A Novel Seahorse XF Assay for Measuring Mitochondrial Function in Matrigel-Embedded Organoids

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Introduction

Organoids offer improved physiological relevance and ethical advantages compared to two-dimensional (2D) cultures and animal models. The Food and Drug Administratoin (FDA) supports the use of validated New Approach Methodologies (NAMs), including organoids, to reduce animal testing and enhance translational research.

In alignment with FDA priorities, a new assay workflow was developed using the Agilent Seahorse XF Flex analyzer and Agilent Seahorse XF Flex organoid microplate. The XF Flex organoid microplate allows organoids to be cultured in extracellular matrix (ECM) gels like Matrigel for several days before metabolic or imaging assays.

The new plate and workflow enable real-time measurement of key metabolic parameters—such as mitochondrial health, toxicity, and glycolysis—in physiologically relevant three-dimensional (3D) models. The unique design, which incorporates a highly transparent well bottom, also enables high-magnification images, including confocal, to be captured in the same well.

Experimental

Seahorse XF Organoid Assay

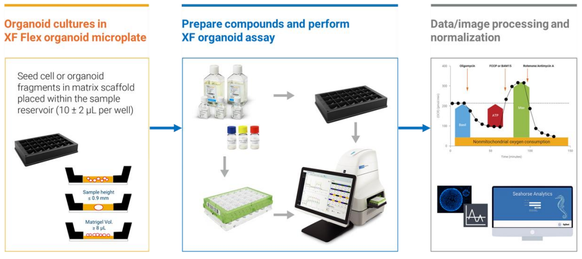


Figure 1. Seahorse XF Flex organoid assay workflow using the Agilent Seahorse XF Flex organoid microplate. Cells or organoids embedded in Matrigel (10 µL per well) are cultured for several days in an XF Flex organoid microplate to assess their metabolic profile using the Agilent Seahorse XF Flex analyzer. The intact organoids can subsequently be used for additional image analysis.

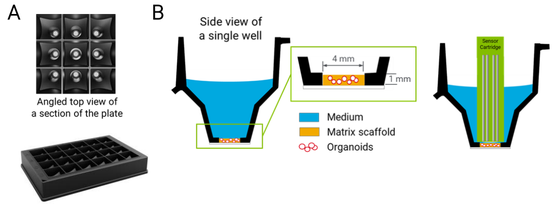
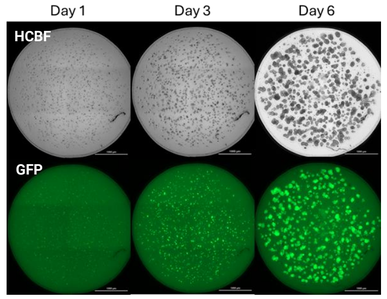


Figure 2. Illustration of the Agilent Seahorse XF organoid microplate. (A) The XF Flex organoid microplate is a 24-well microplate with a black side wall and a clear/thin film bottom is compatible with high-resolution imaging. (B) Organoids embedded in a matrix scaffold, such as Matrigel, are grown in the sample reservoir of 1 mm (height) x 4 mm (diameter) allowing for matrix volume of 10 ± 2 µL per well to be placed securely within the microchamber formed with the sensor probe.

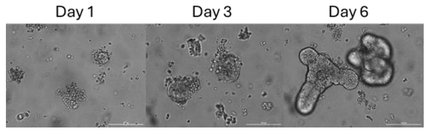
Results and Discussion

Organoid culture on Agilent Seahorse XF Flex organoid microplates

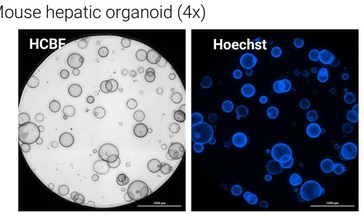
A. HCT116-H2B-GFP (4x)



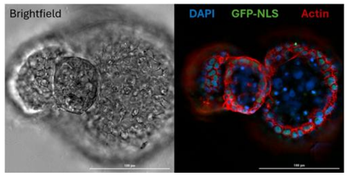
B. Mouse intestinal organoids (20x)



C. Mouse hepatic organoid (4x)



D. MCF10A (confocal, 60x)



E. MCF10A (live vs. fixed, 40x)

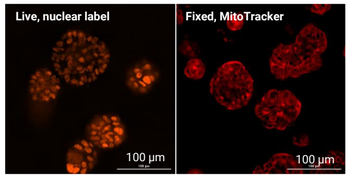


Figure 3. Imaging of organoids cultivated in the Agilent Seahorse XF Flex organoid microplates. (A) HCT116-H2B-GFP colorectal cancer cell line-derived organoid cultured for six days. (B) Mouse intestinal organoids cultured from organoid fragments for six days (STEMCELL Technologies). (C) Mouse hepatic organoids cultured from organoid fragments for three days (STEMCELL Technologies). (D and E) MCF10A cell line-derived organoids imaged using the Agilent BioTek Cytation C10 Confocal imaging reader. Organoids can be imaged live or after fixation.

