Technical Overview

Cell Metabolism

Agilent

Trusted Answers

Measuring Mitochondrial Function of Matrix-Embedded Organoids Using the Agilent Seahorse XF Flex Analyzer

Introduction

The ability to assess mitochondrial function has significantly advanced our understanding of metabolism's central role in cellular physiology, disease pathology, and etiology. With increasing interest in physiologically relevant three-dimensional (3D) models, organoids have emerged as powerful tools in preclinical research. By more accurately replicating the structural and functional characteristics of real organs, organoids overcome many of the limitations associated with conventional two-dimensional (2D) cell cultures and even live-animal models. Notably, the Food and Drug Administration (FDA) roadmap to reduce animal testing highlights the importance of validated New Approach Methodologies (NAMs), including the use of organoids, to improve translational relevance and ethical standards in biomedical research.

In alignment with FDA priorities focused on new approach methodologies, we developed an assay workflow to interrogate energy metabolism in organoid cultures using the Agilent Seahorse XF Flex organoid microplate (XF Flex organoid microplate) and the Agilent Seahorse XF Flex analyzer (XF Flex). The XF Flex organoid microplate (Figure 1) contains a sample reservoir of 1 mm (height) x 4 mm (diameter) that enables culturing of organoids embedded in a scaffold structure (such as Matrigel or other hydrogel containing extracellular matrix) for days before being used in Agilent Seahorse XF assays or high-resolution imaging assays. The Seahorse XF Flex organoid workflow enables researchers to obtain key metabolic parameters that are vital indicators of mitochondrial health, toxicity, glycolysis, and overall cellular (dys)function, using physiologically more relevant models.

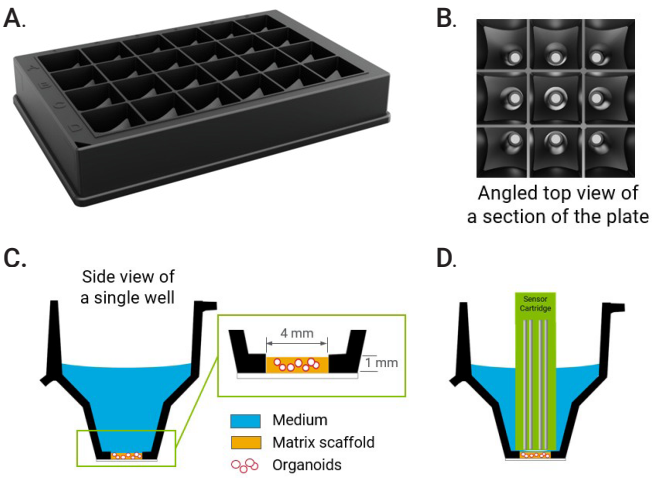

Figure 1. Illustration of the Agilent Seahorse XF organoid microplate, a 24-well microplate with a black side wall and a clear/thin film bottom (A and B), compatible with high-resolution imaging. Organoids embedded in a matrix scaffold, such as Matrigel, are grown in the sample reservoir of 1 mm (height)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

This technical overview provides detailed procedures and guidelines for establishing and maintaining organoid cultures within the Seahorse XF Flex organoid microplates and performing Seahorse XF assays. It includes proof-of-concept examples of using this workflow to determine organoid metabolic profiles and guidance on data normalization considerations. Given the variability in organoid culture conditions depending on the sample source and characteristics, users should optimize protocols and procedures using the provided guidelines.

Seahorse XF Flex assay workflow for organoids cultured in matrix scaffold

The assay workflow illustrated in Figure 2 outlines the key steps for performing the Seahorse XF assay with organoid samples, from organoid preparation to data analysis. Organoids can be cultured from single cells, organoids, or tissue fragments for days in the sample reservoir filled with a matrix scaffold, such as Matrigel. The sensor cartridge must be hydrated one day prior to performing Seahorse XF assays. The details of the steps involved in this workflow are described in the following sections. Users may adjust this workflow based on the type of organoids, matrix scaffold, and research objectives.

Prepare organoid cultures in the XF Flex organoid microplate

Organoids can be cultured in the XF Flex organoid plate from cells or organoids, obtained in vitro, or isolated from organisms or biopsy materials. The organoid culture conditions may vary depending on the user's experimental needs. Therefore, user organoid-specific protocols and user optimal matrix scaffold type and concentration can be adapted. The protocol example introduced here is for organoid cultures in 50 to 100% Matrigel.

The cells or organoids, suspended in Matrigel, may be seeded in the XF Flex organoid microplate, using the single-step strategy or the two-step strategy (Figure 3). The first strategy involves a simple and direct placement of a 10 µL of cell or organoid/Matrigel suspension into each sample reservoir (Figure 3A). This can be done manually or by automated bioprinting equipment. The second strategy involves a coating

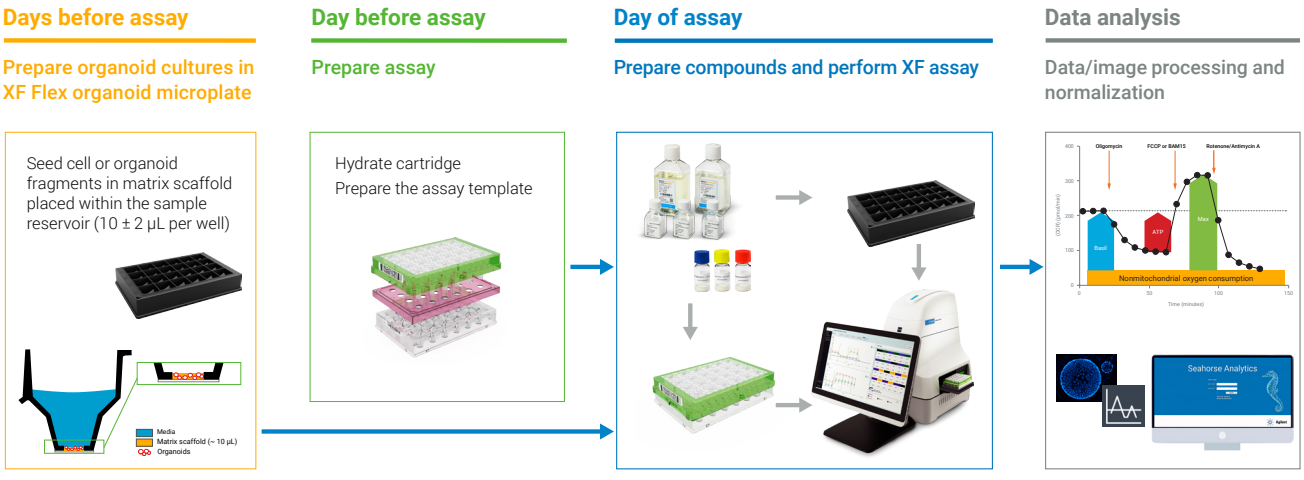

Figure 2. Seahorse XF Flex organoid assay workflow using the Agilent Seahorse XF Flex organoid microplate.

