



Plate-based analysis of isolated mitochondria

Introduction

Mitochondrial dysfunction is implicated in numerous disease states and is also a major mechanism of drug-induced toxicity. Oxygen consumption is one of the most informative and direct measures of mitochondrial function. Traditional methods of measuring oxygen consumption are hampered by the limitations of low throughput and high complexity. The MitoXpress™-Xtra assay from Luxcel, solves these limitations by allowing convenient, plate-based analysis of mitochondrial function. The assay employs MitoXpress, one of a family of phosphorescent oxygen sensitive probes developed by Luxcel. The assay is based on the ability of O₂ to quench the excited state of the MitoXpress probe. As the test material respire (e.g., isolated mitochondria, cell populations, small organisms, tissues and enzymes), O₂ is depleted in the surrounding solution/environment, which is seen as an increase in probe phosphorescence signal. Changes in oxygen consumption reflecting changes in mitochondrial activity are seen as changes in MitoXpress™ probe signal over time.

The assay is non-chemical and reversible, a decrease in oxygen consumption (an increase in O₂ levels) is seen as a decrease in probe signal.

MitoXpress™ is analysed on standard fluorescent plate readers using standard 96- and 384-well microtitre plates. MitoXpress™ assays combine the high data quality and information content of the oxygen electrode approach, with the throughput and convenience of microtitre plate based assays. These capabilities facilitate analysis of the effect of dose, substrate or ADP on observed toxicity; parameters which are critical to the determination of mechanism of action. They also allow easy IC₅₀ generation and the application of structure-activity relationship approaches.

Protocol

Plate Preparation

- Prepare plates on a plate heater equilibrated to 30°C.
- Reconstitute MitoXpress™ probe in 1 ml H₂O to give a 1 µM stock solution. Dilute 1:10 in measurement buffer (250 mM sucrose, 15 mM KCl, 1 mM EGTA, 5 mM MgCl₂, 30 mM K₂HPO₄, pH 7.4) and add 100 µl to each well.
- For compound testing, add 1 µl of compound in an appropriate solvent to test wells.
- Dilute Mitochondria to the desired concentration in measurement buffer and add 50 µl to test well. Dissolve substrate (succinate or glutamate/malate) and ADP in measurement buffer and add 50 µl of this solution to test wells giving a final substrate concentration of 25 mM

(succinate) or 12.5/12.5 mM (glutamate/malate) and a final ADP concentration of 1.65 mM.

- Add 100 µl of mineral oil (preheated to 30°C) to each well. This increases assay sensitivity by minimising interference from ambient oxygen.

Note: Plate preparation time should be kept to a minimum.

Measurement

- Insert the prepared plate into a fluorescence plate reader pre-set to 30°C.
- Measure probe signal at 1.5 min intervals for 10-30 min using excitation and emission wavelengths of 380 nm and 650 nm respectively. (Readers equipped with a time-resolved mode, may achieve improved performance using delay and gate time of 30 and 100 µsec respectively).

Note: Mitochondria are freshly prepared as per user's protocol and should not be left on ice for longer than recommended in the literature. Measurement buffers should be prepared freshly on the day of measurement.

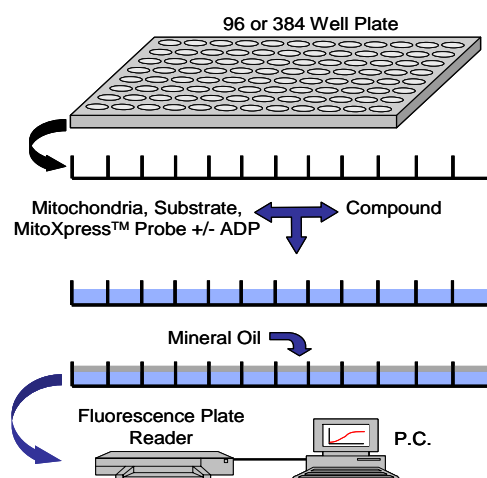


Fig. 1: Schematic diagram of mitochondrial toxicity analysis using MitoXpress™.

Data analysis

- For semi-quantitative analysis the rate of signal increase can be used to compare treated and untreated samples or to compile dose response data.
- For more quantitative analysis, fluorescence data may be related to oxygen concentrations [2].



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Results and discussion

The following data was kindly provided by Pfizer Drug Safety Research and Development (DSRD) group, San Diego, CA .

Assay throughput and performance

The data output from the MitoXpress™ assay (96 samples), and polarographic (one sample) analysis of mitochondrial oxygen consumption is contrasted in Fig. 2. Fig 2a shows typical polarographic analysis illustrating initiation of State 2 and State 3 respiration through addition of substrate and ADP respectively. Fig 2b shows oxygen consumption measured using MitoXpress™, with glutamate/malate- (left) and succinate (right) -driven oxygen consumption is measured at decreasing mitochondrial protein concentrations in both State 2 (top) and State 3 (bottom) (n = 4).

