Multiplexed assessment of mitochondrial function with real-time oxygen consumption measurement and live cell image analysis

Yoonseok Kam, Lisa Winer, Natalia Romero
Cell Analysis Division,
Agilent Technologies, MA., USA

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Introduction

Real-time oxygen consumption rate (OCR) measurement is one of the most robust methods to assess mitochondrial functionality, typically requiring complex instrumentation like the Agilent XF Seahorse analyzer. The new Agilent Mito-rOCR assay offers an innovative solution for assessing mitochondrial respiration through relative oxygen consumption rate (rOCR) measurements using a conventional fluorescence plate reader.[1]

Its oil-free design simplifies the workflow and enhances the performance and usability of plate reader-based rOCR measurements, offering numerous advantages, including multiplexed assay capability.[2] Integrating the Mito-rOCR assay with live cell imaging allows for simultaneous analysis of mitochondrial function and other cellular processes. This integration provides real-time OCRs and microscopic image data from a single sample, offering a comprehensive approach to understanding cellular metabolism and function.

In this study, mitochondrial dysfunction induced by FCCP, a known mitochondrial uncoupler, was examined using Mito-rOCR measurements.[3] This included monitoring changes in mitochondrial membrane potential and morphology with fluorescence imaging markers JC-1 and BioTracker Mitochondria.

Experimental

Mito-rOCR Assay

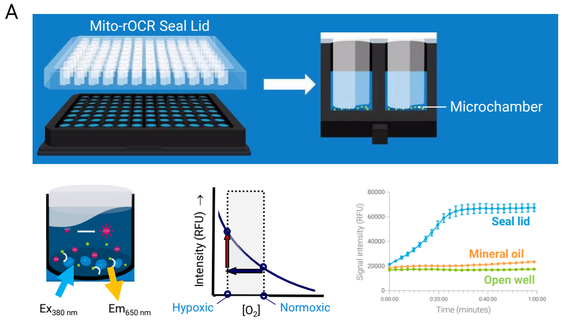

Figure 1. Agilent Mito-rOCR assay principle and workflow
(A) The rOCR reagent, which remains outside the cells, quenches fluorescence in response to oxygen levels. As cells consume extracellular oxygen, the fluorescence intensity rises and is detected by a plate reader. (B) Schematic summary of the Mito-rOCR assay workflow. The assay set up is simplified and experimental run time was significantly reduced by using the seal-lid. Automated data analysis and automated rOCR calculation can be performed within a few minutes using the Mito-rOCR Analysis Module within Seahorse Analytics.

Results and Discussion

Real-time assessment of rOCR by Mito-rOCR assay solution

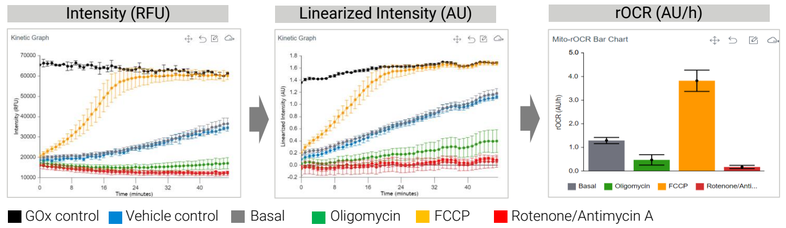

Figure 2. Assessment of rOCR by end-to-end Agilent Mito-rOCR assay solution
A549 cells cultured overnight and the real-time changes in fluorescence intensity (RFU) was measured in the presence of Mito-rOCR reagents with or without mitochondrial modulators (Oligomycin, FCCP and Rotenone/Antimycin) by Agilent Synergy H1 plate reader. The intensity kinetic data was linearized (AU) and the rOCR was calculated automatically by using the Mito-rOCR Analysis Module.

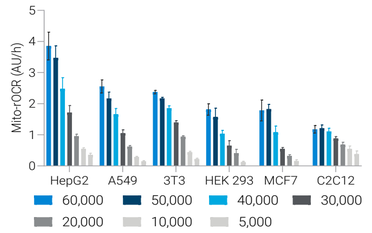

Figure 3. Comparison of signal intensities for different cell types seeded at varying densities
Mito-rOCR (AU/h) from 6 different cell lines seeded at densities decreasing gradually were measured after an overnight culture.

