APPLICATION NOTE

VALA SCIENCES
Unlocking the Cellular Secrets of Disease

Measuring calcium transients in hiPSC-glutamatergic neurons using Kinetic Image Cytometry®

INTRODUCTION

Calcium signaling controls neuronal health and function, including neurogenesis, neurite outgrowth, neurotransmission, synaptic plasticity, gene expression, and apoptosis. Like all cell types, neurons have a network of calcium pumps and regulatory proteins that maintain intracellular calcium concentrations around ~100 nM, about four orders of magnitude less than the extracellular space.[1] Following membrane electrical depolarizations during action potential propagation, calcium enters the neuronal cytoplasm through voltage-gated ion channels, neurotransmitter receptors, and intracellular stores.[2,3] Intracellular calcium concentration peaks throughout the neuron within 100 ms of the action potential but can persist several seconds with a slow decay (Figure 1).[4] Neurons also exhibit action potential-independent calcium waves that can occur over several minutes and remain localized to the site of origin.[5] Calcium peaks and waves induce molecular and structural changes within neurons by activating calcium-sensitive effectors such as calcium/calmodulin-dependent protein kinase II.[6]

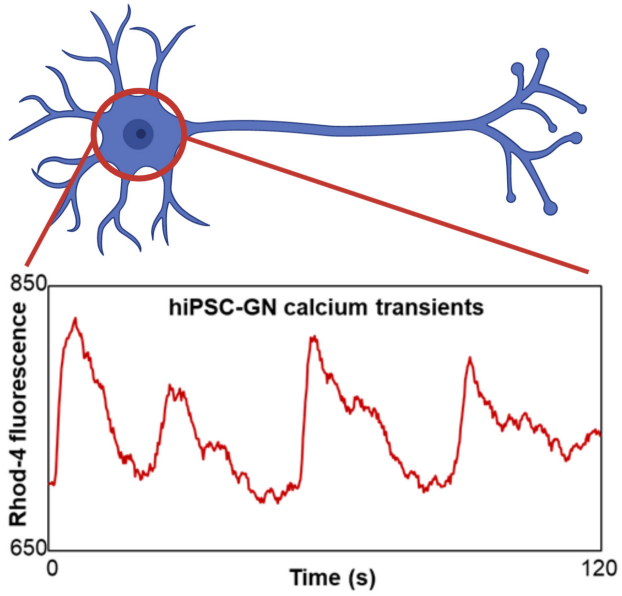


Figure 1. Example trace of intracellular calcium concentration in an active hiPSC-GN. *Neuron was treated with 5 µM kainic acid to increase excitability. Kinetic Image Cytometry® was used to image intracellular calcium concentration based on Rhod-4 fluorescence in the neuronal soma (red circle) at 4 frames per second for 2 minutes. Figure created using BioRender.com.*

Dsyregulation of neuronal intracellular calcium concentrations and signaling occurs in aging, traumatic brain injury, and neurodegenerative diseases such as Alzheimer's, Parkinson's, and Huntington's.[1] Calcium dysregulation may also contribute to pollutant-induced neurotoxicity.[7] In these disorders, neuronal calcium overload typically precedes histopathological markers and clinicial symptoms such as cognitive decline.[8] Calcium overload interferes with neurotransmission and causes neurite retraction, release of toxic compounds (e.g., β-amyloid fragments in Alzheimer's), neuroinflammation, and neuronal cell death.[9] In vitro assays that measure neuronal calcium transients can therefore identify compounds with neurotoxic or neuroprotective effects in models of human diseases.

