APPLICATION NOTE

Analysis of mitochondrial dynamics in human iPSC-derived neurons using the Operetta CLS high-content analysis system.

Key features

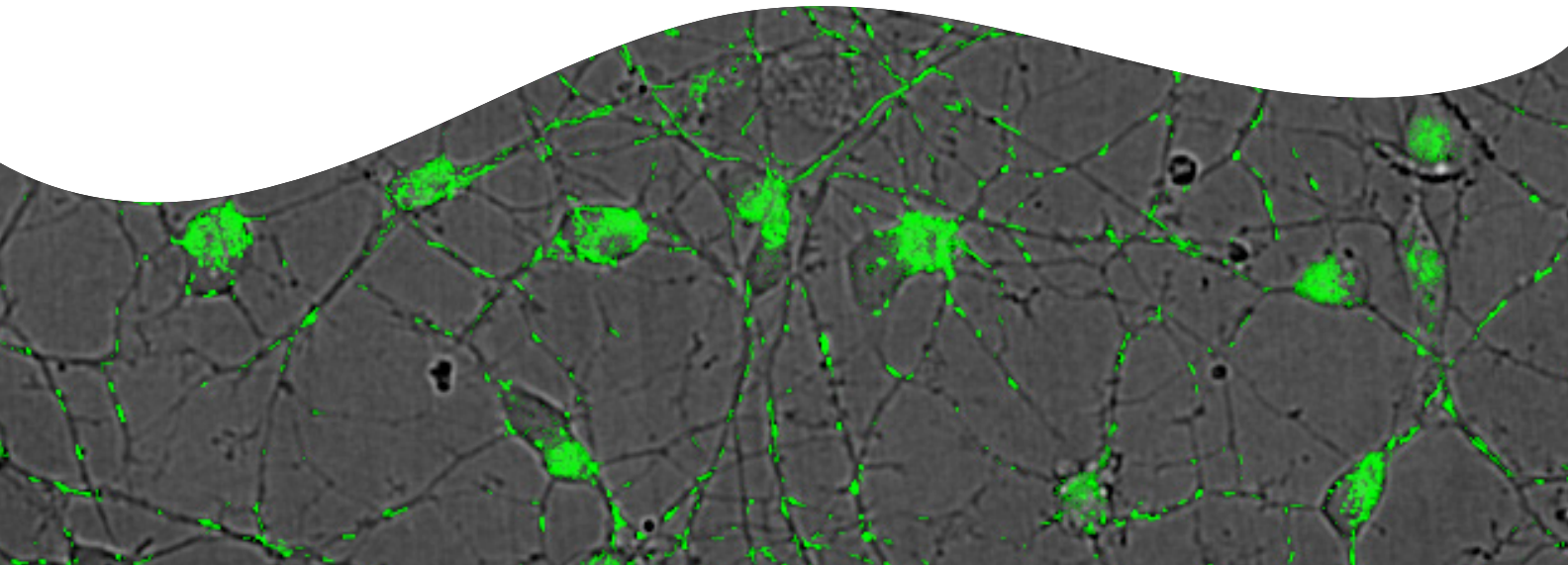
- Fast frame rate imaging to accurately capture rapid cellular responses
- Reliable quantification of mitochondrial dynamics in neurons
- Flexible kinetic acquisition settings for each channel to avoid unnecessary data acquisition

Introduction

Mitochondria primarily supply the cell with the energy required to sustain its defined function within the human body. Their critical role as a production site of ATP through oxidative phosphorylation has implications in energy metabolism and homeostasis; as a result, many in the field are exploring the effect of mitochondrial dysfunction on human disorders, including complex neurological diseases where age tends to be a risk factor.

