Application Note

Cell Analysis

Agilent

Trusted Answers

Multiplexed Assessment of Mitochondrial Function Combining the Agilent Mito-rOCR Assay with Live-Cell Imaging

Author

Yoonseok Kam, Lisa Winer, Natalia Romero

Agilent Technologies, Inc.

Abstract

The Agilent Mito-rOCR assay is an innovative fluorescence plate reader-based solution for measuring relative mitochondrial respiration rates. Its unique design, compatible with multimode plate readers, allows for the simultaneous analysis of mitochondrial function and other cellular processes. This feature is advantageous when combined with microscopy, which provides insights into both the biochemical and morphological characteristics of cells. The multiplexed application of the Mito-rOCR assay enables the extraction of multiple parameters from a single sample, offering a more comprehensive understanding of cellular metabolism and function. This application note outlines the basic workflow of the multiplexed Mito-rOCR assay with other plate reader-based assays and presents two example applications that combine Mito-rOCR measurements with mitochondrial imaging for deeper characterization of mitochondrial function.

Introduction

Agilent offers a range of advanced cell analysis solutions that enable real-time detection of changes in cellular bioenergetics. These technologies are essential for understanding cellular processes such as activation, proliferation, differentiation, and dysfunction. Notably, the Agilent solutions for in vitro assessment of oxygen consumption rate (OCR) provide valuable insights into mitochondrial function and cellular oxidative energy metabolism.

The Agilent Seahorse XF analyzer and related kits are pivotal in advancing mitochondrial research. The Seahorse XF analyzer's ability to measure OCR offers a direct method to assess mitochondrial respiration, a key indicator of cellular metabolism. Its quantitative nature, with the ability to inject compounds and make repeated OCR measurements, ensures reliable data for robust analyses. The Mito-rOCR assay complements this by offering a more accessible option that can be used with fluorescence plate readers, broadening the scope for research applications. The seal lid technology and automated data analysis further refine the process, facilitating precise assessment of relative OCR (rOCR) changes due to pharmacological interventions or genetic modifications.

The Mito-rOCR assay can be performed on a multimode fluorescence plate reader, making it easy to combine with microscopic imaging analysis. The Agilent 96-well microplate included in the Mito-rOCR assay kit is highly compatible with fluorescence imaging on multimode plate readers, allowing seamless integration with microscopic imaging for multiplexed assays. This integration saves cost and time, conserves samples, and reduces variability between experiments by simultaneous assessment of multiple parameters. This application note introduces two application examples integrating live-cell fluorescence imaging with the Mito-rOCR assay on the Agilent BioTek Cytation 5 cell imaging multimode reader.

Experimental

Cell preparation

A549 cells (ATCC, CCL-185) were maintained in F-12K medium (ATCC, 30-2004) supplemented with 10% FBS. A day before the assay, cells were seeded on an Agilent 96-well microplate at 3×10^4 cells/well excluding control wells assigned for blank, background, and glucose oxidase (GOx).

Reagent preparation

Mito-rOCR reagent working solution was prepared by resuspending the rOCR vial in 1 mL assay media (Seahorse XF DMEM assay medium supplemented with 10 mM glucose, 1 mM pyruvate, and 2 mM glutamine, Agilent, p/n 103680-100). Rotenone/antimycin A (Rot/AA) mix were resuspended in 106 µL assay media. GOx was resuspended in 100 µL water. The Mito-rOCR working solution was further diluted with 5.5 mL assay media prewarmed at 37 °C (final volume = 6.5 mL).

Live-cell labeling

For JC-1 imaging, cells were incubated in the culture media for 20 minutes in the presence of 5 µg/mL JC-1 (Sigma-Aldrich, T4069) and 1 µg/mL Hoechst 33342 (Thermo, #62249). Cells were washed with the assay medium. Since BioTracker Mitochondria (Sigma-Aldrich, SCT136) does not require any washing steps, a separated labeling step was excluded. Instead, 100 nM BioTracker Mitochondria and 1 µg/mL Hoechst 33342 was included in the assay media together with the Mito-rOCR reagent.