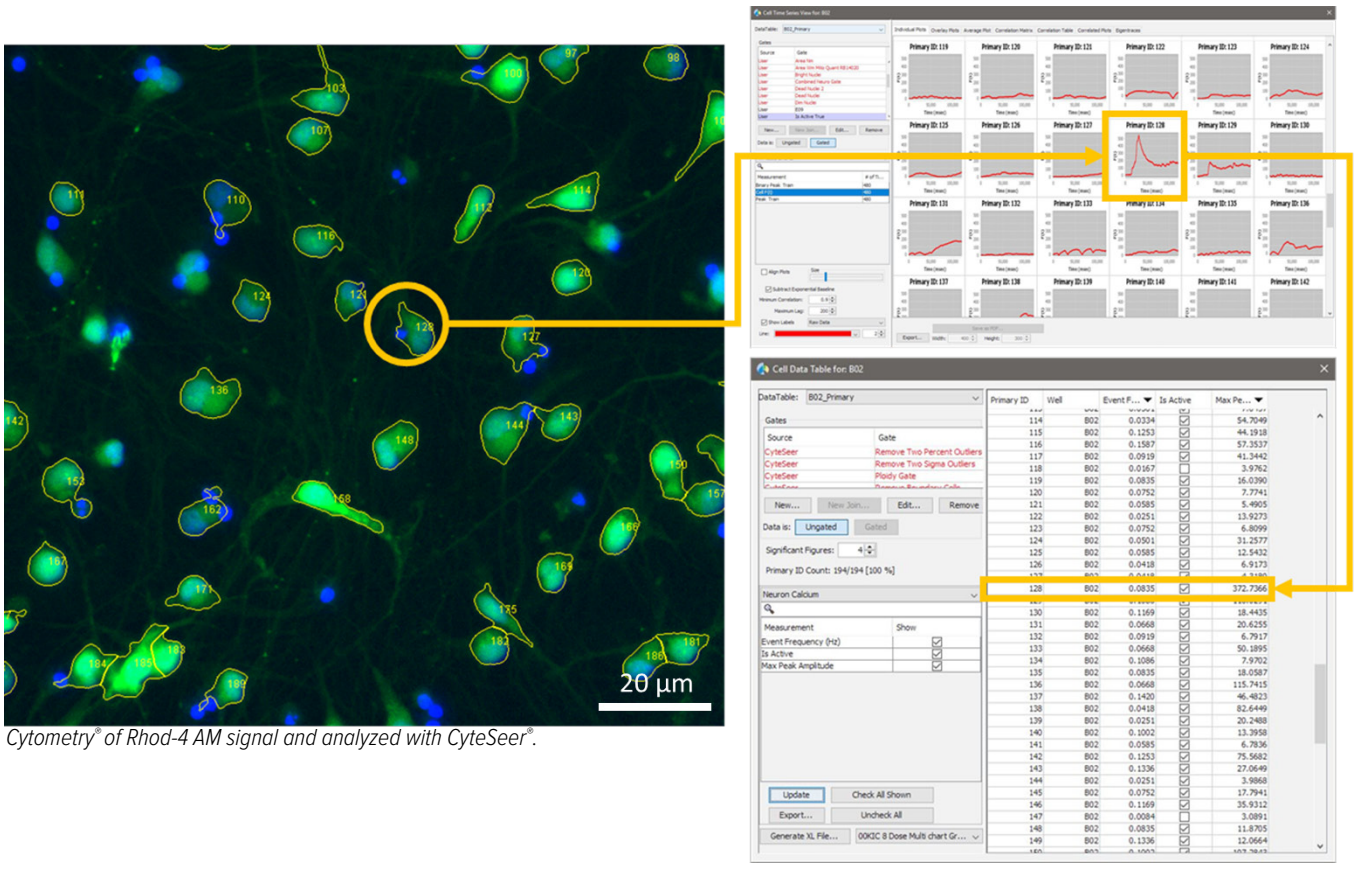
Many in vitro screens measuring compound effects on neuronal function use rodent primary neurons or immortalized human or rodent cell lines. However, these systems incompletely model the gene expression, cell biology, and electophysiology of human neurons and may fail to predict compound safety or toxicity in the human brain.[10] Cortical glutamatergic neurons differentiated from human induced pluripotent stem cells (hiPSC-GNs) exhibit the electrophysiological phenotype of human excitatory neurons and form functional neural networks, synapses, and

calcium signaling pathways in vitro.[11] hiPSC-GNs thus represent a new model system for high-throughput screening of compound effects on neuronal health and function.

