APPLICATION NOTE

Kinetic analysis of calcium flux activity in human iPSC-derived neurons using the Opera Phenix Plus system

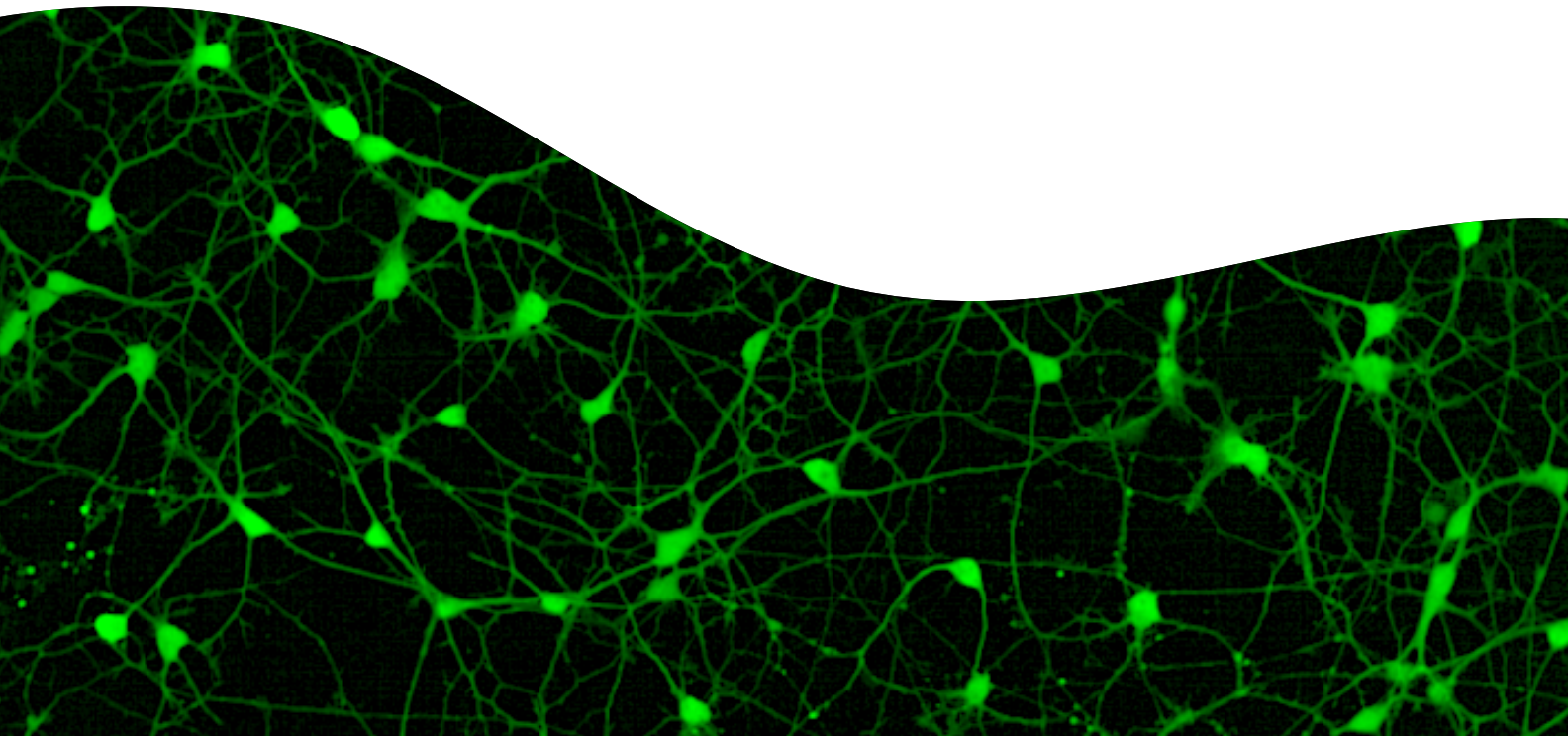
Key features

- Fast frame rate imaging to accurately capture rapid cellular responses
- Reliable quantification of calcium activity in single neurons
- Measure immediate compound responses using onboard liquid handling

Abstract

The study of intracellular dynamic processes is of fundamental importance for unraveling the mechanisms of a broad spectrum of diseases and to develop effective drugs and therapies. Particularly in the field of neuroscience and neurodegenerative diseases calcium signaling is of critical importance. Calcium ions act as second messenger molecules that elicit responses such as neurotransmitter release from synaptic vesicles and altered gene expression. The calcium concentration is highly dynamic due to the presence of intracellular calcium stores and pumps that selectively transport these ions in response to a variety of signals.