Neurodegeneration has been linked to several mitochondrial-dynamic imbalances such as fragmentation of mitochondria, impaired mitophagy, and blocked mitochondrial transport in axons.[1] In fact, their physical transport and localization within the neural network and axonal transport system is required for their functionality within processes such as neurotransmission and generation of membrane potentials.[2]

Mitochondria are dynamic organelles undergoing coordinated cycles of fission and fusion, movement within the neuronal axon and disintegration (mitophagy). These dynamics are essential for energy conversion and neuron survival. Understanding changes in mitochondrial dynamics is therefore crucial in developing mitochondria-based therapy options for complex pathological conditions such as cancer, neurological disorders, and metabolic syndromes. Fast kinetic live-cell imaging combined with high-content screening represents a promising strategy to quantitatively track these changes in real time and at large scale.



In this application note, we show how to investigate mitochondrial dynamics in human iPSC-derived neurons using the Operetta® CLS™ high-content analysis system. Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP), a potent uncoupler of oxidative phosphorylation that dissipates the proton gradient to abolish ATP synthesis, and glutamate, an abundant excitatory neurotransmitter with numerous receptors in the brain, were used to demonstrate the sensitivity of the assay to pharmacological interventions. Using fast kinetic imaging, we developed a high-content imaging assay including an analysis strategy for the quantification of the dynamic movements of mitochondria labeled with PhenoVue™ 551 Mitochondrial stain.

Mitochondrial dynamics assay

Material and methods

Table 1. List of materials and devices used in the mitochondrial dynamics assay.

Cells	Neurosight®-S human iPSC-derived neurons (Nexel, #N-001)
Medium	Neurosight-S Media (Nexel, #NM-001), complemented with 100x Neurosight-S Media Supplement (Nexel, #NS-001)
Compounds	▪ FCCP (Sigma, #C2920) ▪ Glutamate (Sigma, #49621)
Mitochondrial stain	PhenoVue 551 Mitochondrial Stain (Revvity, #CP301)
Microplates	▪ Assay Plate: PhenoPlate™ 384-well (Revvity, #6057300) ▪ Compound Plate: StorPlate™ 384-well V-bottom (Revvity, #6008590)
Plate coating	▪ Laminin (Corning, #354232) ▪ Poly-L-Ornithin (Sigma, #P4957)
Imaging instruments	MuviCyte™ Live-Cell Imaging System (Revvity, #HH40000000) Operetta CLS High-Content Analysis System (Revvity, #HH16000020/1601)

Cell culture

NeuroSight-S human induced pluripotent stem cell-derived (hiPSC) neurons were thawed, plated, and maintained according to the manufacturer's instructions. 1.2 E5 cells in 50 µL plating medium per well were seeded into the wells of a laminin-precoated PhenoPlate 384-well microplate (Figure 1). Medium was exchanged to maintenance medium one day post plating and replaced every other day thereafter. On the day of experiment, medium was renewed 3h before the start of the experiment. hiPSC-derived neurons were allowed to form a continuous growing axon network from day one to six. The mitochondrial dynamics assay on the Operetta CLS system was performed on day 6 post plating.

Compound preparation and cell staining

Compounds were diluted in maintenance medium and stored in a StorPlate 384-well V-bottom microplate (75 µL per well). To minimize evaporation and for light protection, the compound storage plate was pre-equilibrated inside an incubator. Cells were stained with 10 nM PhenoVue 551 mitochondrial stain for 20 minutes and subsequently washed once with 50 µl of maintenance medium prior to the first image acquisition.

Image acquisition

Images were acquired with a temperature- and CO_2- equilibrated Operetta CLS high-content analysis system using the 40x water immersion objective in confocal mode and binning factor 3. This combination allowed imaging with low excitation power (10%) and short acquisition time (100 ms) for the PhenoVue 551 channel. We found that this was the optimal compromise to ensure gentle imaging of mitochondria while still ensuring strong enough signal with good resolution that enabled robust segmentation of objects.

Two channel groups were set up – the 1st group as the PhenoVue 551 (PV) channel and the 2nd group as the Brightfield (BF) channel – to facilitate flexible image acquisition for time series measurements. In each channel group, the frame rate as well as the number of time points can be defined individually.

