Simple High-Throughput Workflows for Assessing Drug-Induced Mitochondrial Dysfunction Using Oxygen Consumption Measurements

Melanie Schwalfenberg, Ryan McGarrigle, Rajas Sane, Nathalia Romero, George W. Rogers, and James Hynes
Bioassay Solutions, Cell Analysis Division
Agilent Technologies.

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Introduction

Impaired mitochondrial function is a significant contributor to off-target drug effects, yet these effects are not readily detected with cytotoxicity assays.

Therefore, there is a need for high throughput *in vitro* assays that can detect acute metabolic liabilities early in the discovery process.

Workflows allowing longer-term drug treatments are also required to detect mitochondrial dysfunction occurring upon extended compound exposure due to bioaccumulation or bioactivation.

Here we describe the development and evaluation of two discrete workflows that address these specific challenges by measuring the impact of drug-treatment on mitochondrial function.

A 384-well workflow facilitates rapid detection of direct mitochondrial insult, while a multiplexed workflow allows longer-term drug treatments, increasing data density and giving additional mechanistic insight.

Both workflows use the MitoXpress Xtra Oxygen Consumption Assay, which facilitates microplate-based analysis of cellular oxygen consumption on fluorescent plate-readers.

A streamlined 384-well workflow

For acute measurements, HepG2 cells cultured in flasks are trypsinized and added to the test wells for measurement. This simplifies cell plating, eliminates washing steps, increases throughput, and improves measurement performance. MitoXpress Mineral Oil is used to seal the sample wells, facilitating automated liquid handling.

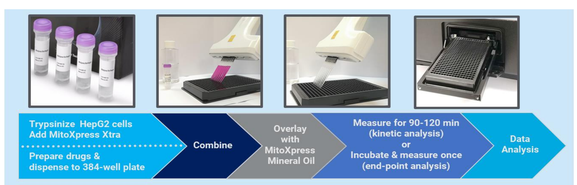

Figure 1: A) MitoXpress Xtra workflow for screening in 384-well plates. Hepg2 cells are trypsinised, added to test wells, and treated with compound. The samples are overlaid with a layer of MitoXpress Mineral Oil to limit oxygen back diffusion and then measured in a fluorescent plate reader. B) Schematic representation of kinetic (slope calculation by linear regression) and end-point (signal intensity at a single timepoint) analysis of the MitoXpress Xtra Assay. C) Schematic illustration of typical heatmap from single concentration screen, with drugs showing an impact on MitoXpress Xtra signal marked in blue.

Performance Assessment & Screening

End-point analysis was evaluated as an alternative to rate data analysis to simplify data reduction and interpretation for high throughput measurements.

Assessment of response consistency and signal separation resulted in Z'–factors ≥ 0.6 for end-point analysis (Fig. 2) and ≥ 0.5 for kinetic data analysis (not shown), indicating good performance for both approaches.

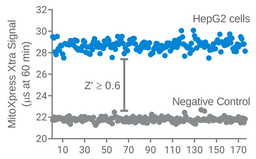

Figure 2: Plate-scatter plot of end-point analysis of typical 384-well plate assay (t: 60 min). Similar results were obtained analyzing the same samples using slope analysis.

Single-concentration screening

To further evaluate this workflow, a training set of 35 compounds were assessed at a single concentration (50 µM, Table 1). This set included known direct mitochondrial toxicants and drugs with more indirect mitochondrial liabilities that are usually only observed after longer-term exposure.

Consistent with previously reported mechanisms and potencies [1-3], the direct mitochondrial toxicants decreased MitoXpress Xtra Signal to below 50% whereas uncouplers increased signal to above 150%.

Compounds with known direct mitochondrial liabilities caused a >50% reduction in MitoXpress signal (>150% increase for uncouplers at optimum concentration) when compared to untreated control. Inactive compounds, and those requiring bioactivation, bioaccumulation, or reportedly acting *via* an indirect means fell outside these categories.

Table 1: Compounds tested in single concentration 384-well format at 50 µM. In comparison to untreated controls Direct mitochondrial toxicants showed >50% reduction in MitoXpress signal (>150% increase for uncouplers). Indirect modulators and drugs that require bioaccumulation or bioactivation fell outside these categories. (n=4).

Direct mitochondrial modulators			Indirect mitochondrial modulators		
Antimycin	Nefadozone	Ketoconazole	5-Fluorouracile	Erythromycin	Monensin
BAM15	Nimesulide	Tamoxifen	Acetaminophen	Methotrexate	Nimodipine
Clotrimazole	Oligomycin	IACS-010759	Buspirone	Mitoxantrone	Phenytoin
FCCP	Sorafenib	Suloductil	Cycloheximide	Valproic acid	Rifampicin
Clofilium tosylate	Pentamidine Isethionate	Flutamide	Diclofenac		

Dose-response data gives additional insight

Compounds that elicited changes in single-concentration screening as per the parameters indicated above were further characterized by establishing dose-response relationships.

The known direct inhibitors of mitochondrial function Flutamide, Nefazodone, Tamoxifen, Clofilium tosylate, and Tamoxifen showed a dose-dependent reduction in MitoXpress signal.

The uncouplers Nimesulide and FCCP showed an increase in MitoXpress Xtra signal.

FCCP shows a typical bell-shaped dose-response curve, which causes it to appear as inhibitor at high concentrations. This underlines the value of dose-response data to gain additional mechanistic insight.