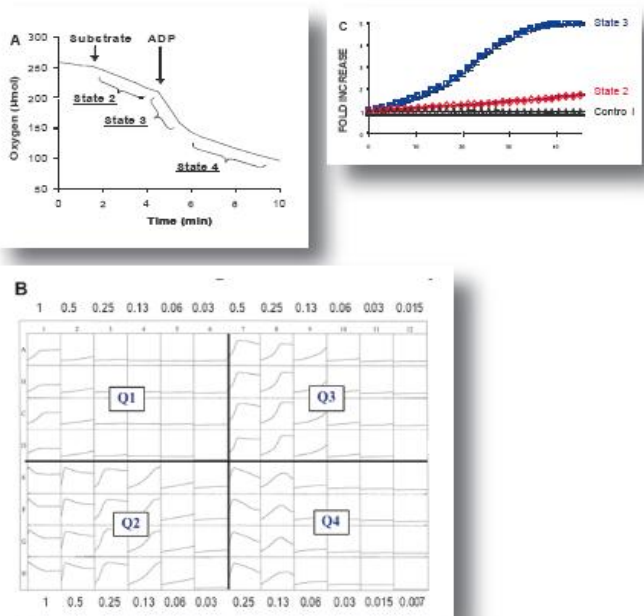


Fig. 2: Analysis of isolated mitochondrial using **A)** conventional polarography and **B)** MitoXpress™. **C)** Data extracted from Fig. 2B illustrating activation of succinate-driven mitochondrial oxygen consumption measured using MitoXpress™ A65N (n = 4, % CV < 3 %)

The compatibility of the MitoXpress™ assay with the microplate format permits analysis under 96 (or 384) discrete conditions. The effectiveness of this level of throughput in analysing isolated mitochondria is highlighted in Fig. 2B which examines increasing mitochondrial protein concentrations on glutamate/malate- and succinate-driven respiration in both basal (State 2) and ADP-activated (State 3) states (all in quadruplicates).

The performance of the MitoXpress™-Xtra assay is highlighted in Fig. 2C, with coefficient of variance below 3%

Assessment of classical mitochondrial effectors

Validation of MitoXpress™ for assessment of mitochondria is illustrated in Fig. 3. These data illustrate the inhibition of mitochondrial function using a panel of classical mitochondrial inhibitors and highlight the dose dependence of this inhibition for KCN.

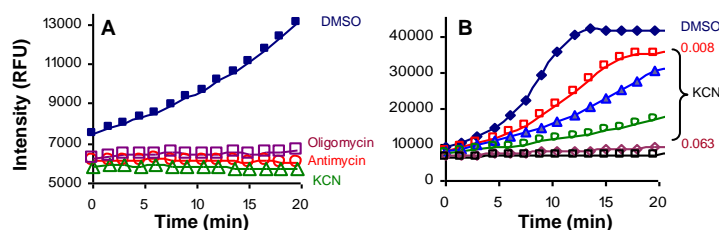


Fig. 3: **A)** Monitoring the effect of a panel of classical ETC inhibitors on mitochondrial function using MitoXpress™, and **B)** dose dependent inhibition of mitochondrial function by KCN.

Compound screening

MitoXpress™ allows screening of compounds at multiple concentrations and in multiple conditions in a single microtitre plate as illustrated in Fig. 4. Such data may be processed further to generate dose response data [1, 2].

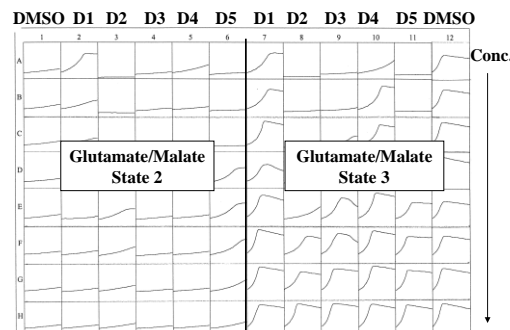


Fig. 4: Effect of test compounds (D01-D05), on both State 2 and State 3 isolated mitochondrial function using MitoXpress™. Some compounds uncouple in a dose dependant manner while others inhibit.

Overall, MitoXpress™ allows highly sensitive high-throughput detection of mitochondrial dysfunction in isolated mitochondria. Use of a 96-well plate format allows screening of 200 compounds per day at a single dose, or acquisition of dose response characteristics for 25 compounds per day. This capability represents a fundamental shift in the capacity for mitochondrial toxicity testing in drug discovery programs, without compromising data quality or information content.

References

1. Hynes J. *et al.*, *Toxicol. Sci.* 2006;92(1):186-200.
2. Will Y., *et al.*, *Nat. Pro.* 2007;1(6):2563-72.
3. Dykens J.A., *et al.*, *Expert Rev Diagn.* 2007; 7(2): 161-175.

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