The PV channel was set up to allow the image acquisition of 50 time points per field at 2 frames per second (fps). Imaging at 2 fps was sufficient to ensure overlapping objects between time points and minimizing the high risk of mitochondrial degradation as a result of phototoxic effects. It could technically be set to a maximum of 100 fps, resulting in a total of 2500 time points if measured using an identical time frame of 25 total seconds which would have been not useful for this application due to oversampling.

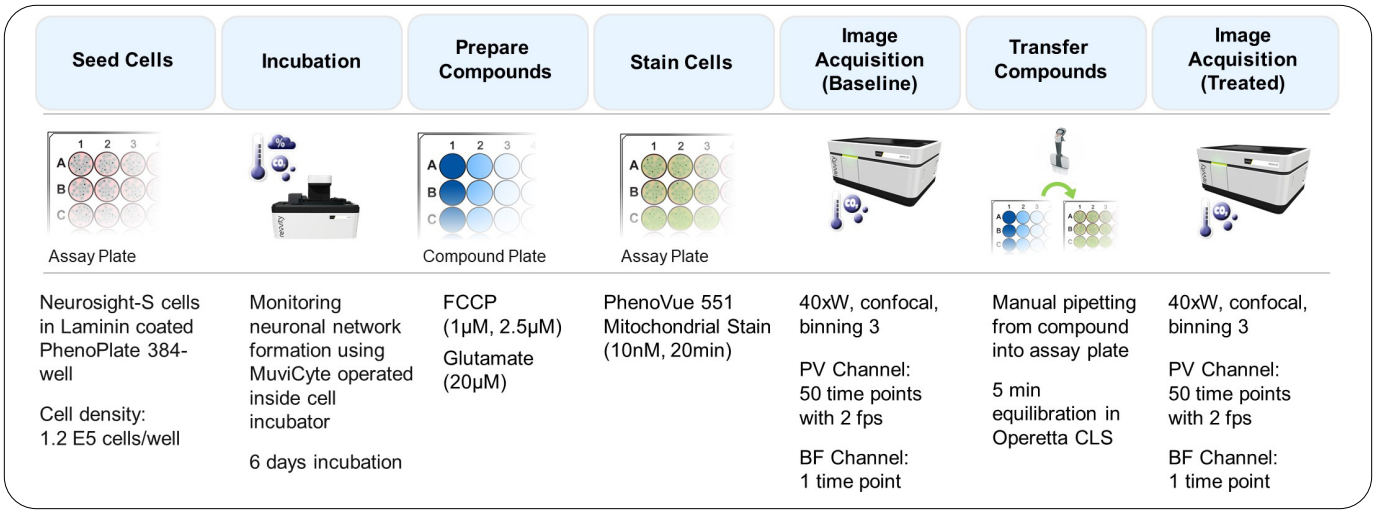


Figure 1. Workflow of the mitochondrial dynamics assay. Cells were plated into the wells of a Laminin-coated PhenoPlate 384-well microplate. The neuronal network formation was monitored using the MuviCyte system operating inside a cell incubator. After a 6 day incubation period, cells were stained with PhenoVue 551 mitochondrial stain. Images of the PhenoVue 551 (PV) channel and Brightfield (BF) channel were acquired using a temperature- and CO_2-equilibrated Operetta CLS high-content analysis system. An additional compound plate was prepared ahead of time for quick and easy compound transfer during the manual pipetting step between measurements. The plate was measured three times using the identical measurement setup: 1st measurement before compound addition (Baseline), 2nd measurement 5 minutes after compound addition, and 3rd measurement 30 minutes after compound addition.

The BF channel was set up as a 2nd channel group to allow the image acquisition of only one time point per field. The BF channel served as a reference to capture the neuronal network structure for subsequent image analysis. Alternatively, the BF channel could have been included in the 1st channel group to be acquired at the same frame rate of 2 fps as part of the PhenoVue 551 channel. However, the morphology of the cells – including the neuronal network – did not change within the given time frame of 25 seconds, so only one image was needed; this saves valuable data space.