Mito-rOCR measurements

The plate medium was replaced with the diluted Mito-rOCR assay media (50 µL/well) except for the two blank wells. The blank wells were filled with 50 µL of the prewarmed assay media without any Mito-rOCR reagent. The dilute Mito-rOCR assay media includes the cell-labelling reagent in the case of BioTracker Mitochondria staining.

GOx (1 µL/well), Rot/AA (1 µL/well), and other test compounds modulating mitochondrial function (≤ 5 µL/well) were added to the corresponding wells and incubated for 10 minutes at 37 °C. The plate lid was replaced with the prewarmed Mito-rOCR seal lid and assembled with the Mito-rOCR magnetic holder as described in the user guide. Oxygen consumption was measured using a premade Gen5 protocol designed to capture time-resolved fluorescence measurements at 1-minute intervals on the Mito-rOCR monochromator assay by Cytation 5.

All media change and seal lid/magnetic holder assembly were performed on a plate heater set at 37 °C. For additional nuclear staining, cells were incubated in the presence of 1 µg/mL Hoechst 33342 after Mito-rOCR measurement.

Mito-rOCR data analysis

Relative oxygen consumption rates were calculated using the Mito-rOCR Analysis View within Agilent Seahorse Analytics following the protocol in the user guide. Briefly, the result file was exported as a text file (.txt) using Gen5 and then uploaded to Seahorse Analytics. The control and experimental group layouts were assigned on the template design page, and kinetic graphs and bar charts were generated. Data comparison and interpretation were performed using the bar charts in Seahorse Analytics or by exporting the data to GraphPad Prism, as illustrated in Figures 1 and 2.

Live-cell imaging

The magnetic holder was removed from the plate for live-cell imaging. The seal lid can be retained during imaging unless doing an additional nuclear staining by 1 µg/mL Hoechst 33342. The seal lid was carefully removed and replaced with the condensation ring lid (the one used during cell culture) after the addition of Hoechst 33342. Cells were incubated for 10 minutes at 37 °C in the presence of Hoechst dye before imaging.

All images were captured at 20× magnification using the BioTek Cytation 5 cell imaging multimode reader. For JC-1 imaging, three fluorescence filter sets—DAPI (Agilent, p/n 1225100), GFP (Agilent, p/n 1225101), and TRITC (Agilent, p/n 1225125)—were used. BioTracker Mitochondria images were obtained using the GFP filter set. The focal point was identified using laser autofocusing (Agilent, p/n 1225010).

Results and discussion

Multiplexing the Mito-rOCR assay with mitochondrial membrane potential assessment

The first multiplexed assay example is to compare the rOCR changes with mitochondrial membrane potential changes induced by well-known mitochondrial modulators: Oligomycin, an inhibitor of ATP synthase, rotenone/antimycin A mix, a combinational inhibitor of complex I and III, and FCCP, a mitochondrial uncoupler. The loss of mitochondrial membrane potential is a common indicator of mitochondrial dysfunction, often induced by drugs. It can be detected by comparing the green and red fluorescence of JC-1, a cationic dye that accumulates in mitochondria. When the membrane potential is intact and sufficiently high, JC-1 forms red fluorescent J-aggregates. However, if the membrane decreases due to mitochondrial perturbation, resulting in membrane depolarization, JC-1 fails to form these aggregates and emits green fluorescence.[1]

Before performing a multiplexed assay, the impact of JC-1 on oxygen consumption rates was assessed. The rOCR of A549 cells was measured in the presence or absence of JC-1 when cells were treated with the mitochondrial modulators oligomycin (0.5 µM), rotenone/antimycin A (0.5 µM each), or FCCP (0.5 µM). These compounds are standard modulators used in mitochondrial assays like the Agilent Seahorse XF Cell Mito Stress Test, which assesses the key parameters of mitochondrial function. The results, depicted in Figure 1A, indicate that the presence of JC-1 does not affect rOCR measurements, and changes in rOCR are consistent with the expected outcomes of mitochondrial inhibition or uncoupling by these compounds. These results suggest that JC-1 can be reliably used in multiplexed assays without interfering with the measurement of mitochondrial respiration.

