Application Note

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Quantitative Assessment of Mitochondrial Toxicity in Hepatic Organoids

Agilent Seahorse XF Mito Tox assay application using the Agilent Seahorse XF Flex

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Abstract

Hepatic organoids have are physiologically relevant in vitro models that more accurately recapitulate liver metabolic functions, tissue architecture, and cellular heterogeneity compared with conventional two-dimensional hepatocyte cultures and some animal models. This application note describes a workflow performing the Agilent Seahorse XF Mito Tox assay using a mouse hepatic organoid system cultured on Agilent Seahorse XF Flex organoid microplates. Using the Seahorse XF Mito Tox assay with the Seahorse XF Flex organoid microplate enables drug-induced mitochondrial dysfunction quantification of organoids grown within matrix scaffolds through the Mito Tox Index (MTI). The MTI reflects the extent of mitochondrial inhibition or uncoupling of drug compounds. Four reference mitochondrial toxicants (three inhibitors and one uncoupler) were evaluated at 10x Cmax. The results confirm the feasibility of the workflow, demonstrating a robust and reproducible platform for quantitatively assessing mitochondrial toxicity, highlighting the potential of organoid based mitochondrial toxicity assays for predicting drug safety.

Introduction

Drug induced liver injury (DILI) remains a critical barrier in drug development and safety assessment, with mitochondrial dysfunction recognized as a major mechanistic contributor. Three-dimensional (3D) hepatic organoids, derived from pluripotent stem cells or primary liver tissue, provide a physiologically relevant model that more accurately reflects liver architecture, cellular heterogeneity, and metabolic activity than conventional two-dimensional (2D) hepatocyte cultures or many animal models.[1, 2] These advantages make organoids an attractive platform for evaluating mitochondrial liabilities during preclinical screening.

The Agilent Seahorse XF Mito Tox assay enables quantification of mitochondrial dysfunction through the Mito Tox Index (MTI), a normalized, unitless metric that distinguishes mitochondrial inhibition from uncoupling and supports reproducible comparison across diverse culture formats.[3, 4] Although the assay has been widely applied to 2D systems to assess mitochondrial toxicity-associated hepatotoxicity (such as, HepG2, primary hepatocytes, HUREL cocultures), its use in 3D matrix-embedded organoids was limited due to incompatibility with microplate formats.

The Agilent Seahorse XF Flex analyzer is designed to measure cellular respiration and glycolytic activity through oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) in multiple 24-well configurations. The accompanying Agilent Seahorse XF Flex organoid microplate supports organoids grown in matrix- embedded conditions, enabling direct metabolic interrogation of 3D liver models, a format widely used in hepatic organoid culture.

In this application, mouse hepatic organoids were successfully reconstituted from frozen organoid fragments directly on the XF Flex organoid microplate, and mitochondrial toxicity was assessed using four model compounds (nefazodone, nimesulide, rosiglitazone, and troglitazone) known to induce mitochondrial dysfunction and hepatotoxicity.[5–7] The integration of the XF Flex organoid microplate with the XF Mito Tox assay provides a robust workflow for evaluating mitochondrial liabilities in 3D liver models, enhancing the utility of the assay utility for predictive DILI risk assessment in preclinical drug development.

Experimental

Materials

Table 1. Material and equipment used in the Agilent Seahorse XF organoid workflow.

Product	Vendor	Part Number
Seahorse XF Flex analyzer	Agilent	S7851A or S7851AN
Seahorse XF Organoid FluxPak		103866-100
Seahorse XF Flex organoid microplates		103865-100
Seahorse XF 3D Mito Stress Test kit		103016-100
Seahorse XF Cell Mito Stress Test kit		103015-100
Seahorse XF DMEM assay medium pack, pH 7.4		103680-100
BioTek Cytation 5 cell imaging multimode reader		
Matrigel matrix	Corning	356231
Mouse hepatic organoids	STEMCELL Technology	70932
Organoid culture plates, 96-well		200-0562
HepatiCult organoid growth medium (mouse)		06030
Hoechst 33342	Thermo Fisher	62249
Nefazodone	Millipore Sigma	N5536
Nimesulide		N1016
Rosiglitazone		R2408
Troglitazone		T2573

Hepatic organoid culture

Frozen mouse hepatic organoids were thawed and initially cultured in a Matrigel dome, then passaged according to the supplier's instructions using HepatiCult organoid growth medium (STEMCELL Technology). For Seahorse XF assays and image analysis, about 50 organoid fragments were seeded in 10 µL of Matrigel per well and cultured for three to four days. The same subculture conditions were applied when organoids were seeded in a 96-well STEMCELL organoid culture plate.