Here we apply fast kinetic imaging using the Opera Phenix Plus high-content screening system to visualize and evaluate spontaneous calcium flux activity in single human iPSC-derived neurons labeled with PhenoVue™ Cal-520 AM. Our approach enables the assessment of calcium flux activity in single neurons, crucial for understanding the fundamental processes of neuronal communication and potential disruptions associated with neurodegenerative disease or neurotoxic insults. We used Gabazine as an antagonist of GABA(A) receptors and Glutamate as an abundant excitatory neurotransmitter to demonstrate the sensitivity of the assay to pharmacological interventions.[1]



Calcium flux assay

Material and methods

Table 1: List of materials and devices used in the calcium flux 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	Gabazine (Sigma, #5059860001) Glutamate (Sigma, #49621)
Calcium indicator	PhenoVue Cal-520 AM (Revvity, #CP125201)
Microplate	Assay plate: PhenoPlate™ 384-well (Revvity, #6057300) Compound plate: StorPlate™ 384-well V-bottom (Revvity, #6008590)
Plate coating	Laminin, 5 µg/ml (Corning, #354232)
Microplate seal for compound plate	Aluminium sealing foil, piercable (Porvair, #229572)
Pipette tips	25 µL, sterile (Revvity, #6001640)
Imaging instrument	Opera Phenix Plus system with liquid handling option (Revvity, #HH14001000)
Secondary analysis	Signals™ VitroVivo (Revvity Signals)