XF 3D Mito Stress Test using organoids

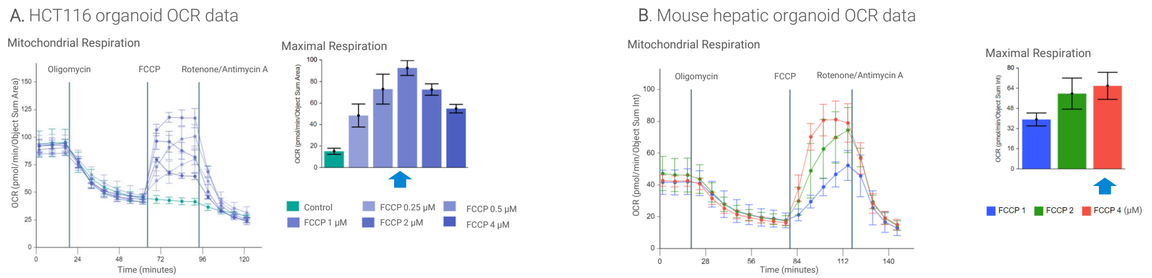


Figure 4. Identification of optimal FCCP dose for organoids.
The dose-dependent uncoupling effects of FCCP on mitochondrial respiration of two different organoid models were measured and analyzed. The OCR data was normalized by total object area or by the integrated fluorescence intensity from Hoechst 33342, which was measured by an Agilent BioTek Cytation cell imaging system.
A. FCCP titration assay identifying 1 µM as the optimal dose for HCT116 cancer organoid model.
B. FCCP titration assay identifying 4 µM as the optimal dose for mouse hepatic organoid model.

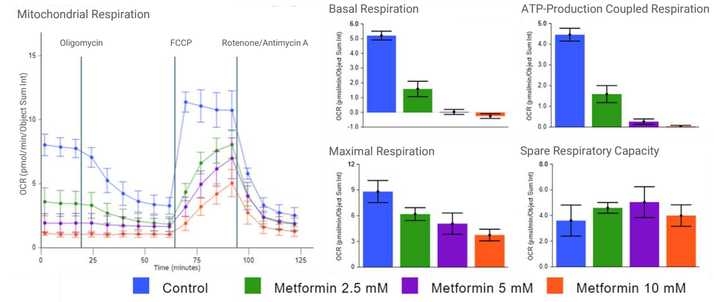


Figure 5. Metformin effect on the mitochondrial respiration in the HCT116-H2B-GFP cancer organoids. HCT116-H2B-GFP cells were cultured in Matrigel (5 mg/mL protein) for six days and exposed to 2.5, 5, or 10 mM metformin overnight before the XF Mito Stress Test was performed. The OCR data was normalized by the integrated fluorescence intensity from Hoechst 33342, measured by an Agilent BioTek Cytation cell imaging system.

Data normalization

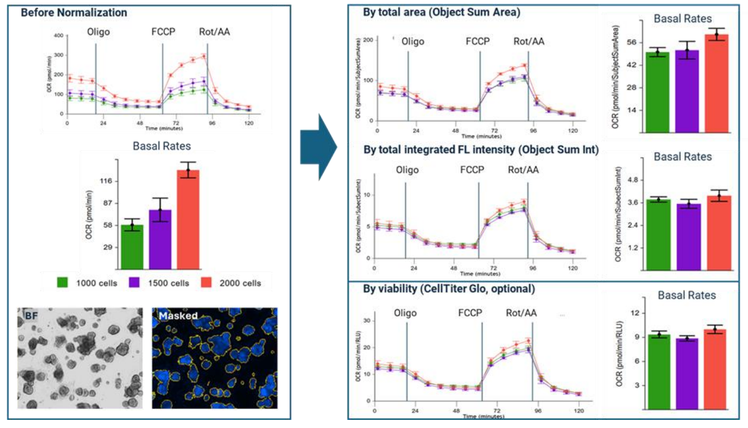


Figure 6. Comparison of normalization methods. OCR data were obtained from organoids of varying amounts, normalized by total object area (Object Sum Area), integrated fluorescence intensity (Object Sum Int), and viability measured using the CellTiter-Glo 2.0 cell viability assay (reported as relative luminescence units, RLU).

Conclusion

The Agilent Seahorse XF organoid assay using the Agient Seahorse XF Flex organoid microplate is an innovative addition to the Seahorse XF technology suite. It extends real-time metabolic analysis beyond traditional 2D cell cultures, enabling robust measurements of drug-induced changes in mitochondrial respiration across diverse 3D organoid models.

The new XF Flex organoid microplate supports long-term culture of matrix-embedded organoids and securely holds 3D samples during metabolic analysis and high-resolution imaging.

Normalization of XF Flex organoid data primarily relies on estimating organoid size and cell number using high-contrast brightfield (HCBF) imaging combined with fluorescence imaging. When image-based size estimation is limited or organoids contain a high proportion of dead cells, viability assays such as CellTiter-Glo (Promega) offer an alternative normalization approach.

The workflow is straightforward, adaptable to a wide range of 3D samples embedded in matrix scaffolds and designed to empower physiologically relevant discoveries across multiple areas of biomedical research.

References

Measuring Mitochondrial Function of Matrix-Embedded Organoids using the Agilent Seahorse XF Flex Analyzer, Agilent Technologies, technical overview, publication number 5994-8742EN, 2025.