Seahorse XF Mito Stress Test

The XF Cell Mito Stress Test kit was used to achieve the optimal concentrations of oligomycin and rotenone/antimycin A (Rot/AA), 1.5 µM and 0.5 µM respectively (data not shown). On the day of assay, the cultured medium was replaced with 500 µL of prewarmed Agilent Seahorse XF DMEM medium (pH 7.4, supplemented with glucose, glutamine, and pyruvate). After washing once by replacing 450 µL of the assay medium, the plate was incubated at 37 °C for 45 minutes in a non-CO_2 incubator. Immediately before starting the assay, the medium was refreshed once and the final volume was adjusted to 500 µL. The stock preparation and injection volume determination of the kit components were performed as described in the Agilent Seahorse XF Cell Mito Stress Test kit user manual.[8]

Seahorse XF Mito Tox assay

Mitochondrial toxicity induced by test compounds was evaluated using the XF Mito Tox assay. The optimal concentrations for the sequential injections of oligomycin and FCCP (1.5 and 4 µM, respectively) were determined by the titration experiment described in the previous section (also see Figure 2). The assay medium replacement is identical to the XF Mito Stress Test described previously, except the degassing step in the non-CO_2 incubator that was reduced to 30 minutes. After the final wash with XF assay medium (final volume = 500 µL), the organoids were incubated with or without the test compounds listed in Table 2 for another 30 minutes at 37 °C in a non-CO_2 incubator. For more details about the principle and applications of this assay, see the Agilent Sesahorse Mito Tox assay white paper and application note.[3,4]

Table 2. Concentration of model compounds for mitochondrial toxicity test.

Compound	10x Cmax	Cmax[9]
Nefazodone	10 µM	1 µM
Nimesulide	100 µM	10 – 40 µM
Rosiglitazone	10 µM	1 µM
Troglitazone	50 µM	5 µM

Image analysis and data normalization

Organoid imaging was performed using the Agilent BioTek Cytation 5 cell imaging multimode reader with 4x PL FL objectives. Brightfield and fluorescence images were captured and z-projected. The total object area (Object Sum Area) or total fluorescence intensity integrated within the objects (Object Sum Int) was quantified using Gen5 software and used to normalize OCR data.

Data analysis

All data was uploaded to and analyzed in Agilent Seahorse Analytics using the companion views of the 3D Mito Stress Test and Mito Tox Screen. Three independent assay results executed using same assay template and instrument were combined using the **Project** feature in the Seahorse Analytics software.

Results and Discussion

Hepatic organoid culture on the Agilent Seahorse XF Flex organoid microplate

Mouse hepatic organoids were successfully regenerated from cryopreserved hepatic progenitor organoids (STEMCELL Technologies) and expanded directly on the Seahorse XF Flex organoid microplate. Organoid fragments formed spherical structures within four days of subculture (Figure 1A), with no detectable differences in morphology, growth rate, or viability compared with standard Matrigel-embedded dome cultures (data not shown). Representative brightfield images from the XF Flex organoid microplate and the STEMCELL organoid culture plate (Figure 1B) demonstrate uniform organoid growth across both plate formats with comparable Matrigel geometry (10 µL per well). These observations confirm that organoids can be reproducibly generated and maintained within the XF Flex organoid microplate without perturbing normal developmental or expansion characteristics, thereby supporting metabolic assays requiring matrix-embedded 3D liver models.