Using this experimental setup, we could image labeled cells 3 distinct times within 1 hour post staining without seeing a significant change in mitochondrial intensity, morphology or dynamics as a control setting (data not shown).

Compound treatment

After the cell staining procedure, the plate was measured for the first time to establish a baseline of the initial mitochondrial dynamic state per well. The plate was removed once from the instrument to subject the cells to subsequent compound treatment with 20 µM Glutamate, 1 µM FCCP, 2.5 µM FCCP or control medium via manual pipetting. In a first step, the supernatant was removed from the assay plate and then replaced with the previously pre-equilibrated compound solutions or control medium. The plate was then put back into the Operetta CLS system. After 5 minutes of equilibration, the plate was measured for a second time. 10 min after the end of the second measurement, the plate was measured for a third time.

The assay layout comprised 32 wells, with 8 replicates per compound. The Operetta CLS system needs 16 minutes to complete the measurement with this setup. As all wells of the plate were treated at the same time by manual pipetting, there is a temporal difference between treatment and measurement for the individual wells. To check for any impact on mitochondrial dynamics due to this slight timing difference, replicates were positioned horizontally, contrary to the measurement meander (Figure 2).

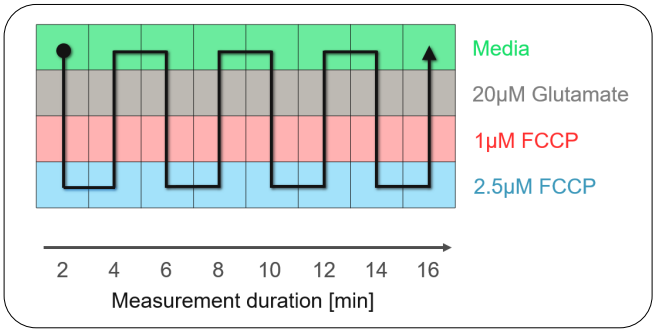


Figure 2. Assay layout and measurement meander. 32 wells comprising 8 replicates per treatment were measured. The measurement started in the upper left well and ended after a reading time of 16 min at the upper right well. Cells were treated all at once during the manual pipetting step. To account for a probable impact on mitochondrial dynamics caused by the temporal difference inherent to this method, replicates were positioned horizontally contrary to the vertical measurement meander.

Analysis

Images from the time series measurement were analyzed using Harmony® imaging and analysis software to evaluate the dynamic mitochondrial fraction over time (Figure 3). To precisely locate mitochondria within the images and exclude artifacts from the background, the image segmentation was conducted in several steps. As a first step, brightfield images were segmented to define the neuronal network region. As a second step, cell bodies were detected in the PhenoVue 551 images to then restrict the cell body mask from the initially-assessed neuronal network region. This final neuronal network region was then used to find mitochondria in PhenoVue 551 images. The dynamic mitochondrial fraction could be identified using a newly calculated image after the subtraction of adjacent PhenoVue 551 time points. The sum area of the remaining pixels is defined as the dynamic mitochondrial fraction.

The final readout is an area ratio between the dynamic mitochondrial fraction and the whole mitochondrial population, which has been calculated for each time point. The area ratio – calculated as a mean value over all time points – represents the mitochondrial dynamic for a given time frame (Figure 4 A). The mitochondrial dynamic fraction was observed to be stable and did not decrease over time within the given time frame of 25 seconds, demonstrating the effectiveness of gentle image acquisition conditions.

As a secondary analysis step, relative mitochondrial movement was calculated from the mean values of the previously established area ratio over time in respect to the first baseline measurement (Figure 4 B). Despite the 16 minutes maximum discrepancy of treatment duration, all replicates showed similar signal levels and calculated values within the same treatment groups. These results suggest that the impact on mitochondrial movement elicited by these respective compounds is rather stable and unchanging within the 5 to 45 minutes treatment time frame.