Multiplexing assessment of mitochondrial membrane potential

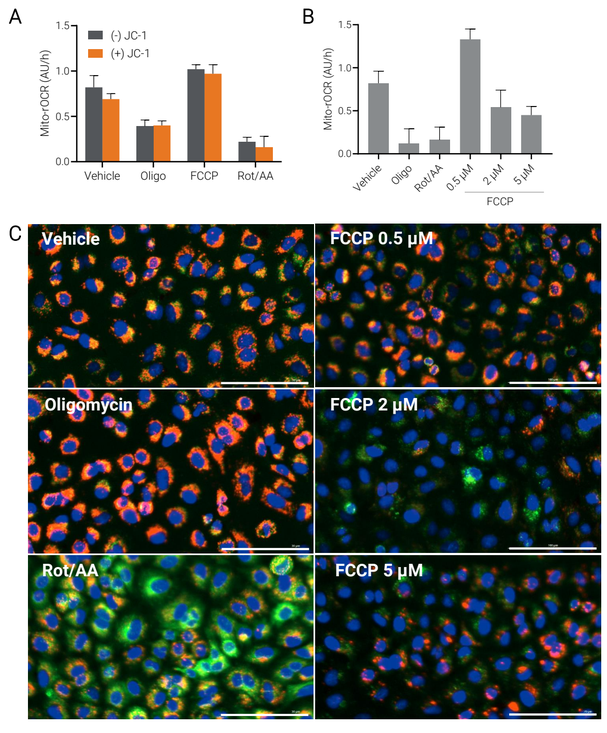

Figure 4. Changes in mitochondrial function (Mito-rOCR) and mitochondrial membrane potential (JC-1) induced by mitochondrial inhibitors and an uncoupler.
(A) The impact of JC-1 on Mito-rOCR was assessed by Agilent Cytation 5 in the presence of vehicle or mitochondrial modulators; oligomycin (Oligo), FCCP, and rotenone/antimycin A (Rot/AA). (B) Mito-rOCR was measured post-JC-1 labeling, both in the presence and absence of mitochondrial modulators. (C) 20× magnification JC-1 fluorescent images were automatically captured following Mito-rOCR measurements by Cytation 5. Nuclei were stained with Hoechst 33342 (blue). JC-1 at high membrane potential is red and JC-1 at low membrane potential is green. (Scale bar = 100 µm).

Multiplexed imaging of mitochondrial structure

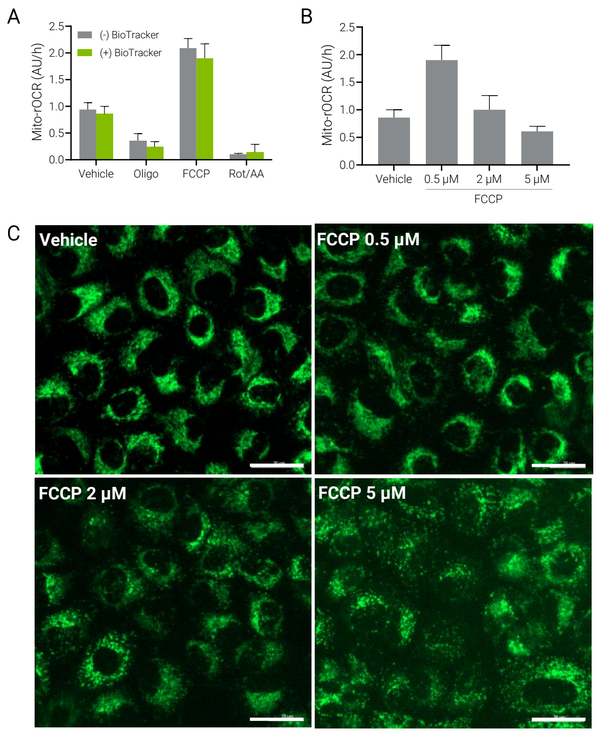

Figure 5. Concentration-dependent differential effect of FCCP on Mito-rOCR and mitochondrial morphology in A549 cells.
(A) The impact of BioTracker Mitochondria on Mito-rOCR was assessed by Agilent Cytation 5 in the presence of vehicle or mitochondrial modulators; oligomycin (Oligo), FCCP, and rotenone/antimycin A (Rot/AA). (B) Mito-rOCR was measured immediately after BioTracker Mitochondria staining, in the presence or absence of FCCP at varying concentrations. (C) 20× magnification BioTracker Mitochondria fluorescent images were automatically captured Cytation 5 following Mito-rOCR measurements. (Scale bar = 30 µm).

Summary

Conclusions

JC-1 and BioTracker Mitochondria do not interfere with Mito-rOCR measurements, enabling the assessment of Mito-rOCR in labeled cells.

The multiplexed Mito-rOCR assay using JC-1 and BioTracker shows that membrane potential and structural integrity of mitochondria were maintained up to the optimal uncoupling concentration of FCCP. However, higher FCCP concentrations resulted in significant reductions in membrane potential and mitochondrial fragmentation, indicating an inhibitory effect on respiration.

Combining the Mito-rOCR assay with multimodal imaging analysis provides enriched and detailed data, enhancing the understanding of mitochondrial functional regulation under cellular stress induced by drugs or gene expression.

Multiplexed Mito-rOCR Assay Workflow

Step 1. Sample (cell) preparation: Cells are seeded on an Agilent 96-well microplate a day before the Mito-rOCR assay. Cells can also be pretreated with test compounds during this preparation step if necessary.

Step 2. Live-cell labeling: On the day of the assay, cells are labeled with molecular markers either before or after the Mito-rOCR assay. If there is no interference between the Mito-rOCR reagents and the live cell marker, cells can be labeled before the Mito-rOCR measurement. If cell labeling potentially interferes with Mito-rOCR measurements or if the Mito-rOCR assay environment affects live cell markers, the Mito-rOCR assay should be completed before cell labeling and imaging.

Step 3. Mito-rOCR assay:. Depending on the live cell markers, the cell labeling and washing steps need to be carefully arranged.. To examine the acute effect of test compounds, they are typically administered to cells along with Mito-rOCR reagents and included during the Mito-rOCR measurement.

Step 4. Live-cell imaging:. It is recommended to remove the magnetic holder from the plate before imaging. If reagent addition or media change is required between the Mito-rOCR assay and imaging step, the lid must be carefully removed to avoid lifting the cells and replaced with the Agilent 96-well microplate lid.

References

1. Agilent Mito-rOCR:Streamlined Assessment of Mitochondrial Function in Live Cells https://www.agilent.com/cs/library/brochures/mini-br-mito-rocr-5994-7406en-agilent.pdf
2. Improved Assay Performance and Workflow with Agilent Mito-rOCR Compared to Oil-Based OCR Assays https://www.agilent.com/cs/library/technicaloverviews/public/te-mito-rocr-performance-5994-7330en-agilent.pdf
3. Multiplexed Assessment of Mitochondrial Function Combining the Agilent Mito-rOCR Assay with Live-Cell Imaging https://www.agilent.com/cs/library/applications/an-multiplexing-imaging-mito-rocr-5994-7900en-agilent.pdf