In this study, we tested if Vala's IC200 Kinetic Image Cytometer® (KIC) automated digital microscopy workstation and CyteSeer® cell image analysis software can quantify the effects of compounds on calcium concentration in hiPSC-GNs. We treated hiPSC-GNs with glutamate, an excitatory neurotransmitter, or with 4-aminopyridine or forskolin, comounds that upregulate calcium transients in some neuronal model systems.[12,13] We loaded neurons in each treatment group with Fluo-4 or Rhod-4, fluorescent intracellular calcium indicators, imaged the neurons with KIC®, and used CyteSeer® to measure the effects of each compound on neuronal intracellular calcium transients. Our results demonstrate that Vala's technology can efficiently and accurately analyze neuronal activity from hundreds of hiPSC-glutamatergic neurons.

METHODS

iiCell® GlutaNeurons (Cellular Dynamics) were thawed and seeded in 384-well plates (Greiner Bio-One) at 60K, 90K, or 120K cells per cm^2 as needed per assay. Neurons were maintained in the media suggested by the manufacturer at 37°C and 5% CO_2 and treated with compounds as described below.



Cytometry® of Rhod-4 AM signal and analyzed with CyteSeer®.

To measure the effects of glutamate on neuronal calcium concentration, iCell® GlutaNeurons were plated on PEI/laminin coated plates at 120K/cm^2 and maintained for 7 days in the manufacturer's suggested medium. Neurons were loaded with a calcium indicator dye solution containing: 5 µM Fluo-4 (Thermo Fisher Scientific), 1X PowerLoad (Thermo Fisher Scientific), 1 µg/mL Hoechst 33342, and 2.5 mM probenecid in phenol red-free BrainPhys (StemCell

Technologies). Glutamate was prepared at a 20X working concentration for dispensing with a liquid handling system (5 µL dispensed into 95 µL well volume for a final concentration of 25 µM). Fluo-4 fluorescence was recorded for 2 minutes at 4 frames per second with glutamate dispensed after 20 seconds of imaging. Calcium time series were analyzed by CyteSeer® using custom algorithms to identify live neurons and to determine their associated soma (Figure 2).

To measure compound effects on calcium transients in hiPSC-GNs, we treated the iCell® GlutaNeurons with 50 µM 4-aminopyridine (4-AP; Cayman Chemical), 25 µM forskolin (Fsk; Sigma-Aldrich), or 0.1% DMSO as a vehicle control on day 12 in culture. On day 14 in culture, we performed live calcium imaging using the IC200 Kinetic Image Cytometer®. Neurons were loaded with a calcium indicator dye solution containing: 5 µM Rhod-4 AM (AAT Bioquest), 1X PowerLoad (Thermo Fisher Scientific), 1 µg/mL Hoechst 33342, and 777 µg/mL probenecid for 40 minutes at 37°C and 5% CO_2. Following dye loading, compounds were re-applied to the wells in phenol red-free BrainPhys (Stemcell Technologies) for imaging at 4 frames per second for 2 minutes in one field of view per well. Calcium time series were analyzed by CyteSeer® using custom algorithms to identify live neuronal nuclei and to determine their associated soma (Figure 2).