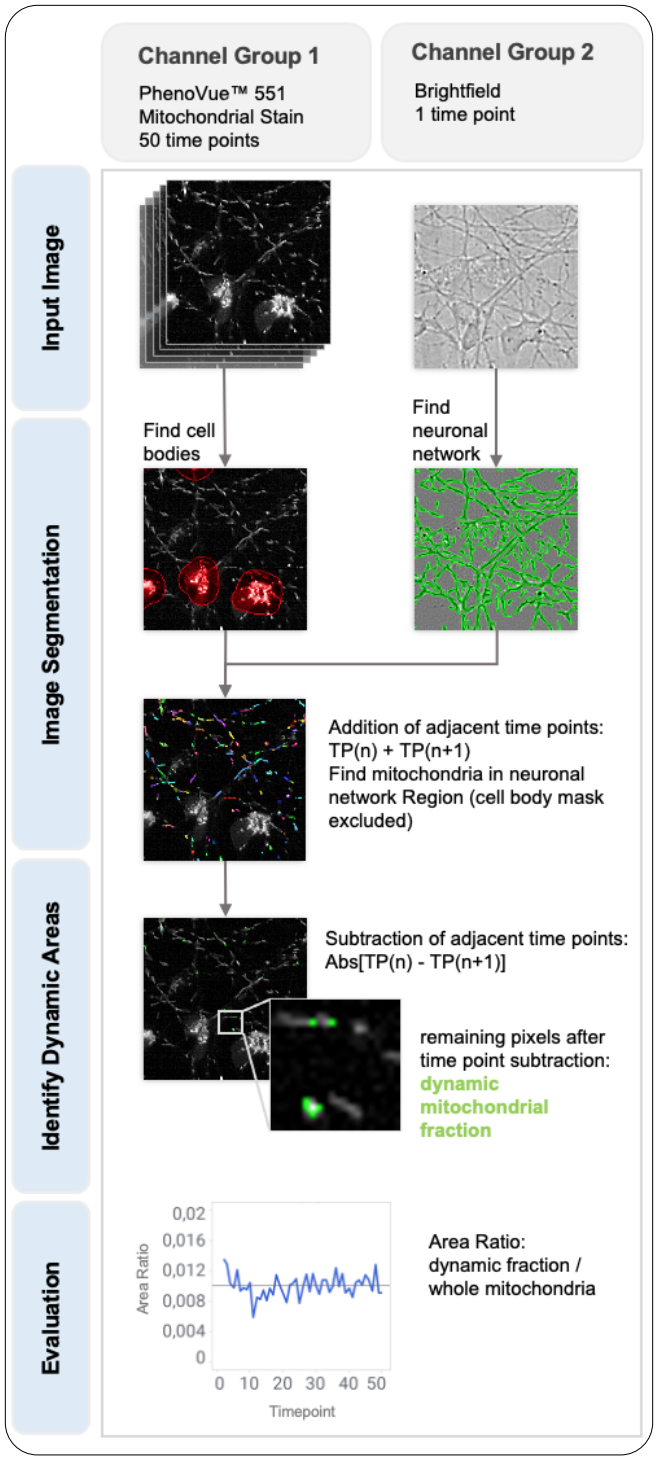


Figure 3. Image analysis sequence in the Harmony software for the evaluation of mitochondrial dynamics over time. Initially, images were segmented into cell bodies and neuronal network. The final neuronal network region (cell body mask excluded) was then used to locate populations of mitochondria. The dynamic mitochondrial fraction could be identified using a newly calculated image after subtraction of adjacent PhenoVue 551 time points. The sum area of the remaining pixels was defined as the dynamic mitochondrial fraction. Finally, the area ratio of the dynamic mitochondrial fraction and whole mitochondria region was evaluated and calculated for each time point.