Next, mitochondrial respiration changes induced by mitochondrial intervention was assessed alongside mitochondrial membrane potential. Since oxygen consumption measurements are not interfered by JC-1 or Hoechst 33342 (data not shown), A549 cells were labeled with JC-1 and Hoechst 33342 for 20 minutes and followed by a wash to remove excess dye. Oxygen consumption was immediately measured over 45 minutes to obtain Mito-rOCR in the presence of inhibitors and the uncoupler. The assay concluded with JC-1 imaging, without any additional washing steps between the Mito-rOCR assay and image acquisition.

Figure 1B shows the Mito-rOCR assay results, indicating rOCR decreases by oligomycin and rotenone/antimycin A and upregulation in the presence of FCCP. Figure 1C presents representative images obtained from the same plate. Although both oligomycin and rotenone/antimycin A similarly lowered the mitochondrial respiration (Figure 1B), the membrane potential change detected by JC-1 fluorescence was markedly different. While oligomycin slightly increased the membrane potential compared to the vehicle control, adding rotenone + antimycin A severely disrupted the membrane potential. When cells were treated with FCCP, they exhibited differential effects depending on the concentration. The rOCR reached its maximal rate at the optimal concentration of 0.5 µM FCCP. However, excessive FCCP lowered the mitochondrial respiration rate, as previously reported. As shown in Figure 1C, the membrane potential remained relatively intact at the optimal FCCP concentration. In contrast, the membrane potential was disrupted with significant morphological changes by treatment with higher FCCP concentrations. This differential effect on rOCR and mitochondrial membrane potential measurements is expected, considering the nature of the methods. The addition of the uncoupler at optimal concentrations results in a small decrease in membrane depolarization, facilitating and increasing electron transport, H^+ pumping to the intramembrane space, and a drastic increase in mitochondrial respiration, which contributes to maintaining the steady state of the membrane potential. The addition of higher concentrations of the uncoupler meanwhile, resulted in respiration inhibition, decreased electron transport and H^+ pumping with the consequential membrane depolarization. These results highlight the value of direct measurements of oxygen consumption when assessing oxidative phosphorylation (OXPHOS).[2]