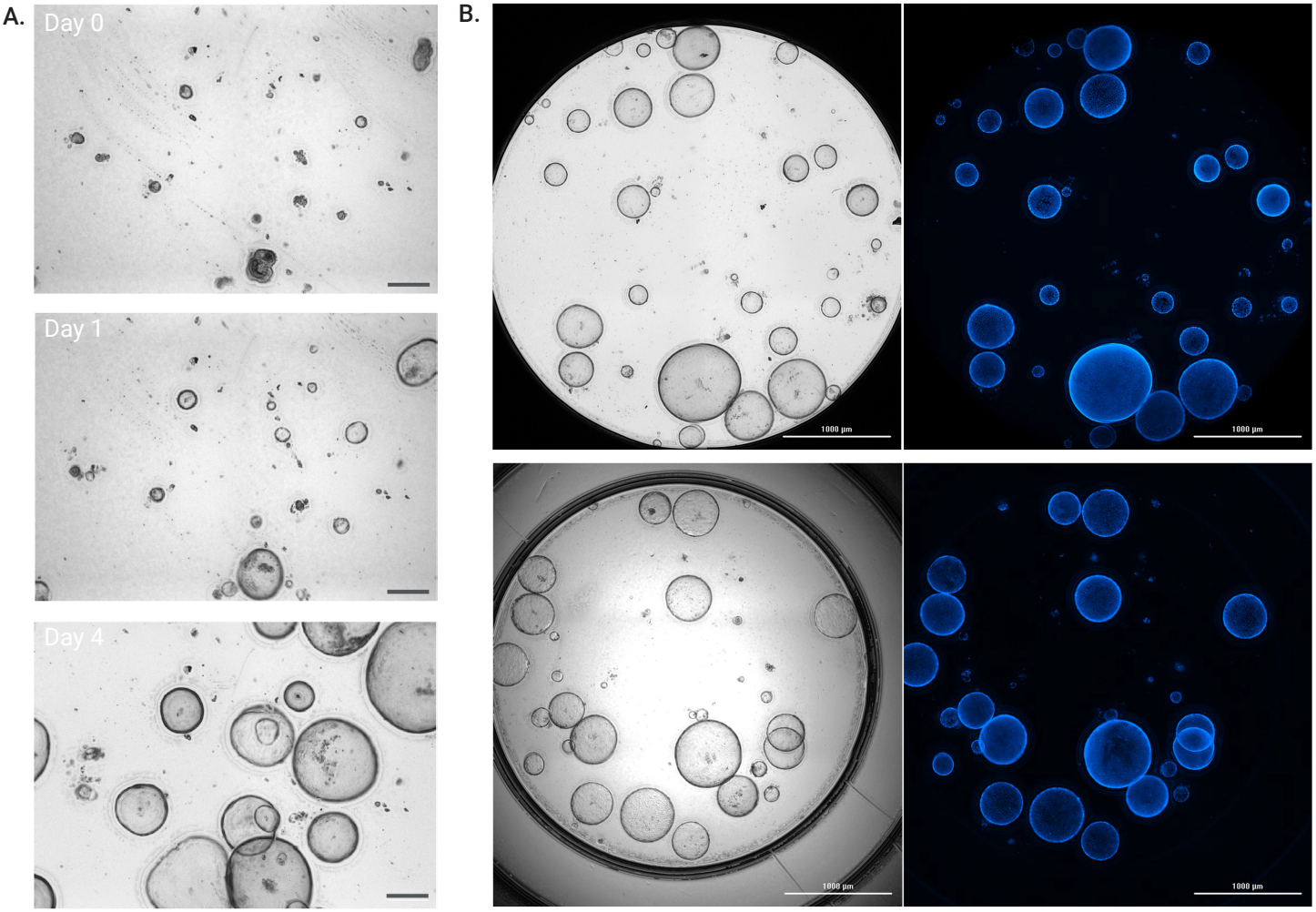

Figure 1. Mouse hepatic organoid growth in Agilent Seahorse XF Flex organoid microplates. A) Mouse hepatic organoid fragments were seeded and grown in 10 µL of Matrigel per well for four days on the Seahorse XF Flex organoid microplate. Z-projected 4x high contrast brightfield (HCBF) images were captured by the Agilent BioTek Cytation 5 cell imaging multimode reader using Gen5 software right after Matrigel crosslinking (Day 0), one (Day 1) and four days after (Day 4). Scale bar = 200 µm. B) Hepatic organoids were cultured for three days on the XF Flex organoid microplate (upper panels) and the STEMCELL organoid culture plate (lower panels) and stained with Hoechst 33342. Z-projected 4x HCBF (left) and DAPI fluorescence image (right). Scale bar = 1,000 µm.

Optimization XF Mito Tox assay condition

To establish optimal XF Mito Tox assay conditions for hepatic organoids, assay FCCP concentration was titrated to achieve maximal OCR. The XF Cell Mito Stress Test was performed on the organoids samples using three FCCP concentrations (1, 2, and 4 µM), while oligomycin (1.5 µM) and Rot/AA (0.5 µM) concentrations were constant. As shown in Figure 2, normalization of kinetic traces to baseline OCR (the final measurement before oligomycin addition) indicated that 4 µM FCCP produced the most robust and reproducible maximal respiration. This optimization establishes appropriate conditions for subsequent quantitative assessment of mitochondrial toxicants using the MTI.

