Multiplexed Screening of Drug-Induced Mitochondrial Dysfunction and Cytotoxicity

Melanie Schwalfenberg, Ryan McGarrigle, Conn Carey, and James Hynes

Biosensor and Assay Solutions, Cell Analysis Division

Agilent Technologies, Ireland.

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Introduction

Drug-induced mitochondrial toxicity

Impaired mitochondrial function has been identified as a significant contributor to drug-induced toxicity, and has been implicated in late stage drug failure, black box warnings and market withdrawal [1,2]. Early detection of mitochondrial liabilities is therefore of particular interest to the pharmaceutical industry.

Conventional cytotoxicity assays are not well suited to the detection of drug-induced mitochondrial dysfunction due to the ability of commonly used cell types to mitigate the consequences of mitochondrial insult [3, 4].

Oxygen consumption measurements provide a direct measure of mitochondrial function, facilitating the identification of drug-induced perturbation immediately post-treatment [2].

Longer term treatments are sometimes preferred in order to reveal toxicity mediated by bioactivation or bioaccumulation, however, it can difficult to determine whether any observed changes in cell respiration are a consequence of direct mitochondrial inhibition or non-specific cytotoxicity.

Here we assess the feasibility of combining cytotoxicity and mitochondrial function measurements into a single, multiplexed 'mix-and-measure' assay, thereby simplifying screening workflows.

We also examine the utility of this multiplexed approach to better delineate drug-induced mitochondrial dysfunction and non-specific toxicity after longer term compound treatments.

Mitochondrial Function was assessed using the **MitoXpress Xtra Oxygen Consumption Assay**. MitoXpress Xtra is an oxygen sensitive fluorescent probe that facilitates microplate-based analysis of cellular oxygen consumption on plate-readers.

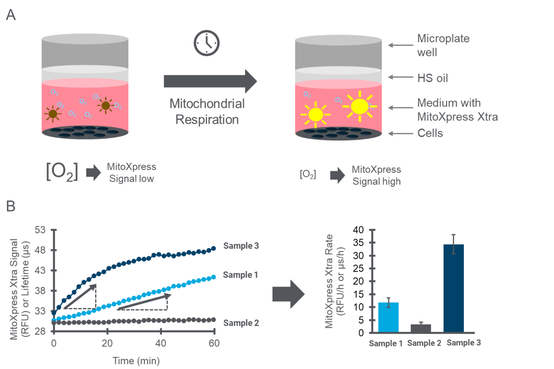

Figure 1: Measuring mitochondrial function using the MitoXpress Xtra Oxygen Consumption Assay. (A) The MitoXpress Xtra reagent is added to cells and overlaid with HS oil. Cellular respiration leads to oxygen depletion in the sample, resulting in increased MitoXpress Xtra signal. (B) This change in signal is measured kinetically on a fluorescence plate reader. The rate of signal increase is calculated by linear regression and is indicative of the rate of cellular respiration.

Cytotoxicity was assessed using **Calcein AM**, a non-fluorescent molecule that is internalized by cells with intact membranes. Intracellularly, Calcein AM is converted to its fluorescent form Calcein and retained; the fluorescence intensity of Calcein is proportional to the number of live cells in the well.

Multiplexing Mitochondrial Function and Cytotoxicity

Mitochondrial function and cytotoxicity measured in the same well

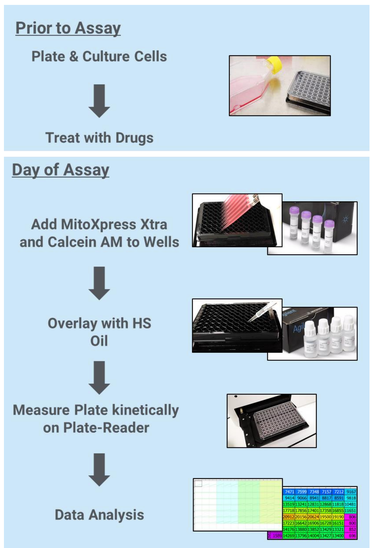

Figure 2: Workflow of the multiplexed assay.