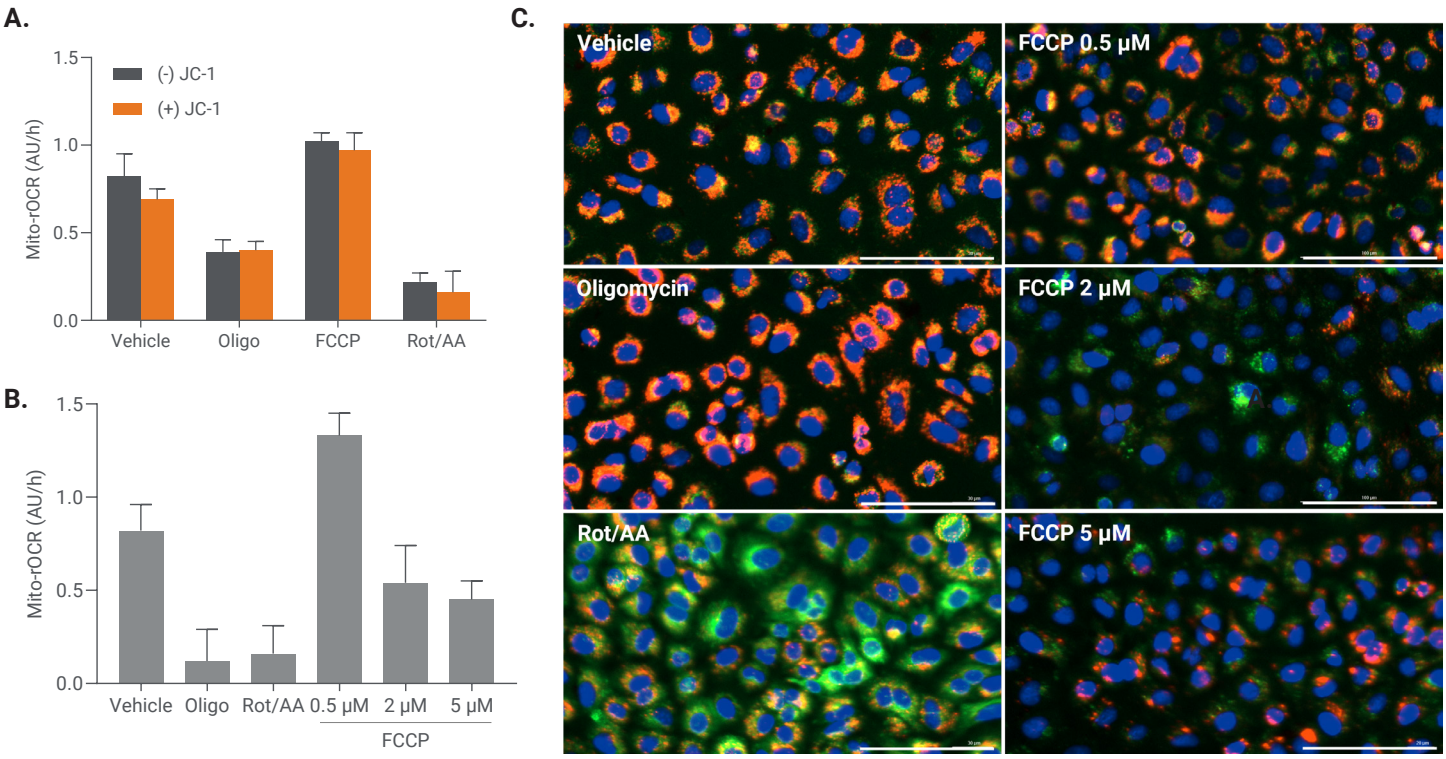

Figure 1. Changes in mitochondrial function (Mito-rOCR) and mitochondrial membrane potential (JC-1) induced by mitochondrial inhibitors and an uncoupler. (A) The impact of JC-1 on Mito-rOCR was assessed in the presence of vehicle or mitochondrial modulators; oligomycin (Oligo), FCCP, and rotenone/antimycin 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. 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

The second multiplexed assay example shows the structural changes in mitochondria associated with respiration rate. Mitochondrial morphology is another phenotypic change closely associated with the mitochondrial function. As shown in the multiplexed Mito-rOCR assay with JC-1 staining, uncoupling with excessively high concentrations of FCCP can cause mitochondrial morphological defects. These observations align closely with the earlier report indicating that FCCP can induce mitochondrial fragmentation by disrupting mitochondrial membrane potential and by associating with OPA1 cleavage.[3]

The mitochondrial structural change caused by FCCP at different concentrations was monitored by BioTracker 488 Green Mitochondria dye along with the Mito-rOCR measurements. BioTracker Mitochondria dyes are membrane permeable fluorogenic stains which become brightly fluorescent upon accumulation in the mitochondrial membrane. The fluorescence signal depends on mitochondrial mass not on mitochondrial membrane potential, and it is lost if mitochondrial membrane integrity is compromised by cell death or any other damage.

The possible adverse effect of BioTracker Mitochondria dye on the oxygen consumption measurement was examined similarly to the previously mentioned JC-1 experiment. There was no significant change in rOCR caused by the presence of BioTracker Mitochondria dye (Figure 2A). The FCCP-concentration-dependent Mito-rOCR elevation was repeatedly observed as shown Figure 2B. The BioTraker Mitochondria staining pattern in Figure 2C also shows FCCP-concentration dependent loss of mitochondria integrity loss, with increase on punctate morphology and expected decrease in Mito-rOCR. This morphological change corresponds well to the membrane potential disruption monitored previously (Figure 1C).