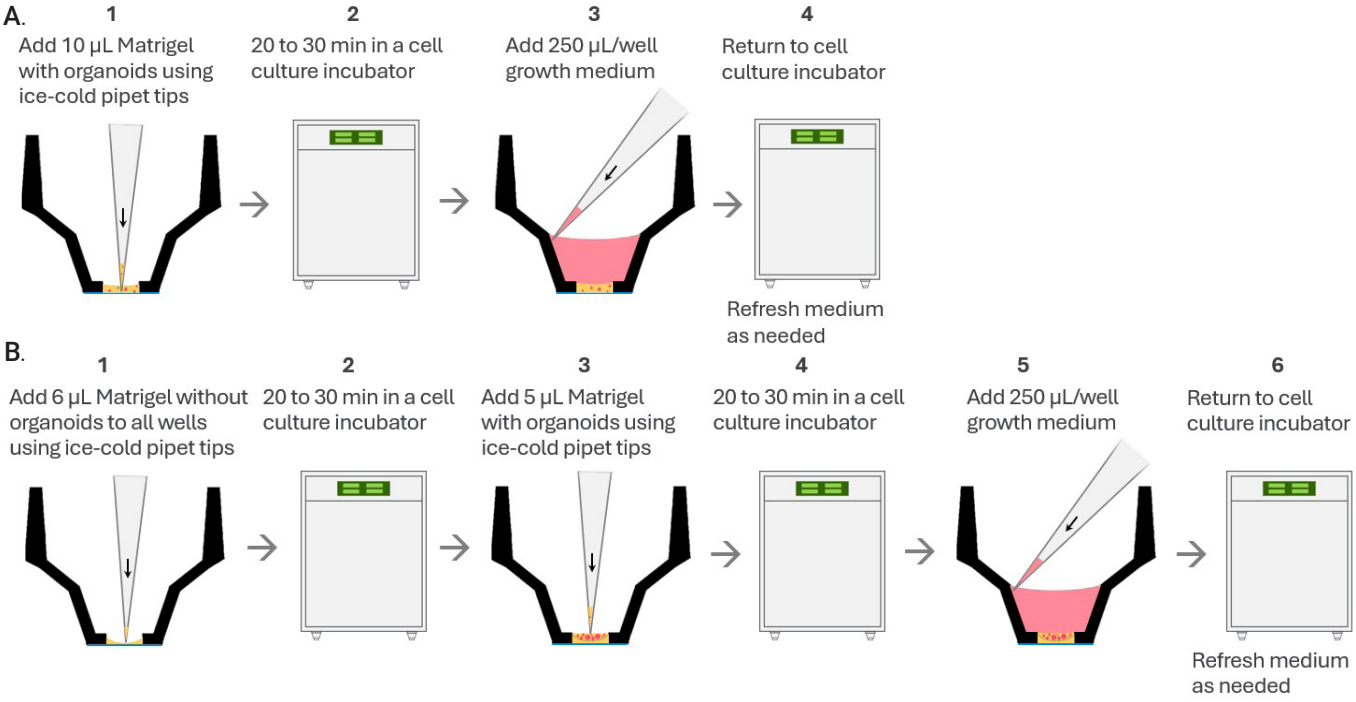

Figure 3. Organoid seeding procedure. A. Single-step seeding procedure. B. Two-step seeding procedure. The organoids are cultured until they are ready for Agilent Seahorse XF assays.

step with plain Matrigel before adding organoid materials (Figure 3B). This strategy can prevent unintended 2D growth of cells under the Matrigel and organoid clustering at the edge of wells.

Follow the steps outlined to complete the organoid culture preparation in the XF Flex organoid microplate.

Single-step seeding procedure

1. Resuspend cells or organoids in ice-cold 50% Matrigel diluted in culture medium or phosphate-buffered saline (PBS). Use ice-cold pipette tips to maintain temperature and prevent premature polymerization.
2. Carefully dispense 10 µL of the Matrigel-cell/organoid suspension into the sample reservoir of each well. Gently swirl the pipette tip to ensure even distribution of the material. **Do not seed wells A1 and D6, as these will serve as background wells during the Seahorse XF assay.**
3. Add 10 µL of diluted Matrigel without any cell or organoid **in wells A1 and D6** in the same way.
4. Incubate the plate in a humidified CO_2 incubator at 37 °C for 20 to 30 minutes to allow Matrigel to polymerize.
5. Gently add 250 µL of prewarmed growth medium to each well to cover the polymerized Matrigel with sample.
6. Maintain the organoid cultures as required. Refresh the medium according to the user's specific organoid culture condition.

Two-step seeding procedure

1. Dispense 6 µL/well of the diluted Matrigel without any cells or organoids in the sample reservoir of each well. Gently swirl the pipette tip to ensure the Matrigel evenly coats the bottom and edge of the well.
2. Incubate the plate in a humidified CO_2 incubator at 37 °C for 20 to 30 minutes to allow the Matrigel to polymerize.
3. Resuspend cells or organoids in ice-cold Matrigel diluted in culture medium or PBS by using cold pipette tips. Carefully dispense 4 to 5 µL of the Matrigel–cell/organoid suspension on top of the polymerized Matrigel layer. **Do not seed wells A1 and D6 as these will serve as background wells during the Seahorse XF assay**.
4. Add 4 to 5 µL of the diluted Matrigel without any cells or organoids in the background wells in the same way.
5. Incubate the plate again in a humidified CO_2 incubator at 37 °C for 20 to 30 minutes to allow the top Matrigel layer to polymerize.
6. Gently add 250 µL of prewarmed growth medium to each well to cover the polymerized Matrigel layer.
7. Maintain the organoid cultures as required. Refresh the medium according to the user's specific organoid culture condition.

Note: Minimally, two **background** wells (typically A1 and D6) are required for each Seahorse XF Flex assay. So, a total of 22 samples can be analyzed in each assay. The background wells must not contain any cells or organoids. It is necessary to fill these wells with Matrigel at the same concentration and volume as in the sample wells.