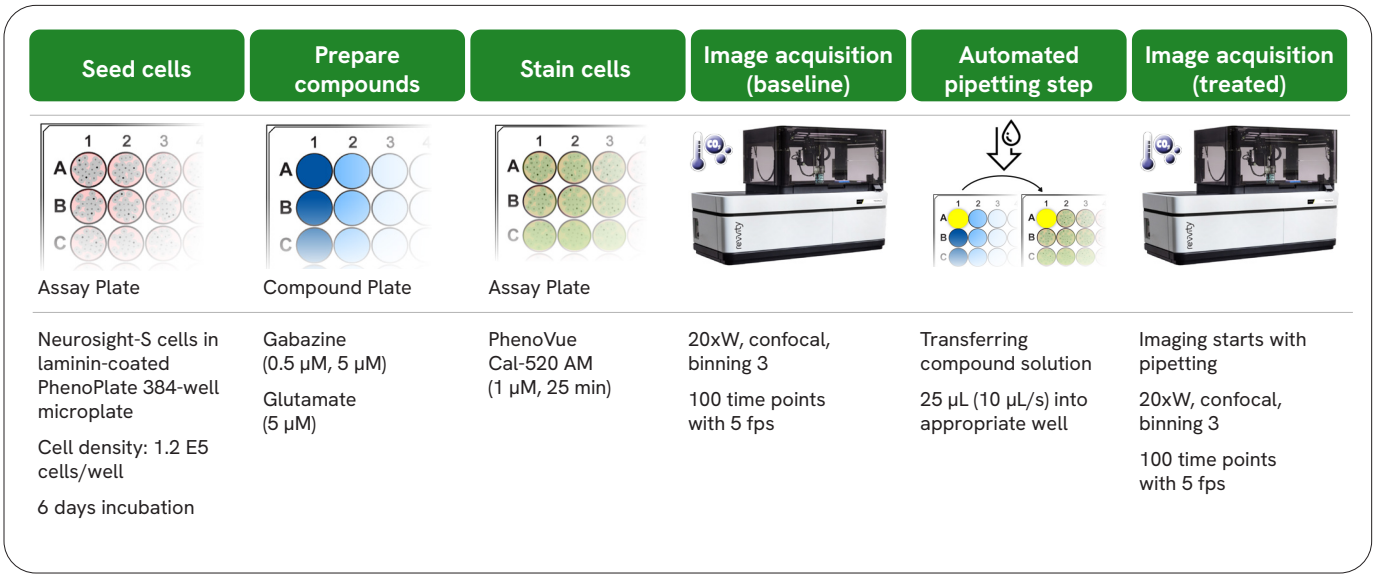


Figure 1: Workflow of the calcium flux assay. Cells are plated into the wells of a laminin-coated PhenoPlate 384-well microplate. The neuronal network formation was monitored using a standard live-cell microscope. After a 6 day incubation period, cells are stained with PhenoVue Cal-520 AM. Images are acquired using a temperature- and CO_2 - equilibrated Opera Phenix Plus high-content screening system. In addition, a 384-well compound plate is prepared for the automated compound transfer step during the measurement. The assay plate was measured with one field and plane per well with a time series measurement in well-repeat mode. Each well was imaged with two consecutive image acquisition sequences with one pipetting step between the two sequences. The image acquisition of the second sequence was synchronized with the start of the compound transfer procedure.

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 32 wells of a laminin-coated PhenoPlate 384-well microplate (Figure 1). Media was exchanged to maintenance media one day post plating and then replaced every other day thereafter. On the day of the experiment, media 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 calcium flux assay on the Opera Phenix Plus system was performed on day 6 post plating.

Compound preparation and cell staining

Compounds were diluted in maintenance media and stored in a StorPlate 384-well V-bottom (50 µL per well). The compound plate layout included two different Gabazine concentrations (1.5 µM and 15 µM), Glutamate (15 µM) and medium control each with 8 replicates. The compound solutions were provided with 3 times higher concentration to ultimately reach final concentrations in the wells of the assay plate after compound transfer. The well positions used in compound and assay plate were identical due to the automated 1:1 compound transfer from one well of the compound plate into the corresponding well of the assay plate. To minimize evaporation and for light protection, the compound plate was heat-sealed as well as pre-equilibrated inside an incubator. Cells were stained with 1 µM PhenoVue Cal-520 AM stain for 25 minutes and subsequently washed once with 50 µl of maintenance media prior to image acquisition.

Image acquisition

Images were acquired with the temperature- and CO_2 - equilibrated Opera Phenix Plus high-content screening system using the 20x water immersion objective in confocal mode and binning factor 3. This combination allowed for imaging with low excitation power (5%) and short acquisition time (80 ms) for the PhenoVue Cal-520 AM channel.

We found that this was the optimal setup to allow gentle imaging of neurons while still ensuring a sufficient signal for robust segmentation of single cell bodies and neurite segments.

The time series measurement was set up in well-repeat mode. Each well was processed with two consecutive image acquisition sequences with one pipetting step between the two sequences. This allowed for imaging of 100 time points with 5 fps in the first sequence to record the baseline of spontaneous calcium flux activity in neurons. Subsequently the automated pipetting step transferred 25 µL compound solution with a dispense speed of 10 µL/s into the corresponding well of the assay plate. The fast kinetic image acquisition of the second sequence was synchronized with the start of the compound transfer to record the change of calcium flux activity upon treatment. In each imaging sequence, the frame rate as well as the number of time points can be defined individually facilitating flexible image acquisition for time series measurements. For this application the same parameters were used as for the first imaging sequence, 100 time points at 5 fps.

Analysis

Images from the time series measurement were analyzed using the Harmony image and analysis software. To assess the calcium flux activity in neurons the active area was evaluated for each time point (Figure 2). In a first step, the images were segmented to define a population of single cell bodies and another population of single neurite segments.

Subsequently, the Cal-520 intensities of single cell bodies or neurite segments were normalized against the median intensity per well of all objects at the first timepoint of the image sequence. Due to well-to-well staining intensity variations, normalization is essential for global threshold definition and active cell body and active neurite segment identification.