A multiplexed protocol was developed and validated (Fig. 2).

Cells are grown in 96-well microtiter plates and treated with test drugs for the desired time.

On the day of measurement, spent growth medium is removed and replaced with fresh medium containing MitoXpress Xtra and Calcein AM.

No additional wash steps or loading periods are required, delivering a **simple 'mix-and-measure' protocol**.

Samples are then overlaid with HS oil to limit back diffusion of atmospheric oxygen.

In the same measurement run, MitoXpress Xtra signals are measured kinetically and a single Calcein data set is taken automatically 30 min post reagent addition.

Multiplexing does not impact assay performance

Multiplexed measurement of MitoXpress Xtra and Calcein AM does not adversely impact individual MitoXpress (Fig. 3A) or Calcein (Fig. 3B) outputs.

Evaluation of assay performance demonstrated a good signal separation and low signal variation for the multiplexed assay. Generally, Z' values for both parameters exceed 0.6. Representative MitoXpress Xtra data are presented in Fig. 3C.

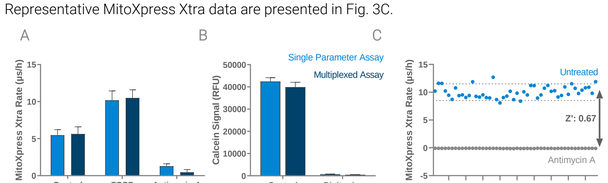

Figure 3: Assay performance analysis for the multiplexed assay. A) MitoXpress Xtra rates and response to FCCP and Antimycin A treatment and B) Calcein signal in untreated or Digitonin treated HepG2 cells are not adversely impacted by multiplexing. C) Typical result from Z' factor analysis. MitoXpress Xtra rates of Antimycin A treated HepG2 cells were compared to rates from untreated cells, revealing a Z' factor of 0.67. Dotted lines represent +/-15% of the average MitoXpress Xtra rate.

Compound Screening

MitoXpress Xtra enables the detection of direct mitochondrial inhibitors (Fig.4 A&B) and uncouplers (Fig. 4C) immediately post treatment (0h). No Cytotoxicity is observed.

Cytotoxicity is observed after 24h; but, is far more pronounced for non-specific mitochondrial insult (Fig. 4E) than for more specific inhibition (Fig. 4D) or uncoupling (Fig. 4F).

This **differential dose response** is consistent with reported mode-of-actions [3,5] and provides an opportunity to more confidently detect specific mitochondrial dysfunction after prolonged exposure times.

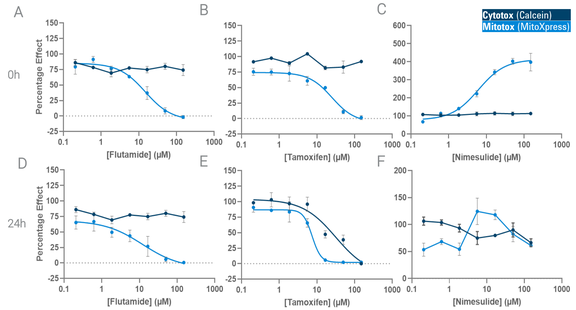

Figure 4: Multiplexed mitochondrial respiration and viability assessment in HepG2 cells after treatment with modulators of mitochondrial function. Cells treated acutely (upper panel) or for 24h (lower panel). Responses are shown as % of controls.

Similar responses were observed for additional compounds with reported mitochondrial liabilities (Fig. 5), further supporting this multiplexed concept as a means of **combining Mitotox and Cytotox measurements in a single well** while also better **delineating the involvement the mitochondria** in any observed toxicity.