The origin and culture conditions of the organoids may require optimization to fit within the sample reservoir and may be embedded in a total of 10 ± 2 µL of matrix scaffold per well. The type and concentration of the matrix scaffold may vary depending on the user's protocol and organoid characteristics. It is essential that the scaffold maintains its gel state during Seahorse XF analysis at 37 °C and does not alter the pH of the assay medium.

Prepare assay one day before assay

Cartridge hydration

Fill each well of the utility plate with 1 mL of XF calibrant solution and place the cartridge and utility plate ensemble in a 37 °C, non-CO_2 incubator overnight.

Note: Power on the Seahorse XF Flex analyzer to allow the temperature to stabilize overnight.

Template design

A Seahorse XF assay template contains the instrument protocol that instructs the Seahorse analyzers to collect measurements and execute injections. It also contains assay design information (such as plate layout) that is required to support downstream data transformation within Agilent Seahorse Analytics. The Seahorse XF assay template must be prepared prior to the assay, which can be accomplished using the Agilent Seahorse XF Flex Controller or Wave Pro software. Agilent Seahorse Analytics, a web-based tool, can also be used for template creation.

A default assay template for performing the Agilent Seahorse XF Organoid Mito Stress Test using the XF Flex organoid microplate (XF Organoid Mito Stress Test) is provided in the software. The instrument protocol in this template contains a 3-minute mix, 0-minute wait, and 4-minute measurement in each measurement cycle (Figure 4). A total of 18 cycles (three cycles for baseline measurement, six cycles after oligomycin injection, four cycles after FCCP injection, and five cycles after Rot/AA injection) are included. It is not recommended to change the parameters within a measurement cycle. However, the number of cycles may

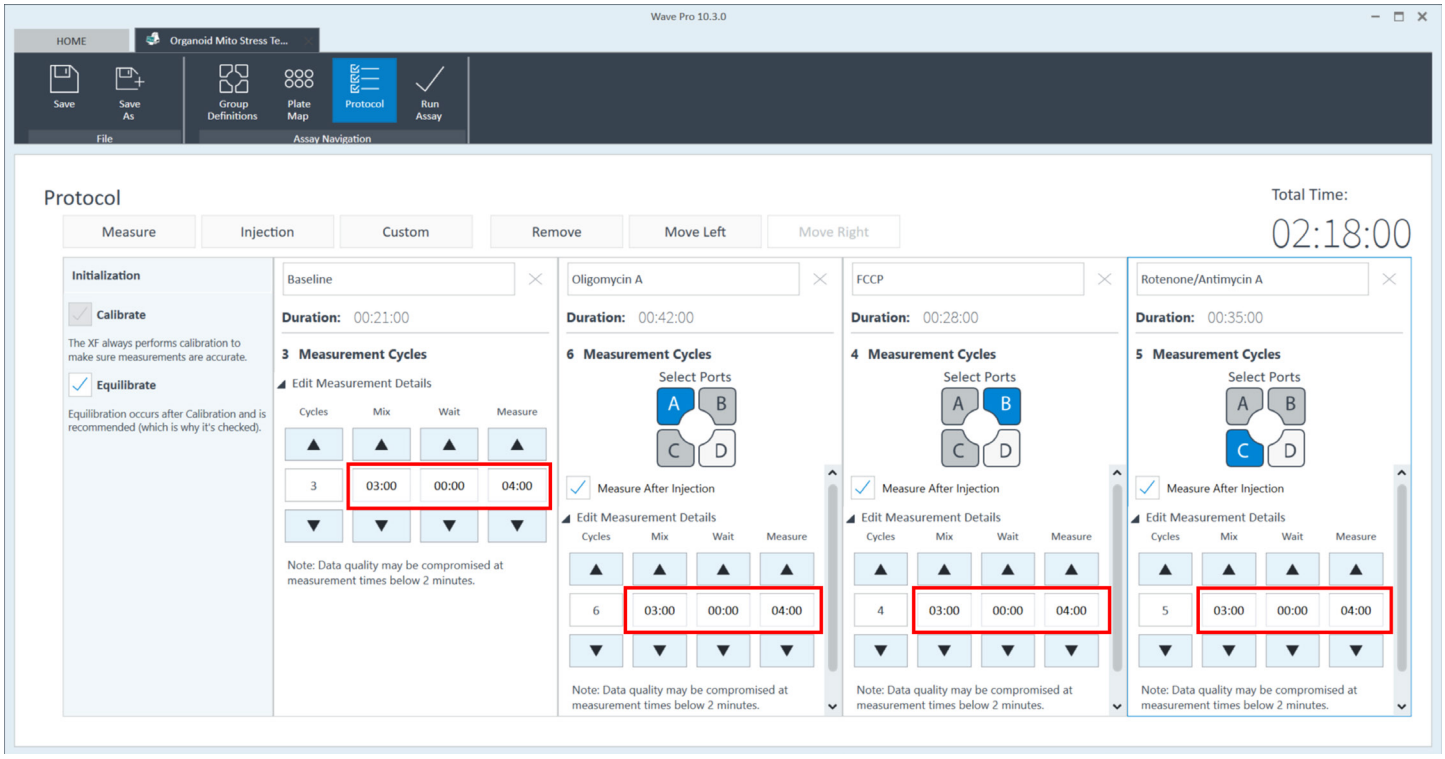

Figure 4. Agilent Seahorse XF Flex instrument protocol in the default **XF Organoid Mito Stress Test** template.

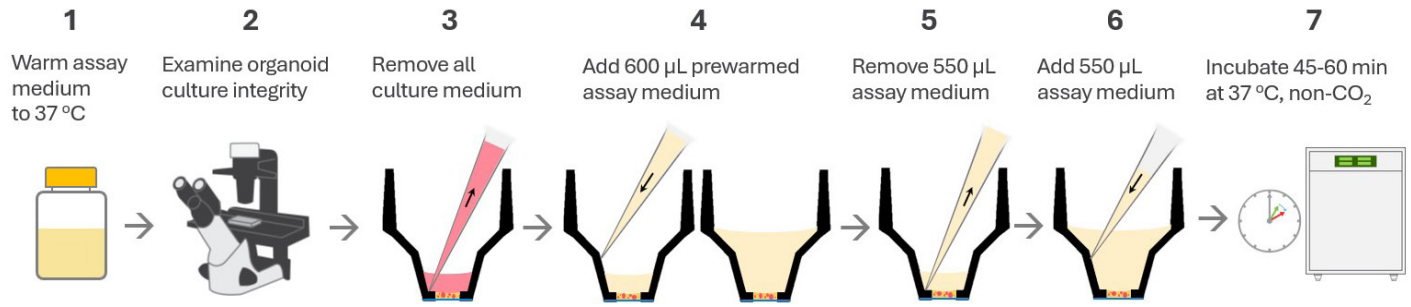

Figure 5. Replace the culture medium in Agilent Seahorse XF Flex organoid microplate to prewarmed Agilent Seahorse XF assay medium.

be modified as needed. For instance, if the response to a mitochondrial modulator does not reach the maximum response within the programmed measurement cycles, it may be necessary to extend the number of measurement cycles to capture the full response.