Performance Assessment & Screening

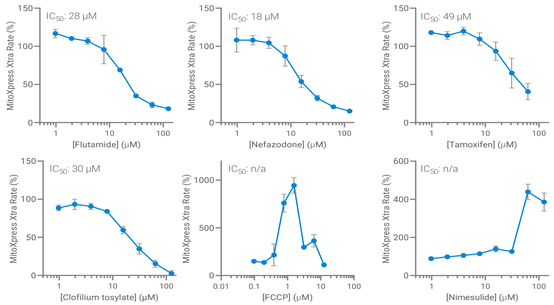

Figure 3: Sample dose-response data and apparent IC_{50} values from selected hits from the single-concentration screen measured using the 384-well workflow and kinetic data analysis.

Multiplexed long-term treatment workflow

Detection of drug-induced metabolic perturbation caused by bioactivation or bioaccumulation can require longer-term drug treatments. However, it can be difficult to interpret whether such metabolic dysfunction is a cause or a consequence of broader cellular dysfunction.

To enable more informed analysis of long-term drug treatments, a multiplexed workflow was developed using adherent cells in 96-well plates. Mitochondrial function and cytotoxicity are measured in the same well using MitoXpress Xtra and Calcein AM respectively. Both reagents are added simultaneously and measured sequentially, thereby minimizing handling and wash steps.

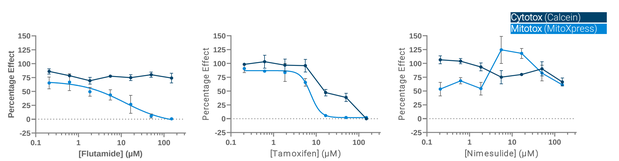

Figure 4: Multiplexed mitochondrial respiration (light blue) and viability (dark blue) assessment in HepG2 cells after treatment with modulators of mitochondrial function. Cells were treated for 24h.

Mitochondrial respiration was reduced after 24h treatment with Flutamide and Tamoxifen, the uncoupler Nimesulide caused a bell-shaped like dose-dependent response. Viability was only significantly reduced in the Tamoxifen-treated samples.

This differential dose-response suggests that cytotoxicity observed for Tamoxifen is multifactorial, while Flutamide causes a more specific mitochondrial insult. This is consistent with reported mode-of-actions[1-3]. The absence of a strong cytotoxic response for Nimesulide also suggests a specific mitochondrial insult, while the 24h MitoXpress data highlight the necessity for acute measurement when screening for uncouplers (See Fig. 3).

Conclusions

We have developed two discrete workflows to address specific challenges in identifying drug-induced mitochondrial dysfunction.

Streamlined 384-well workflow to detect direct drug-induced mitochondrial dysfunction

- The use of trypsinized HepG2 cells, MitoXpress Mineral Oil and end-point data analysis increase throughput while assay performance remains high.
- Single concentration screening of a training set of compounds demonstrates the utility of this workflow as screening tool.
- Further evaluation of hits using dose-response assays allows to determine drug potency and delivers additional insight.

Multiplexed workflow to detect mitochondrial toxicity apparent after longer treatments

- Using adherent cells, longer-term Mitotox and Cytotox assessment can be performed in the same well in 96-well plates using MitoXpress Xtra and Calcein AM.
- MitoXpress Xtra and Calcein AM are added to the well and measured at the same time, minimizing washing and reagent addition steps.
- Differential responses can contextualise observed metabolic perturbations subsequent to long term treatments and provide additional mechanistic insights.
- This facilitates a more complete characterisation of compound toxicity without the need to run additional assays.

Material & Methods

384-well plate workflow: Drugs were diluted in DMEM from 10mM DMSO stock solutions and pre-dispensed in 10x concentration (5 µL, solubility permitting) into a black walled, clear bottom 384-well plates.

HepG2 were detached from the culture vessel by trypsinization, washed, and re-suspended in full DMEM containing MitoXpress Xtra reagent (Agilent Technologies, #MX-200-4; $1.7*10^6$ cells/ml). 45 µL of this suspension were transferred into the plate on a plate-block heater at 37°C. After a brief shake, the wells were overlaid with 50 µl of pre-warmed MitoXpress Mineral Oil (Agilent Technologies, #MO-200L-1) using a multichannel pipette and reverse pipetting. Plates were immediately transferred into the plate-reader for kinetic measurements over 90-120 min.

Multiplexed-workflow: Cells were plated and treated with drugs as described previously in 96-well plates. Immediately before measurement, spend culture medium was replaced by DMEM containing MitoXpress Xtra (Agilent technologies, #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 the plate-reader

Plate reader settings and data analysis: The MitoXpress Xtra Signal was detected using dual-read time-resolved fluorescence detection using a CLARIOstar plate reader (BMG Labtech) with suitable filter set (TREX-L filter and 645-BP20 filter, BMG Labtech) at 37°C per manufacturer's instructions

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 calculating fluorescence lifetime of the MitoXpress Xtra signal. End-point (µs at indicated timepoints)was used for single concentration screen. Kinetic analysis (µs/h) was performed for IC_{50} measurements and multiplexed assay,

MitoXpress Xtra Endpoints and rates were normalised to on-plate controls (Vehicle treated = 100%, 1 µM Antimycin A = 0%) Calcein intensity measured at t:30 min was used for viability assessment.

References

[1] Hynes *et al.*, (2006) *Toxicol Sci,* 92(1): p. 186-200.; [2] Marroquin *et al.*, (2007) *Toxicol Sci*, 97(2): 539–547.; [3] Hynes *et al.*, (2013) *Toxicol in vitro*, 27(2): p. 560-569.