Prior to measuring the toxicity of the test compounds, assay robustness was evaluated by Z' factor analysis (Figure 3). Z' values for detection of mitochondrial inhibitors exceeded 0.5 in two independent experiments, indicating high assay quality for detection of mitochondrial inhibition. Z' values for MTI uncoupler mode did not reach the 0.5 threshold, suggesting that for high throughput screening of uncoupling compounds, the assay may be less robust, but can still provide reliable mechanistic assessment when interpreted cautiously.

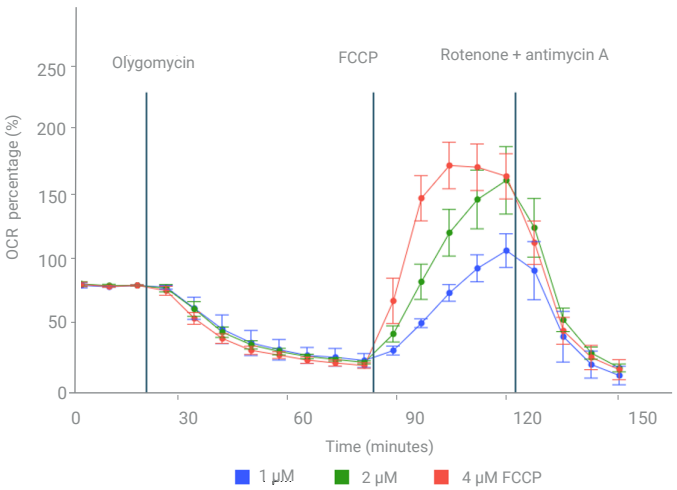

Figure 2. Optimization of FCCP injection concentration. The OCR of mouse hepatic organoids was measured under varying FCCP concentrations, while oligomycin and Rot/AA were held constant. Baseline normalized kinetic traces indicate that 4 µM FCCP yields the maximal OCR response.

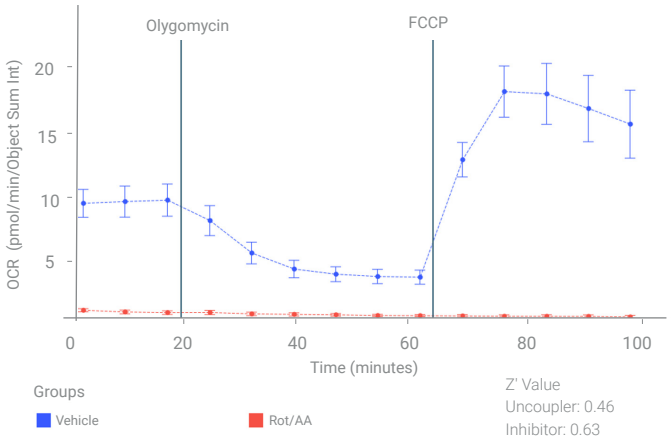

Figure 3. Assay performance evaluation by Z′ value. The robustness of the Agilent Seahorse XF Mito Tox assay using hepatic organoids was evaluated by Z′ calculation. All data were normalized by the integrated fluorescence intensity of Hoechst 33342 staining (Object Sum Int) measured from images captured using the Agilent BioTek Cytation 5 cell imaging multimode reader.

Mito Tox Index evaluation of model compounds

The MTI values of four mitochondrial toxicants (nefazodone, nimesulide, rosiglitazone, and troglitazone) were measured across three independent experiments using hepatic organoids derived from different batches (Figure 4A). Across all experiments, mechanistic classification was consistent: nefazodone, rosiglitazone, and troglitazone exhibited mitochondrial inhibition, whereas nimesulide induced mitochondrial uncoupling. When data from all runs were combined (Figure 4B), both the magnitude and direction of MTI responses were concordant with prior reports describing the mitochondrial liabilities of these compounds.

Among the inhibitors tested, troglitazone exhibited the strongest inhibitory MTI at 10x Cmax, consistent with its known mitochondrial toxicity and its withdrawal from clinical use due to severe DILI. Rosiglitazone, which has a lower or conditional mitochondrial risk,[7,10–12] elicited a moderate MTI response, whereas nefazodone demonstrated strong inhibitory effects consistent with its reported hepatotoxicity. Together, these findings demonstrate that the XF Mito Tox workflow in hepatic organoids can distinguish compound-specific mitochondrial liabilities in accordance with historical toxicological data.