Day of assay

Assay medium preparation

Prepare the assay medium following the information in Table 1. No pH adjustment to the assay medium is necessary when standard Agilent Seahorse XF supplement concentrations are used. However, the assay medium composition can be modified if desired.

Table 1. Agilent Seahorse XF assay medium preparation

Component	Volume (mL)	Final Concentration (mM)
Seahorse XF DMEM medium, pH 7.4 or XF RPMI medium, pH 7.4	97	–
Seahorse XF 1.0 M glucose solution	1	10
Seahorse XF 100 mM pyruvate solution	1	1
Seahorse XF 200 mM glutamine solution	1	2

Note: BSA and serum should not be included in the medium or in the injection ports.

XF Flex organoid microplate preparation

Replace the organoid growth medium with prewarmed (37 °C) Seahorse XF assay medium using the technique illustrated in Figure 5.

1. Remove the growth medium without touching the organoid culture layer by positioning the pipette tip on the side wall of each well (Step 3).
2. Gently add 600 µL of prewarmed Seahorse XF assay medium (Step 4).
3. Remove 550 µL Seahorse XF assay medium and add 550 µL of Seahorse XF assay medium. (Step 5 and 6)
4. Place the XF Flex organoid microplate in 37 °C non-CO_2 incubator and keep it for 45 to 60 minutes. (Step 7) for plate degassing.

Mito Stress Test compound preparation

Note: Use compounds on the same day that they are reconstituted. Discard any remaining compound solutions. Do not freeze and re-use.

Note: The last injection (e.g. Rot/AA) solution may include 10 µM Hoechst 33342 (1 µM well final concentration) to stain nuclei for imaging and data normalization purposes.

Resuspend the contents of each vial using prewarmed assay medium in the volumes indicated in Table 2. Vortex for 30 seconds or gently pipette the solution up and down to fully dissolve the contents. The resulting stock solutions may require further dilution prior to port loading as exemplified in Table 3. Optimal reagent concentrations for each reagent can vary depending on the organoid size and type. Therefore, it is recommended to perform titration assays to determine the appropriate working concentrations, particularly for FCCP which can exhibit variable responses across different organoid models.

Table 2. Preparation of stock solutions for the Agilent Seahorse XF 3D Mito Stress Test kit.

Reagent	Cap Color	Assay Media to Add (mL)	Injection Solution Concentration (X)	Stock Solution Concentration (µM)	Final Well Concentration (µM)
Oligomycin A	Blue	2.7	9	270	30
FCCP	Yellow	2.7	10	200	20
Rot/AA	Red	2.7	11	110	10

Table 3. Preparation of injection solutions for the Agilent Seahorse XF 3D Mito Stress Test kit. The concentration and loading volumes correspond to a starting volume of 600 µL per well in an Agilent SeahorseXF Flex organoid microplate.

Injection Solution	Stock Solution (µL)	Assay Medium (µL)	Concentration in Well (µM)	Loading Port and Volume
Oligo A (9 x)	1000	2000	10	Port A: 75 µL
	2000	1000	20	
	2700	0	30	
FCCP (10 x)	750	2250	5	Port B: 75 µL
	1500	1500	10	
	2250	750	15	
	2700	0	20	
*Rot/AA (11 x)	2700	0	10	Port C: 75 µL

Note: The optimal reagent concentrations for some small-sized organoids can be supported by the Agilent Seahorse XF Cell Mito Stress kit (103595-100). In these cases, follow the instructions provided in the Agilent Seahorse XF Cell Mito Stress kit user guide to prepare the assay medium and injection solutions.

Note: Some cell or organoid types have better uncoupling responses to the uncoupler Bam15 than to FCCP (e.g. T cells, NK-T cells, MCF-10A, etc.). Agilent Seahorse XF T Cell Metabolic Profiling Assay Kit (103772-100) is recommended to be used for those types of samples.

To perform a modified assay which includes an additional injection of a test compound prior to oligomycin injection (3D Mito Stress Test (Acute)), use a starting volume of 525 µL in the well and the loading port volumes shown in Table 4.

Table 4. Preparation of injection solutions for the modified assay. The concentration and loading volumes correspond to a starting volume of 525 µL per well in an Agilent Seahorse XF Flex organoid microplate.

Injection Solution	Port Concentration (Fold)	Loading Port and Volume
Test compound	8x	Port A: 75 µL
Oligomycin A	9x	Port B: 75 µL
FCCP	10x	Port C: 75 µL
Rot/AA	11x	Port D: 75 µL

Loading injection ports

1. Remove the assembled sensor cartridge with the Seahorse XF hydrobooster and utility plate from the incubator. Place the sensor cartridge upside down, next to the utility plate. Hold the utility plate steadily with one hand and use the other hand to remove the hydrobooster by lifting it from one corner. Place the sensor cartridge back onto the utility plate. Remove the growth medium without touching the organoid culture layer by positioning the pipette tip on the side wall of each well (Step 3).
2. Carefully dispense the port injection solution(s) into each port as indicated in Table 3 or Table 4.
3. Visually inspect the injection ports for even loading. The liquid should be in the port. Make sure there are no residual drops on top of the cartridge.

Performing Seahorse XF assay

Note: The measurement cycles must be composed of 03:00 minute mix, 00:00 minute wait and 04:00 minute measurement (Figure 4).

1. Select the right assay template from the list of available templates and follow the instrument prompts to perform the assay.
2. Review the groups (cell types or conditions), plate layout map, and instrument protocol for your assay; modify if desired.
3. Click Start Run to start the Seahorse XF assay.
4. When prompted, remove the cartridge lid, and place the loaded sensor cartridge with the utility plate on the thermal tray. Ensure correct plate orientation and that the cartridge lid has been removed. Then, click I'm Ready. Calibration takes approximately 15 to 30 minutes.
5. After completing calibration, click Open Tray to eject the utility plate and load the cell plate. Ensure that the lid is removed from the cell plate before loading.
6. Click Load Cell Plate to run the assay.