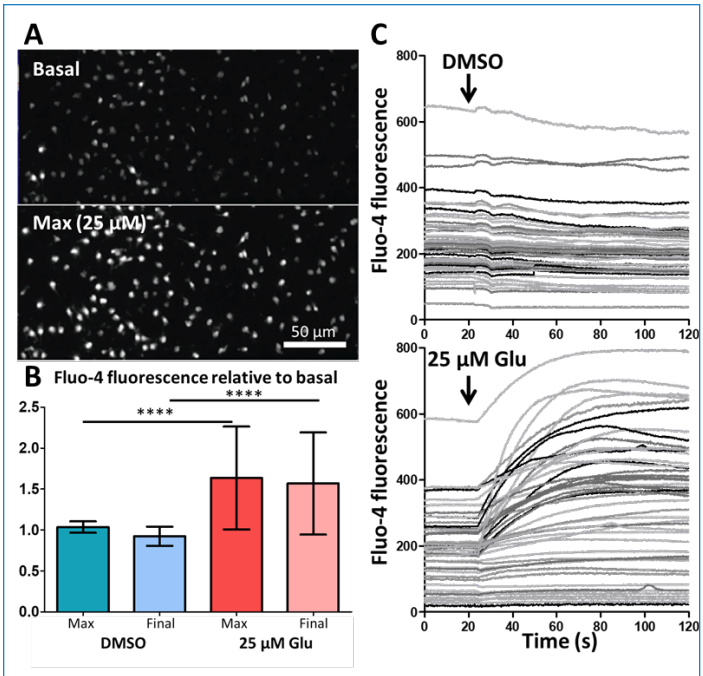


Figure 3. Intracellular hiPSC-GN calcium concentration increases in response to glutamate. *(A) Representative images of Fluo-4-loaded hiPSC-GNs at baseline and after addition of 25 µM glutamate. (B) Maximum and final Fluo-4 fluorescence relative to starting basal fluorescence in neurons treated with DMSO or 25 µM glutamate. DMSO: n = 989 cells; Glu: n = 855 cells. Data represented as mean ± SD. Statistical analyses by t-tests with Welch's corrections. (C) Calcium traces for 50 representative cells per condition before and after dispensing DMSO alone (top) or 25 µM glutamate (bottom). Solutions were dispensed after 20 seconds of imaging. Total imaging time: 120 seconds.*

RESULTS

In the absence of stimulus, some hiPSC-GNs display small soma calcium transients in Fluo-4 traces (Figure 3). After 20 seconds of imaging, we dispensed either DMSO alone or 25 µM glutamate. While the calcium levels in DMSO-treated neurons continued to display small fluctuations, the levels in glutamate-treated neurons increased to 1.5 times the basal level within 30 seconds. The calcium levels in some glutamate-treated neurons decreased over time but remained higher than that of the DMSO-treated neurons by the final frame of imaging.

Figure 4 confirms that DMSO-treated hiPSC-GNs exhibit transient spikes in soma calcium levels that vary in magnitude and frequency on a cell-by-cell basis, as seen in typical neurons. The IC200 KIC® and CyteSeer® could resolve calcium transients for each cell in the field of view. As expected, 50 µM 4-aminopyridine (4-AP) increased the frequencies and peak amplitudes of neuronal calcium transients, as evident in individual traces (Figure 4) and in averages across hundreds of hiPSC-GNs (Figure 5).[13] Forskolin (25 µM) dampened hiPSC-GN average calcium transient frequency and peak amplitude.

DISCUSSION

Progressive neurodegenerative diseases such as Alzheimer's currently have no cures, affect tens of millions of people worldwide, and cost hundreds of billions of dollars in annual patient care.[9] The data above demonstrate that Vala's instrumentation (the IC200 KIC®) and image analysis software (CyteSeer®) can rapidly quantify compound effects on calcium transients in hundreds of hiPSC-GNs. High-throughput assays on hiPSC-GNs using Vala's technology can increase the likelihood of discovering effective treatments by increasing the efficiency and accuracy of screens for neuroprotective or neurotoxic compounds.