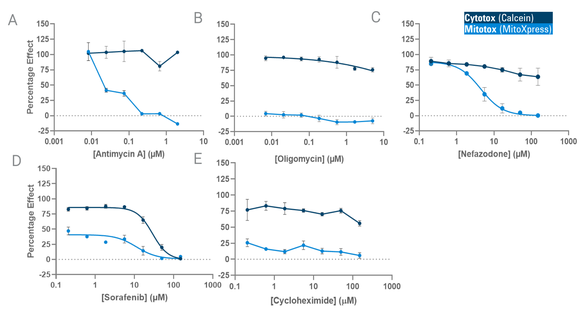

Figure 5: Multiplexed dose-response data generated after 24h treatment with model compound. Shown as % of untreated controls.

Conclusions

- Standard **cytotoxicity** assays are not well suited to the detection of mitochondrial toxicity.
- MitoXpress Xtra can detect direct drug-induced mitochondrial dysfunction immediately post treatment.
- By multiplexing MitoXpress Xtra and Calcein AM assays into as single, convenient, plate reader based workflow, longer-term Mitotox **and Cytotox assessment can be performed in the same well**, thereby streamlining testing procedures and increasing the output of relevant *in vitro* data.
- Multiplexing does not impair the functionality and assay performance of individual readouts.
- Mitotox and Cytotox dose response assessments are easily performed on the same plate.
- Differential responses can contextualise observed toxicity, with data suggesting that;
  - Compounds with a prominent mitochondrial liability (e.g. Flutamide, Nefazodone, Nimesulide) exhibit a differentially stronger MitoTox response than Cytotox response.
  - Compounds exhibiting with a non-specific or multifactorial toxicity (eg. Tamoxifen) will show significantly less separation in dose response.
- This multiplex approach helps contextualise observed metabolic perturbations subsequent to long term treatments and can provide additional mechanistic insights into observed drug induced metabolic dysfunction. This facilitating a more complete characterisation of compound toxicity without the need to run additional assays.

Material & Methods

Cell plating and assays

HepG2 cells were seeded at 30,000 – 80,000 cells/well (dependent on treatment duration) in black-wall, clear-bottom 96 well plates and allowed to adhere over night before treatment with drugs or vehicle at the indicated timepoints.

Immediately before measurement, spend culture medium was replaced by DMEM containing MitoXpress Xtra (reconstituted as described in User Manual; Agilent, #MX-200-4) and 1.25 µM Calcein AM and drugs were re-administered. Samples were sealed with a layer of pre-warmed HS Oil and immediately transferred into a CLARIOstar (BMG Labtech), preheated to 37°C.

Both parameters were measured kinetically for 90-120 min using the scripting function of the instrument software.

Plate reader settings and data analysis

The MitoXpress Xtra Signal was detected using dual-read time-resolved fluorescence detection with Ex: 340 ± 50 nm (TREX-L filter, BMG Labtech) and Em: 645 ± 20 nm (645-BP20 filter, BMG Labtech). The following integration and delay times were used: Integration start 1: 30 µs, Integration time 1: 30 µs; Integration start 2: 70 µs, Integration time 2: 30 µs.

Calcein fluorescence intensity was measured using the following settings: Ex: 483 ± 14 nm, Ex: 530 ± 30 nm, Dichroic: 502.5

Data analysis was performed by fluorescence lifetime calculation for the MitoXpress Xtra signal and linear regression over the kinetic signal trace (integrated into MARS data analysis software, BMG Labtech). Calcein intensity values measured at t:60 min were used for viability assessment.

References

[1] Wallace, (2008) *Trends in Pharma Sciences*, 29 (7), 361.; [2] Hynes et al, (2006) *Toxicol Sci*, 92(1): p. 186-200.; [3] Marroquin et al, (2007) *Toxicol Sci*, 97(2): 539–547.; [4] Hynes and Will, (2018) The Evolution of Mitochondrial Toxicity Assessment in *Industry. Mitochondrial Biology and Experimental Therapeutics*.; [5] Hynes et al, (2013) *Toxicol in vitro*, 27(2): p. 560-569.

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