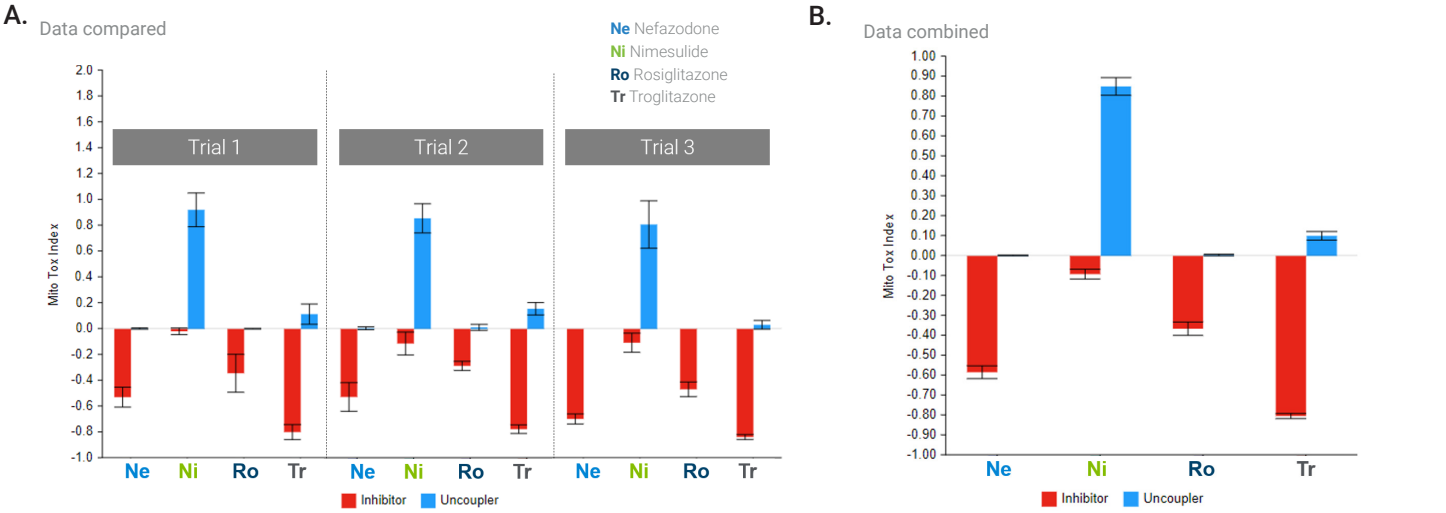

Figure 4. Mitochondrial toxicity of model compounds in mouse hepatic organoids measured using the Agilent Seahorse XF Mito Tox assay. Three independent XF Mito Tox assays were performed using each model compound at 10x Cmax. MTI values from the individual runs are shown side by side (A), and combined results are displayed in panel (B), as analyzed using Agilent Seahorse Analytics software.

Conclusion

This study demonstrates the feasibility and robustness of integrating the Agilent Seahorse XF Mito Tox assay with the Agilent XF Flex organoid microplate for use in hepatic organoids. Mouse hepatic organoids were reliably regenerated directly within the XF Flex organoid microplate without compromising growth or morphology, enabling direct real-time metabolic analysis of matrix-embedded 3D liver models. Optimization of assay conditions, particularly FCCP concentration, established parameters suitable for quantifying mitochondrial responses in organoids.

Using four well-characterized mitochondrial toxicants, the workflow generated reproducible MTI values that reflected expected mechanistic profiles, including clear differentiation between mitochondrial inhibition and uncoupling. The strong concordance with previously reported mitochondrial and hepatotoxicity profiles highlights the quantitative capability and biological relevance of the platform.

Together, these results support the XF Flex organoid microplate-based XF Mito Tox workflow as a robust and predictive tool for assessing compound-induced mitochondrial dysfunction in hepatic organoids. This organoid culture platform expands the applicability of the XF Mito Tox assay to 3D culture systems and enhances its relevance for preclinical DILI risk assessment, offering improved physiological relevance compared to traditional 2D systems.

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Products used in this application

Agilent products

Agilent Seahorse XF Flex analyzer

Agilent Seahorse XF Flex organoid FluxPak

Agilent Seahorse 3D Mito Stress Test kit

Agilent Seahorse XF Cell Mito Stress kit

Agilent Seahorse XF DMEM assay medium pack

Agilent BioTek Cytation imaging multimode reader

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