CONCLUSIONS

The neuronal activity assay presented here can be expanded to include simultaneous measurement of hiPSC-GN calcium transients and action potentials using FluoVolt™, a small molecule fluorescent voltage sensor.[14] CyteSeer® can report many calcium and voltage parameters (e.g., maximum peak amplitude, mean peak area, and peak width at half-maximal amplitude) that can be combined with multidimensional analysis methods, machine learning, and artificial intelligence to detect changes relevant to neuronal function. Vala's technology can also quantify action potentials and/or calcium transients in other disease-relevant cell types, including cardiomyocytes, skeletal muscle, and pancreatic beta cells. Thus, the IC200 KIC® is a versatile automated microscopy workstation with broad applications across biomedical and cell biology research.

CONTACT US

Please contact Vala Sciences, Inc at www.valasciences.com/contact, info@valasciences.com, or toll-free at (858) 742-8252 for more information about purchasing an instrument, which includes a copy of CyteSeer®. We also provide drug discovery or safety screening services using our technologies.

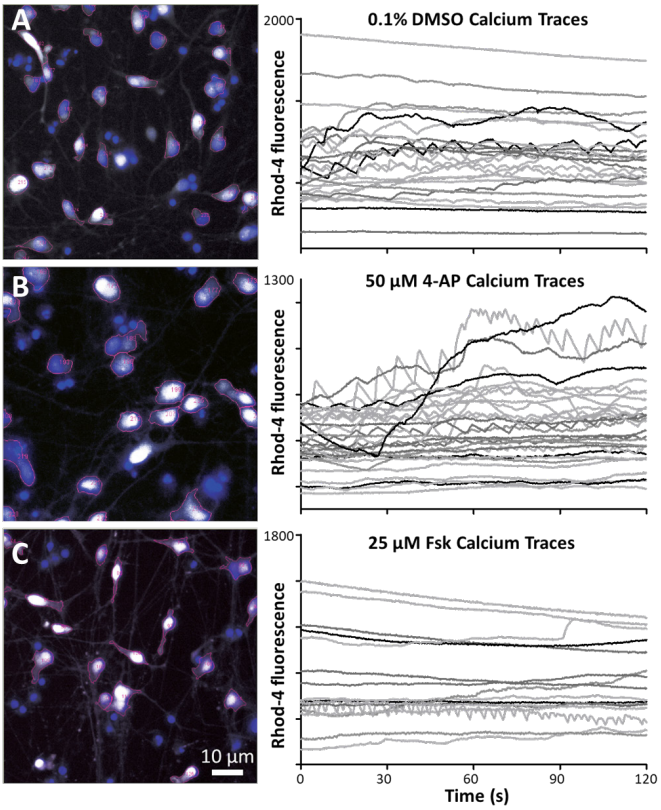


Figure 4. Single-neuron calcium readouts from CyteSeer®. *Calcium time series were analyzed by CyteSeer® using algortithms to identify live neuronal nuclei and determine their associated soma. (A-C, left) Examples of Rhod-4 fluorescence in neurons (white) and Hoechst-stained nuclei (blue). Soma masks are outlined in magenta. These pictures are a ~200 x 200 µm ROI of the full field of view (830 x 700 µm). (A-C, right) Rhod-4 fluorescence on individual soma masks over time. Each trace represents a single neuron in the ROIs in the corresponding panels on the left.*

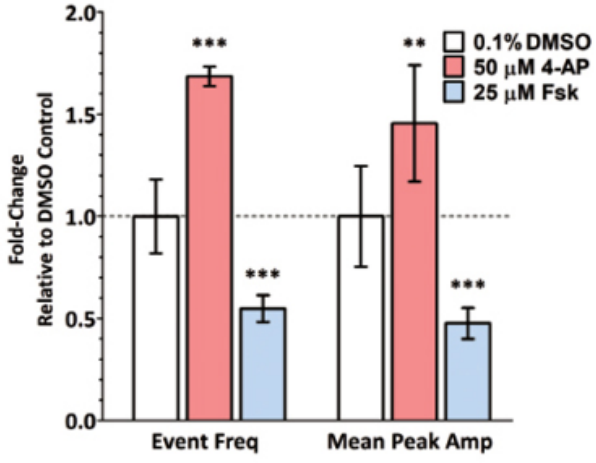


Figure 5. 4-aminopyridine (4-AP) increases calcium transient frequency and amplitude while forskolin (Fsk) attenuates calcium transients. *For both 4-AP and Fsk, n = 6 wells; for DMSO, n = 10 wells. Data were pooled from two separate representative experiments (see Figure 4 for example). Bars and error lines represent mean ± SD. Statistical analysis by ANOVA followed by Dunnett's post-test; **p<0.01, ***p<0.001.*