Note: Post Seahorse XF assay, the organoid samples can be further processed by either imaging-based or other methods to quantify the amount organoids in each well for data normalization purpose. See Data normalization section at the end of the document for more details.

Data analysis

Seahorse assay result files can be uploaded in Seahorse Analytics for further data analysis. Figure 6 shows a typical OCR kinetic graph from 'Standard Companion View'. The Seahorse XF organoid microplate is compatible only with selective Agilent Seahorse XF assays (see Table 6 in the Materials and equiment section). Users can assess glycolysis only by measuring extracellular acidification rates (ECARs). The PER or glycoPER calculation is not available for the data generated with XF Flex organoid microplate due to the variation in proton diffusion rate caused by Matrigel.

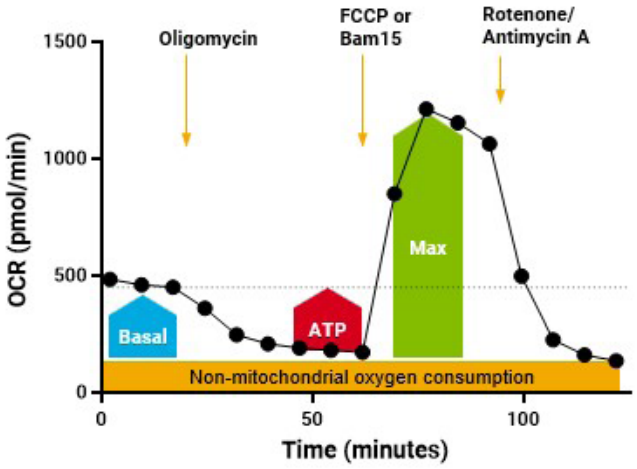

Figure 6. A schematic example of Agilent Seahorse XF Mito Stress Test using organoid cultures.

Materials and equipment

Table 5. Material and equipment used in XF organoid workflow.

Material	Vendor	Part Number
Seahorse XF Flex analyzer	Agilent Technolgies	S7851A or S7851AN
Seahorse XF Flex organoid FluxPak		103866-100
Seahorse XF Flex organoid microplates		103865-100
Seahorse XF 3D Mito Stress Test kit[1]		103016-100
Seahorse XF Cell Mito Stress Test kit[2]		103015-100
Seahorse XF DMEM assay medium pack, pH 7.4		103680-100
BioTek Cytation cell imaging multimode reader		
Matrigel matrix	Corning	356231
Cell Recovery Solution	Corning	354253
Mouse hepatic progenitor organoids	STEMCELL Technology	70932
HepatiCult Organoid Growth Medium (Mouse)	STEMCELL Technology	06030
DMEM/F-12 with 15 mM HEPES	STEMCELL Technology	36254
HCT116-H2B-GFP		
DMEM	Gibco	11995-065
Hoechst 33342	Thermo Fisher	62249

1. Some cell types have better uncoupling response to Bam15 than FCCP (such as T cells, NK-T cells, MCF-10A). The Agilent Seahorse XF T Cell Metabolic Profiling Assay kit (103772-100) can be used for organoids composed of those cell types.
2. The Seahorse XF Cell Mito Stress Test kit (103595-100) can be an alternative for use with small organoids which do not require high compound concentrations.
3. For additional Seahorse assays, please refer to the Seahorse XF assay kits and reagents brochure.

Applications

Hepatic organoids: Single-step seeding procedure

The metabolic phenotype was assessed in functional organoids generated from mouse hepatic progenitor organoids (STEMCELL, part number 70932). Briefly, organoid fragments were subcultured by resuspending in 100% Matrigel according to the manufacture's guidelines and 10 µL per well of Matrigel-organoid suspension was dispensed in the XF Flex organoid microplates using the one-step seeding procedure (Figure 3A). Stable spherical organoids are formed within three days and can be used up to five days after seeding. Z-projected brightfield and fluorescent images of the three-day old mouse hepatic organoids are shown in Figure 7.

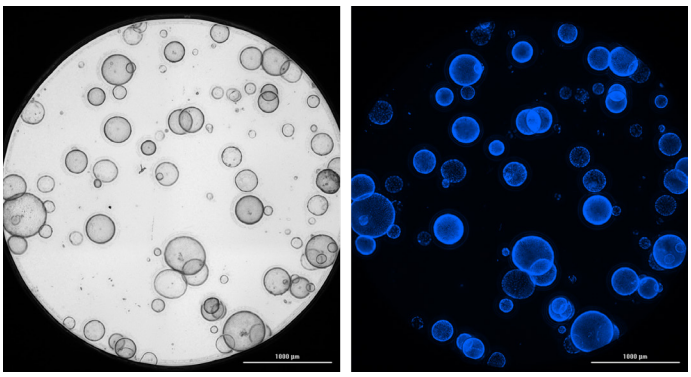

Figure 7. Three-day mouse hepatic organoids cultured in the Agilent Seahorse XF Flex organoid microplate. Brightfield (left) and fluorescent (right) images were captured by the Agilent Biotek Cytation cell imaging system using a Gen5 software protocol including z-stacking with five slices over an 800 µm thickness (z-projected), post XF Mito Stress Test. Hoechst 33342 was injected together with rotenone/antimycin in the last step of the assay.

Figure 8 shows the result of a typical XF Mito Stress Test with different FCCP concentrations. The data clearly demonstrates the feasibility of using the XF Flex organoid plate and XF Flex analyzer to perform metabolic analysis in organoids, with robust responses observed to all modulators. This FCCP titration experiment reveals that 4 µM is the concentration that results in the maximal OCR after uncoupler injection. The optimal concentrations of oligomycin and rotenone/antimycin A are also determined to be 1.5 and 0.5 µM, respectively (data not shown). Please note that the maximal responses to mitochondrial modulators are not reached immediately, especially for oligomycin which does not reach plateau within six measurements. Thus, a total of eight measurement cycles were applied after oligomycin injection to assess the correct ATP-linked OCR and other parameters. This is frequently observed as the presence of Matrigel can delay compound penetration into organoids.