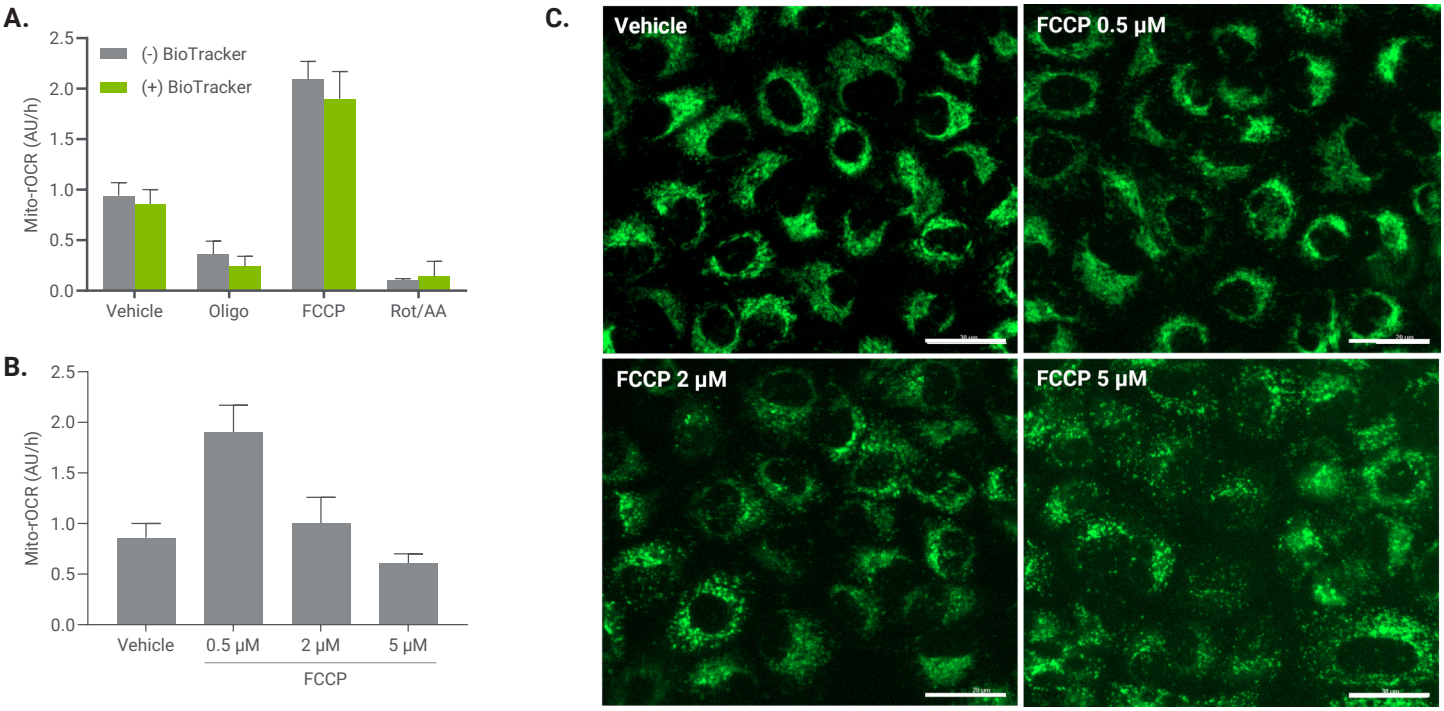

Figure 2. Concentration-dependent differential effect of FCCP on Mito-rOCR and mitochondrial morphology in A549 cells. (A) The impact of BioTracker Mitochondria on Mito-rOCR was assessed in the presence of vehicle or mitochondrial modulators; oligomycin (Oligo), FCCP, and rotenone/antimycin A (Rot/AA). (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 following Mito-rOCR measurements. (Scale bar = 30 µm).

Multiplexed Mito-rOCR assay workflow

Combining Mito-rOCR measurement with imaging data enables a more comprehensive understanding of mitochondrial respiration and related cellular functions. For instance, a decrease in OCR due to excessive FCCP can be linked to defects in the physical state of mitochondria, as demonstrated in the two application examples in this note.

When designing a multiplexed Mito-rOCR workflow that incorporates fluorescence imaging data analysis, several factors must be considered:

1. Fluorescence spectrum compatibility: The fluorescence spectrum of the imaging marker must be distinct from that of the oxygen sensor used in Mito-rOCR (Ex 380 nm/Em 650 nm), and the fluorophore should not interfere with Mito-rOCR measurements. The oxygen sensor's spectrum in the Mito-rOCR kit is distinct from many commonly used fluorescence markers such as GFP, RFP, and DAPI, making it suitable for multiplexing (data not shown). However, it is recommended to check for any potential interference. Figures 1A and 2A provide examples of how this can be tested.
2. Timing compatibility: The Mito-rOCR assay requires measurements over 45 minutes while maintaining a microchamber to limit oxygen supply. Therefore, the labeling procedure must be compatible with this requirement. Also, Mito-rOCR measurement must begin immediately after the Mito-rOCR reagent is administered, so any labeling or imaging steps that delay measurement must be avoided.
3. Media compatibility: The multiplexing workflow must be designed with media compatibility in mind. For live cell markers that need to be measured in serum- or phenol red-free conditions, such as HBSS, it is recommended to start with Agilent Seahorse XF assay media supplemented with metabolic fuel to avoid acidification in the microchamber under non-CO_2 conditions.