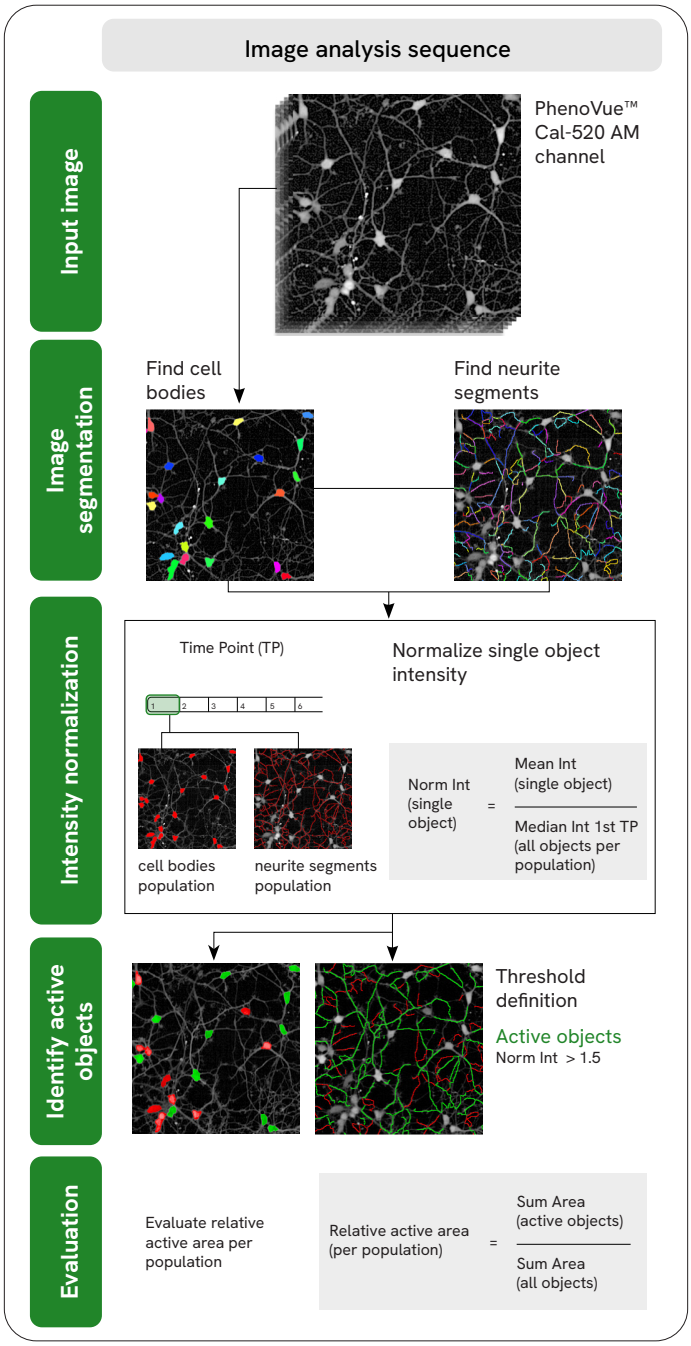


Figure 2: Image analysis strategy in the Harmony software for the evaluation of calcium flux in neurons. Initially, images are segmented into cell bodies and neurite segments. The single object mean intensities are normalized against the median intensity of all objects in the corresponding population at the first time point. This allows for a global threshold definition to identify calcium flux in active cell bodies and neurite segments throughout the assay plate. The calcium response state of a single object is defined as "active" if the normalized intensity exceeds the value of 1.5, 50% above defined ground state activity. Finally, the relative active area of cell bodies and neurite segments are evaluated for each time point.

The first time point intensity per population and well serves as a calcium flux ground state activity in cells and neurite segments. We defined the calcium response state of a single object as "active" if the normalized intensity exceeded the value of 1.5, which corresponds to 50% above defined ground state activity.

The final readout is the area fraction of active objects in relation to all objects of the corresponding population calculated for each time point (Figure 3 A). The fraction of active objects was observed to be stable and did not decrease significantly over time within the given time frame of 20 seconds before treatment indicating the gentleness of imaging conditions. However, the calcium response activity changed immediately upon compound addition. Within the first seconds the fraction of active objects increased substantially after Glutamate treatment and declined after Gabazine treatment.

As additional readout, the relative calcium response after treatment was calculated from the mean values of all time points pre- and post- compound addition (Figure 3 B). Again, Glutamate leads to a significant increase in relative calcium response activity. The relative calcium response level throughout the cell bodies is almost a factor of 2 larger compared to neurite segments. The treatment with Gabazine reveals an inhibition of calcium response activity up to a factor of 4 compared to baseline level response in both populations. Neurite segments were more sensitive compared to the cell body region, as they showed response to 0.5 µM Gabazine already. The control treatment with media already showed a decrease of the relative calcium response in both the cell bodies (20% decline) and neurite segments (50% decline).