REFERENCES

1. Wegierski, T. & Kuznicki, J. Neuronal calcium signaling via store-operated channels in health and disease. Cell Calcium 74, 102-111, doi:10.1016/j.ceca.2018.07.001 (2018).
2. Brini, M., Cali, T., Ottolini, D. & Carafoli, E. Neuronal calcium signaling: function and dysfunction. Cell Mol Life Sci 71, 2787-2814, doi:10.1007/s00018-013-1550-7 (2014).
3. Lai, H. C. & Jan, L. Y. The distribution and targeting of neuronal voltage-gated ion channels. Nat Rev Neurosci 7, 548-562, doi:10.1038/nrn1938 (2006).
4. Smetters, D., Majewska, A. & Yuste, R. Detecting action potentials in neuronal populations with calcium imaging. Methods 18, 215-221, doi:10.1006/meth.1999.0774 (1999).
5. Rosenberg, S. S. & Spitzer, N. C. Calcium signaling in neuronal development. Cold Spring Harb Perspect Biol 3, a004259, doi:10.1101/cshperspect.a004259 (2011).
6. Fink, C. C. & Meyer, T. Molecular mechanisms of CaMKII activation in neuronal plasticity. Curr Opin Neurobiol 12, 293-299, doi:10.1016/s0959-4388(02)00327-6 (2002).
7. Radio, N. M. & Mundy, W. R. Developmental neurotoxicity testing in vitro: models for assessing chemical effects on neurite outgrowth. Neurotoxicology 29, 361-376, doi:10.1016/j.neuro.2008.02.011 (2008).
8. Stutzmann, G. E. Calcium dysregulation, IP3 signaling, and Alzheimer's disease. Neuroscientist 11, 110-115, doi:10.1177/1073858404270899 (2005).
9. Brawek, B. & Garaschuk, O. Network-wide dysregulation of calcium homeostasis in Alzheimer's disease. Cell Tissue Res 357, 427-438, doi:10.1007/s00441-014-1798-8 (2014).
10. Hunsberger, J. G. et al. Induced Pluripotent Stem Cell Models to Enable In Vitro Models for Screening in the Central Nervous System. Stem Cells Dev 24, 1852-1864, doi:10.1089/scd.2014.0531 (2015).
11. Miki, D. et al. Characterization of Functional Primary Cilia in Human Induced Pluripotent Stem Cell-Derived Neurons. Neurochem Res 44, 1736-1744, doi:10.1007/s11064-019-02806-4 (2019).
12. McDonough, P. M., Prigozhina, N. L., Basa, R. C. B. & Price, J. H. Assay of Calcium Transients and Synapses in Rat Hippocampal Neurons by Kinetic Image Cytometry and High-Content Analysis: An In Vitro Model System for Postchemotherapy Cognitive Impairment. Assay Drug Dev Technol 15, 220-236, doi:10.1089/adt.2017.797 (2017).
13. Pena, F. & Tapia, R. Seizures and neurodegeneration induced by 4-aminopyridine in rat hippocampus in vivo: role of glutamate- and GABA-mediated neurotransmission and of ion channels. Neuroscience 101, 547-561, doi:10.1016/s0306-4522(00)00400-0 (2000).
14. Miller, E. W. et al. Optically monitoring voltage in neurons by photo-induced electron transfer through molecular wires. Proc Natl Acad Sci U S A 109, 2114-2119, doi:10.1073/pnas.1120694109 (2012).