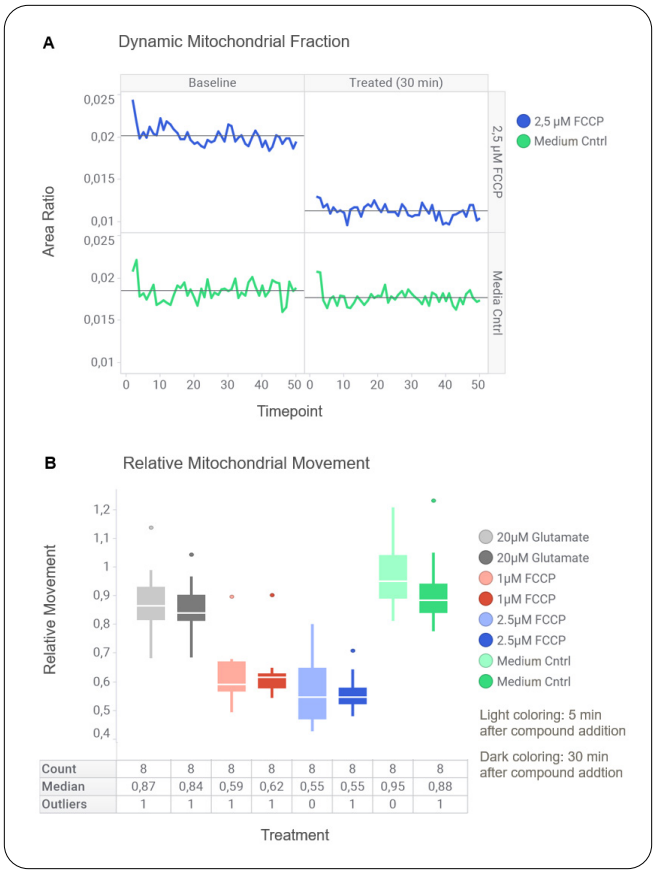


Figure 4. Evaluation of mitochondrial dynamics. (A) The ratio of dynamic mitochondrial fraction over time is shown for two representative wells before and after treatment: untreated medium control in green and FCCP-treated sample in blue. Before compound addition (Baseline), the mean area ratio was about the same level (0.02) in both wells. Treatment with 2.5 µM FCCP for 30 min led to a significant decrease in mitochondrial dynamics. (B) Box plot of the relative mitochondrial movement compared to baseline levels calculated from the mean values of the area ratio over time. Besides untreated medium control (green), cells were treated with 20 µM Glutamate (gray), 1 µM FCCP (red) or 2.5 µM FCCP (blue). Values from two measurements are shown: 5 minutes after compound addition (light coloring) and 30 minutes after compound addition (dark coloring). Glutamate treatment did not reveal a significant change in relative mitochondrial movement. However, the relative mitochondrial movement after FCCP treatment declined substantially.

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Conclusion

Understanding pathological changes in mitochondrial functions is crucial in developing mitochondria-based therapy options for complex diseases such as cancer, neurological disorders, and metabolic syndromes. In this application note, we show how to investigate mitochondrial dynamics in human iPSC-derived neurons using the Operetta CLS high-content analysis system.

Establishing a high-content imaging assay to analyze the dynamics of mitochondria in neurons requires high frame rate imaging. A performance-verified, bright mitochondrial dye and a sensitive imaging instrument allow appropriate image resolution for the reliable tracking of mitochondria while keeping excitation power and time low to avoid phototoxic effects. Utilizing the PhenoVue 551 Mitochondrial stain combined with gentle, high-resolution imaging enables the reliable detection of mitochondrial dynamics for a time-period of up to 1 hour without compromising mitochondrial function. In total, 70 wells could be used with this assay setup comprising baseline measurement, compound addition, and final measurement. With further image acquisition optimization, for example reducing fast kinetic imaging from 25 seconds to 10 seconds, throughput could be increased even more to 180 wells within the hour timeframe.

Hence, live-cell imaging combined with high-content screening represents a promising strategy to analyze mitochondrial dynamics in real time and large scale.

References

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