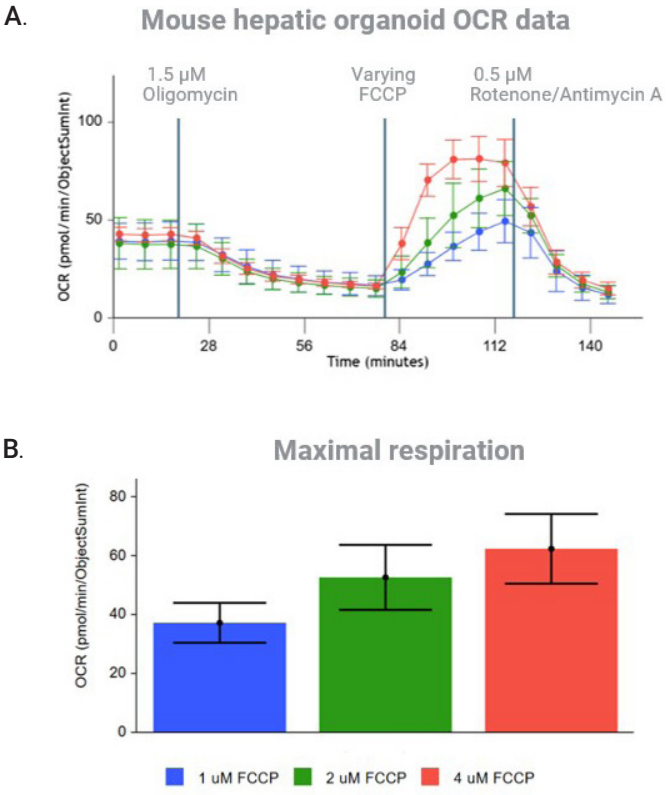

Figure 8. FCCP titration assay in mouse hepatic organoids. A. OCR kinetic graph illustrating responses to increasing FCCP concentrations. B. Maximal respiration values from Panel A. OCR data were normalized by the integrated fluorescent intensity (ObjectSumInt) from Hoechst 33342 nuclear staining, which was measured by an Agilent BioTek Cytation cell imaging system. For detailed methodology, refer to the 'Data normalization' section.

Cancer cell-originated organoids: Two-step seeding procedure

Tumor organoid culture can be initiated from in vitro cultured primary cells or tissue biopsies. This example used was performed using the HCT116-H2B-GFP cell line grown with Matrigel in the XF Flex organoid microplate for five to six days. Briefly, cells were resuspended in ice-cold growth medium at 1 x 10^5 to 5 x 10^5 cells/mL and mixed with Matrigel at a 1:1 ratio (50% Matrigel with ~ 5 mg/mL final protein concentration). Five µL of the mixture was placed on top of the preformed Matrigel layer in each well, following the two-step seeding procedure (Figure 3B), which results in a 500 to 2,000 cells/well seeding density. The images in Figure 9 show a five-day organoid culture generated using a seeding density of 1,000 cells/well in the XF Flex organoid microplate. Multiple organoids in the culture are evident with sizes ranging from 50 to 150 µm. This type of organoid culture (small with relatively consistent size) responds well to low concentrations of mitochondrial modulators, 1.5 µM oligomycin and 0.5 µM rotenone/antimycin mix.

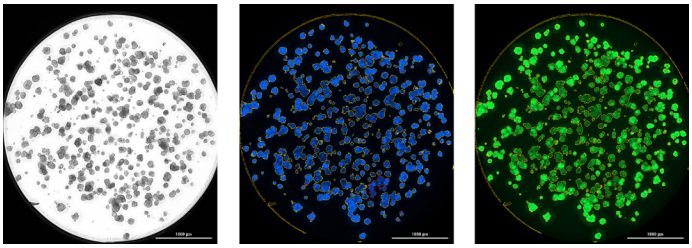

Figure 9. Five-day HCT116-H2B-GFP organoids culture in the Agilent Seahorse XF Flex organoid microplate. Brightfield (left) and fluorescence images (middle and right) were captured by an Agilent BioTek Cytation cell imaging system using a Gen5 software protocol that includes z-stacking with five slices over an 800 µm thickness (z-projected), post XF Mito Stress Test. Hoechst 33342 was injection together with rotenone/antimycin A in the last step of the assay.

In the FCCP titration assay, six concentrations ranging from 0.25 µM to 4 µM were evaluated. Data was normalized by the total object area identified by the brightfield images (for details, see the **Data normalization** section). As shown in Figure 10, a typical bell-shaped OCR response to FCCP was observed in the concentration range tested, and the maximal response is obtained at 1 µM FCCP.

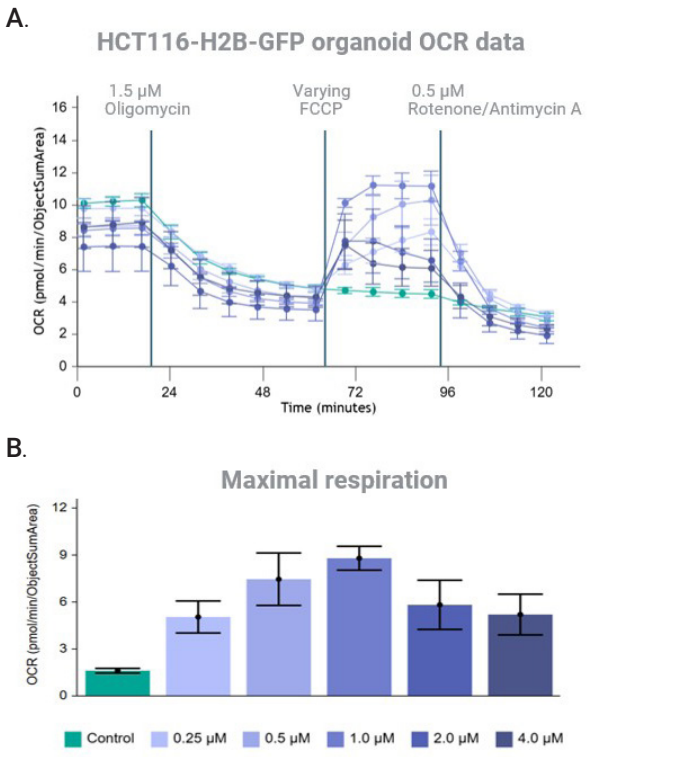

Figure 10. FCCP titration for HCT116-H2B-GFP organoids cultured in an Agilent Seahorse XF Flex organoid microplate. HCT116-H2B-GFP cells were seeded at 1,000 cells embedded in 11 µL of 50% Matrigel per well and then cultured for five days. A. OCR kinetic graph normalized by the brightfield total object area. B. Maximal respiration calculated by Agilent Seahorse Analytics software.

The data in Figure 11 demonstrates an approach to evaluate the effect of compounds that target mitochondria function using organoid cultures. Metformin is well known to suppress mitochondrial respiration. As shown, overnight exposure of organoid cultures to metformin results in a concentration dependent inhibition of mitochondrial respiration in cancer organoids.