Figure 3. Two general workflows of multiplexed live-cell imaging accompanied with Mito-rOCR assay. The cells are labeled with live cell markers before the Mito-rOCR assay (A) or after (B). Test compound may be applied to cells before this workflow for a long-term pretreatment assay or included the Mito-rOCR preparation for an acute treatment assay.

Considering these factors, the multiplexed Mito-rOCR workflow can be composed of four key steps: (1) sample (cell) preparation, (2) live-cell labeling, (3) Mito-rOCR assay, and (4) cell imaging. Two possible variations of this workflow are schematically illustrated in Figure 3. If the live-cell labeling is fully compatible with the Mito-rOCR measurement conditions and stable for 45 minutes or longer, the workflow shown in Figure 3A is recommended, as introduced by the two application examples in this note. The alternative workflow in Figure 3B should be considered for any cell imaging analysis not compatible with Mito-rOCR assay conditions.

Step 1. Sample (cell) preparation:

Cells are seeded on an Agilent 96-well microplate a day before the Mito-rOCR assay. However, the culture conditions and duration may vary depending on the cell type. The seeding density and culture conditions may need to be optimized for 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. The protocols for live-cell labeling and imaging vary depending on the targets of interest and the nature of the markers. If there is no interference between the Mito-rOCR reagents and the live cell marker, cells can be labeled before the Mito-rOCR measurement (Figure 3A). 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:

Mito-rOCR can be obtained. Depending on the live cell markers, the cell labeling and washing steps need to be carefully arranged. The media should be changed to one compatible with the live-cell imaging markers. The Mito-rOCR assay requires fluorescence measurements at 1-minute intervals for at least 45 minutes. 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:

The Mito-rOCR seal lid and Agilent 96-well microplate are compatible with inverted microscope imaging. Therefore, automated brightfield and fluorescence imaging is feasible with any compatible multimode imaging plate reader. The magnetic holder can be retained for low magnification imaging. However, it increases the lens-to-object distance and may interfere with high magnification microscopy for outer wells. It is recommended to remove the magnetic holder from the plate before imaging. There is no direct interference with inverted microscopy caused by the seal lid. However, 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.

Conclusion

The Agilent Mito-rOCR assay, a fluorescence plate reader-based assay, offers the advantage of multiplexed analysis of mitochondrial metabolism and function by integrating its workflow with imaging. This multiplexed workflow enables a more comprehensive analysis by combining mitochondrial function with other related cellular activities, using the Agilent BioTek Cytation 5 cell imaging multimode reader.

References

1. Perelman, A.; Wachtel, C.; Cohen, M.; Haupt, S.; Shapiro, H.; Tzur, A. JC-1: alternative excitation wavelengths facilitate mitochondrial membrane potential cytometry. *Cell. Death. Dis*. **2012**, *3*(11), e430. DOI: 10.1038/cddis.2012.171
2. Kowaltowski, A.J.; Abdulkader, F. How and when to measure mitochondrial inner membrane potentials. *Biophys. J*. **2024**. DOI: 10.1016/j.bpj.2024.03.011
3. van der Stel, W.; Yang, H.; le Dévédec, S. E.; van de Water, B.; Beltman, J. B.; Danen, E. H. J. High-content high-throughput imaging reveals distinct connections between mitochondrial morphology and functionality for OXPHOS complex I, III, and V inhibitors. *Cell. Biol. Toxicol.* **2023**. *39*(2), 415-433. DOI: 10.1007/s10565-022-09712-6

www.agilent.com/lifesciences/mito-rocr

RA45611.1230902778

Published in the USA, November 20, 2024
5994-7900EN

Agilent
Trusted Answers