With further image acquisition optimization, for example reducing fast kinetic imaging from 20 seconds to 10 or even 5 seconds, throughput could be further increased.

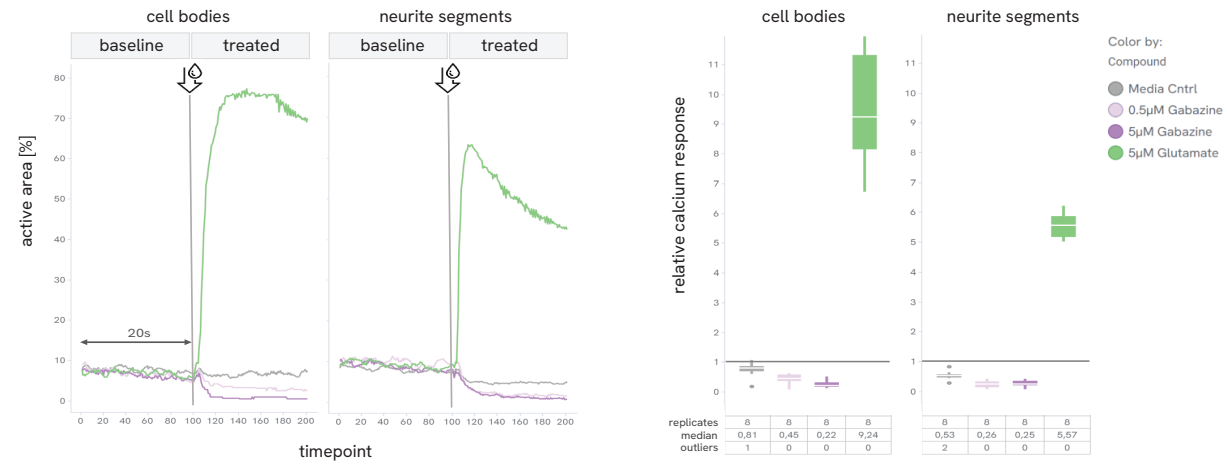


Figure 3: Calcium flux activity in iPSC-derived neurons upon treatment with Glutamate and Gabazine. (A) The relative active area in both populations is shown for representative wells before and after treatment. Before compound addition (Baseline), about 9% of cell bodies and neurite segments show active calcium signals. Treatment with 5 µM Glutamate immediately activated about 75% of cell bodies and up to 65% of neurite segments. Treatment with 0.5 µM or 5 µM Gabazine led to a decrease of the active area for both populations. (B) Box plot of the relative calcium response after treatment. Values from 8 replicates per compound are shown. A calcium response value of 1 (black line) represents no calcium flux alteration after treatment. Glutamate treatment increases the relative calcium response activity by a factor of 9 in cell bodies and by a factor of 5.5 in neurite segments. The treatment with Gabazine inhibits the calcium response activity up to a factor of 4 compared to baseline level response in both populations. Analysis performed in Signals *VitroVivo* software.

Conclusion

In this application note, we show how to investigate compound effects on spontaneous calcium signaling events in human iPSC-derived neurons using the Opera Phenix Plus high-content screening system. Combining the PhenoVue Cal-520 AM stain with gentle, fast kinetic high-resolution imaging enables the reliable detection of calcium flux without compromising neuronal function.

As a prospect this experimental approach could advance to three-dimensional organotypic cell models which are supposed to provide more predictive results for therapeutic development.[2]

In summary, fast kinetic live cell imaging combined with high-content screening represents a promising strategy to analyze calcium response activity in real time and at large scale.

References

1. Galiakberova AA, Surin AM, Bakaeva ZV, Sharipov RR, Zhang D, Dorovskoy DA, Shakirova KM, Fisenko AP, Dashinimaev EB. IPSC-Derived Human Neurons with GCaMP6s Expression Allow *In Vitro* Study of Neurophysiological Responses to Neurochemicals. Neurochem Res. 2022 Apr;47(4):952-966. doi: 10.1007/s11064-021-03497-6. Epub 2021 Dec 2. PMID: 34855047; PMCID: PMC8891101.
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