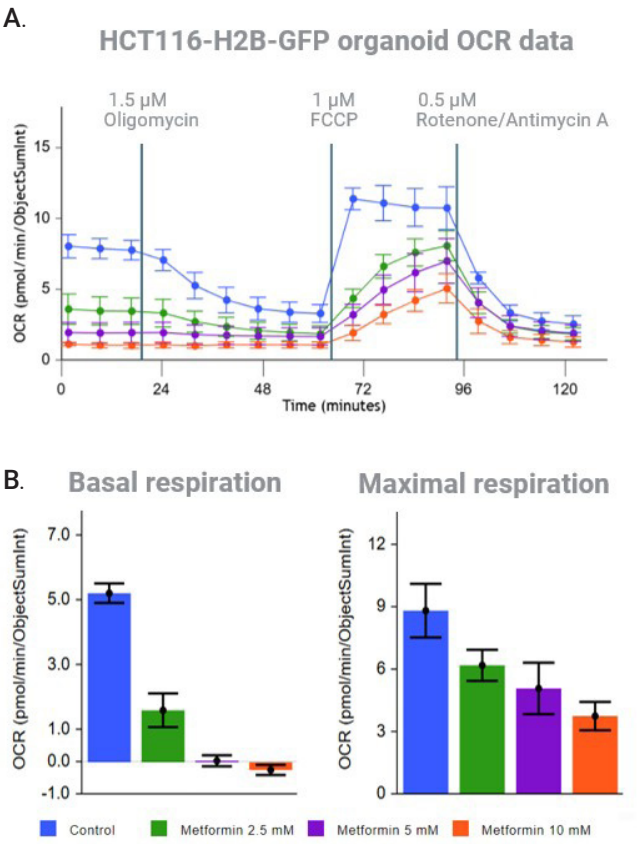

Figure 11. Metformin effect on the mitochondrial respiration in the HCT116-H2B-GFP cancer organoids. HCT116-H2B-GFP cells were cultured in Matrigel (5 mg/mL protein) for six days and exposed to 2.5, 5, or 10 mM metformin overnight before the XF Mito Stress Test was performed. The OCR data was normalized by the integrated fluorescence intensity from Hoechst 33342, measured by an Agilent BioTek Cytation cell imaging system using the DAPI channel.

Data normalization

Organoid culture often relies on a multiday process, and includes complex growth and differentiation, which may cause large well-to-well variation in the organoid number, size, and morphology. Since counting of individual cells is not feasible, an orgnaoid-specific approach for sample quantification is required for data normalization. Here listed are two approaches that can be applied to a wide range of orgnaoid models. The first one, is an image-based method that quantifies the amount of organoid material in a well using the sum of area from objects idenfied by z-projected bright field images (Object Sum Area) or fluorescene intensity integrated in the area (Object Sum Int). The second one, is an enzymatic method that quantifies the amount of viable cells present in the well at the end of the Seahorse XF assay using a viability assay, such as the CellTiter-Glo cell viability assay (Promega).

Organoid quantification using imaging-based methods

The microscopic imaging is a generally applicable method for sample size/quantity estimation. Figure 12 shows an example of identifying organoids using 4x high contrast brightfield (HCBF) and flourescent images obtained using the Cyation cell imaging multimode reader. A total of five different focal images were captured and projected to a single plane image (Figure 12A). Hoechst 33342 images were collected and projected simultaneously (Figure 12B). Organoid objects were idenfied by Gen5 software, shown by the yellow boundary lines in Figure 12B. The total area (Object Sum Area) of all organoid objects can be used as a denominator to normalize the assay data. In order to account for the cell density variation in each organoid object, the total fluorescence intensity integrated in the object boundary (Object Sum Int) can be measured as shown in Figure 12B.

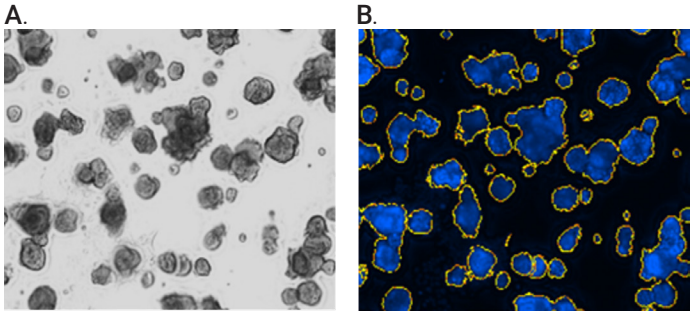

Figure 12. Example of image-based organoid identification and flourescence intensity detection. A. Brightfield image projected from five z-stack slices. The objects and object boundaries were defined from this projected image. B. Fluorescent image of the same well, stained wiht Hoechst 33342 nuclear dye. The yellow lines indicate the object boundaries defined from the z-projected brightfield image. The fluorsecence intentity within the boundaries can be enumerated by Gen5 software.

Organoid quantification using viability assays

Luminescence-based ATP quantification is a widely used method for assessing cell viability by measuring luminescent signal proportional to ATP level. The CellTiter-Glo 2.0 Cell Viability Assay (Promega, part number G9241) and CellTiter-Glo 3D Cell Viability Assay (Promega, part number G9681) are commonly applied for this purpose. To assess viability in

