased from Invitrogen and prepared according to manufacturer's instructions.

**XF Bioenergetic Assay.** A549 cells were seeded in XF 24-well cell culture microplates at 2.0 to 3.0 x 10<sup>4</sup> cells/well (0.32 cm<sup>2</sup>) in 200 - 500  $\mu\text{l}$  growth medium and then incubated at 37°C for 20 to 24 hours. Assays were initiated by removing the growth medium and replacing it with 600 to 900  $\mu\text{l}$  of assay medium (A low-buffered RPMI 1640 medium, 1 mM phosphate) pre-warmed to 37°C. The cells were incubated at 37°C for 30 minutes to allow media temperature and pH to reach equilibrium. Prior to each rate measurement the XF24 Analyzer mixed the assay media for 10 minutes to allow the oxygen partial pressure equilibrate. Following mixing, OCR and ECAR were measured simultaneously for 2 to 4 minutes to establish a baseline rate. The assay medium was then mixed again for 2 - 4 minutes between each rate measurement to restore normal oxygen tension and pH. After the baseline measurement, 75 to 200  $\mu\text{l}$  of a testing agent prepared in assay medium was then injected into each well to reach the desired final concentration. This was followed by mixing for 5 to 10 minutes to expedite compound exposure to cellular proteins after which OCR and ECAR measurements were then

made. Generally, 2 to 3 baseline rates and 2 or more response rates were measured and the average of two baseline rates or test rates was used for data analysis. The values of OCR and ECAR reflect both the metabolic activities of the cells and the number of cells being measured. For relative measurements comparing metabolic rate after compound exposure to a pre-exposure baseline, that is, when data are expressed as a percentage of OCR or ECAR change over baseline, the number of cells present in a well is not relevant since the same cell population is assayed. In the present study, the acute responses to pharmacological modulators of energy metabolism by cancer cells were determined, and the responses were expressed as a percentage of baseline rate prior to compound exposure.

Typically, at the end of each assay cells were detached by incubating with 0.25% trypsin (Invitrogen) and the number and percentage of viable cells were determined after by trypan blue exclusion assay using a ViCell (Beckman-Coulter). Cell viability of all treated samples was similar compared to control cells in acute assays lasting one hour or less.

**Calcein AM cell viability assay.** The viability of the test culture following exposure to compounds or vehicle was determined using the Calcein AM assay. A549 cells were seeded in black 96 well tissue culture microplates at 1.0 x 10<sup>4</sup> cells/well and incubated in the 37°C incubator for 24 hours. Cells were treated with vehicle or compound at the indicated concentrations for 30 minutes. Calcein AM staining solution was prepared by dissolving Calcein AM (50  $\mu\text{g/vial}$  special package) in 100  $\mu\text{l}$  of DMSO followed by diluting to 1  $\mu\text{M}$  in Hank's balanced salt solution (HBSS) just before using. The assay was performed by first removing from each well the culture medium containing the compounds and then washing each well with 200  $\mu\text{l}$  of HBSS. 100  $\mu\text{l}$  of 1  $\mu\text{M}$  Calcein AM staining solution was then added to each well. After incubation at 37°C for 30 minutes the, fluorescent intensity of each well was measured at a wavelength of 530 nm using an excitation wavelength of 480 nm on a FLUOstar Optima plate reader (BMG Labtech).

### References

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