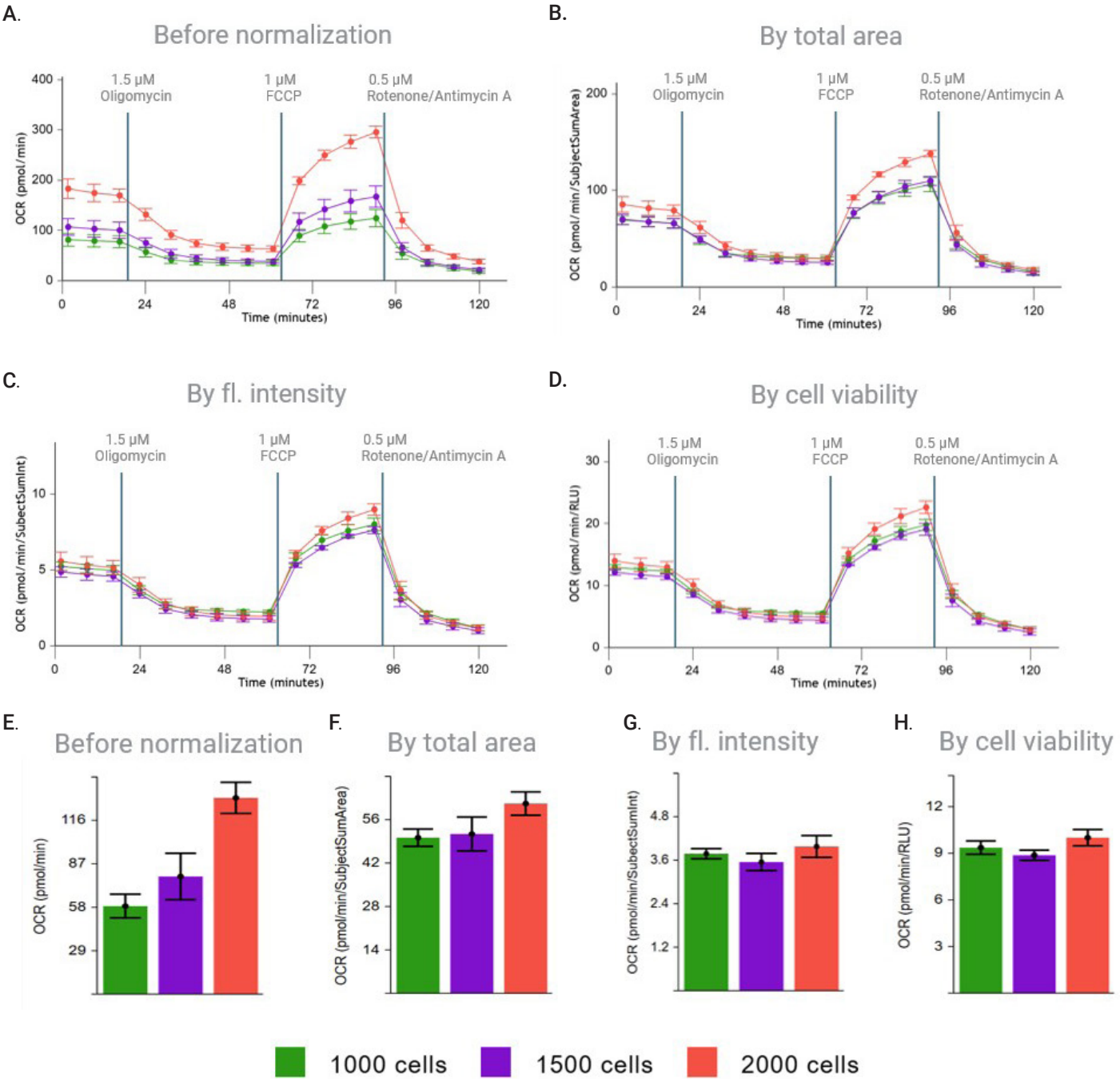

Figure 13. Comparison of normalization methods by total object area (ObjectSunArea), integrated fluorescence intensity (SubjectSumFlt) and viability measured by the CellTiter-Glo 2.0 cell viability assay (RLU). A to D. Kinetic OCR graphs before and after normalization. E to H. Basal OCR before and after normalization.

samples embedded within a matrix scaffold (like Matrigel), appropriate post-Seahorse XF assay procedures are required, including matrix dissolution and sample recovery prior to proceedign with the cell viability assay.

Below is a brief procedure used in this study to obtain of organoid sampe vaibility measurements , expressed as relative luminescent intensity. Further optimization may be necessary depending on the specific organoid type and matrix scaffold used.

1. Post Seahorse XF assay, remove XF assay medium thoroughly, as illustrated in Figure 5 (step 3).
2. Add 30 µL ice-cold Corning Cell Recovery Solution or similar Marigel resolving reagent.
3. Incubate the plate on ice, or at 4 °C for 30 minutes.
4. Add 160 µL Seahorse XF assay medium, or PBS, and recover organoids from Matrigel by pipetting up and down repeatedly.
5. Add 200 µL CellTiter-Glo working reagent, mix well, and incubate 10 minutes at room temperature.
6. Read the luminescent intensity in the XF Flex organoid microplate with a plate reader.

Normalization method selection

The choice of normalization method should be guided by both the characteristics of the organoids and the specific research objectives. The two approaches introduced above do not provide an absolute cell count within organoids, which limits the ability to compare data across independent experiments. To address this, it is recommended to include a reference control group in each experiment or to use a standard curve when available. Further refinement and optimization of normalization strategies may be necessary to achieve reliable and reproducible results.

In the example presented in Figure 13, the three data normalization methods discussed above are applied. All of them result in a reduction of well-to-well variability introduced by sample size/quantity variability across wells. Here, the three experimental groups represent HCT116-H2B-GFP organoids created from three different initial seeding density; 1000, 1500 and 2000 cells/well and the Seahorse XF Mito Stress Test was performed using 1.5 µM oligomycin, 1 µM FCCP and 0.5 µM rotenone/antimycin A. HCT116-H2B-GFP organoids showed basal OCR increments corresponding to the seeding densities (from 80 to 180 pmol/min). After normalizaiton, the basal OCR signals for different seeding densities are brought within 15% of each other (Figure 13 E to H). In addition, all three methods significantly decreased the OCR variation and can be considered to be suitable for data normalization.

Conclusion

The Agilent Seahorse XF Flex organoid workflow presented in this technical overview is an innovative addition to the suite of tools offered by the Seahorse XF technology that enables researchers to expand real-time metabolic analysis beyond the traditional two-dimensional cell cultures. This workflow, enabled by the Agilent Seahorse XF Flex organoid microplate, allows for culturing organoids embeded in matrix for days and to securely holding organoid samples (or other 3D samples) in place when metabolic analysis and high-resolusion imaging assays are performed. It is easy to follow, adaptable to many organoid types, and can produce robust metabolic measurements, as demonstrated by the examples provided here. This new capability of Seahorse XF technology will empower physiologically relevant discoveries in a wide range of biomedical research areas.

Given the variability in organoid origin, culture methods, and research objectives across different users, it is strongly recommended to perform optimization studies prior to routine implementation of this assay. Following the guidelines outlined in this technical overview will help ensure robust and reproducible results tailored to specific experimental needs.

Products used in this application

Agilent products

Agilent Seahorse XF Flex analyzer

Agilent Seahorse XF Flex organoid FluxPak

Agilent Seahorse XF Flex 3D Mito Stress Test kit

Agilent Seahorse XF Flex Cell Mito Stress Test kit

Agilent Seahorse XF DMEM assay medium pack

Agilent BioTek Cytation imaging multimode reader

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