Introduction

A major impediment to studying diseases affecting the human nervous system is the ability to monitor, analyze, and quantify the activity of neuronal cell populations that accurately represent human phenotypes. These limitations are the consequence of minimal access to cells from human patient tissue, as well as a lack of purpose-built instrumentation enabling functional measurements from neuronal cells at sufficient throughput to permit full phenotypic characterizations. With the advent of cellular reprogramming technologies, there has been abundant research toward protocol development to differentiate human induced pluripotent stem cells (hiPSCs) into multiple cell populations found in the brain (e.g. neuronal, glial, immunological, etc.). This has resulted in the generation of many different neuronal cell models (e.g. dopaminergic, GABAergic, glutamatergic, peripheral, etc.), most of which remain poorly characterized. This imparts a requirement to better understand in vitro cellular models and identify means by which they could be refined. The IncuCyte® S3 Live-Cell Analysis System for Neuroscience technology, methodology, and applications described within this application note were designed with these issues in mind. That is, to provide researchers with a set of automated tools in order to facilitate the evaluation, characterization, and validation of complex neuronal models.
Assay Principle

While measuring morphological features of neurons (e.g., neurite outgrowth) can provide insight into their structure, neuronal activity assays provide a more exquisite and sophisticated understanding of how neurons function, form synaptic connections with other neurons, and how they respond to their environment. In this application note, we describe an integrated solution for long-term neuronal activity measurements based on IncuCyte® NeuroBurst Orange, a new neuronal specific, live-cell genetically-encoded calcium indicator (GECI) and the IncuCyte® S3 for Neuroscience. Along with new system capabilities and analysis tools provided by the IncuCyte® S3 Neuronal Activity Analysis Software Module, this system enables automated quantification of calcium oscillations and morphological monitoring of thousands of functional neurons within a culture over long periods of time—days, weeks, and months (Figure 1). This approach provides researchers the opportunity to better understand how and when network connections are made between cells in culture, and how the environmental context (e.g., drug treatments, stromal cells, media formulations, etc.) can alter their behavior.

Materials and Methods

IncuCyte Neuroburst Transduction

IncuCyte NeuroBurst Orange is a lentiviral-based reagent that encodes a GECI under the control of the neuronal specific synapsin promoter. The transduction protocol for expression of IncuCyte Neuroburst Orange in neuronal/astrocyte co-cultures is summarized in Figure 2a: tissue culture plates were coated with biomatrix, neuron and astrocytes were plated sequentially, and then NeuroBurst Orange Reagent (Essen BioScience Cat. No. 4586) was added after two days in culture. The following day, the NeuroBurst Orange Reagent was removed, anti-proliferative compounds were added to restrict glial cell division, and IncuCyte scans were initiated to assess neuronal activity.

Cell Plating Methods

Plate coating primary rat co-culture model: 96-well plates were coated with 0.1 mg/mL Poly-D-Lysine for 20 hrs at room temperature. Plates were washed 2X with sterile water and allowed to dry before plating.

Plate coating IPS-derived neurons: 96-well plates were coated with 2.2 mg/mL polyethyleneimine (PEI, Sigma Cat. No. 408727) in 0.1 M sodium borate solution (Thermo Fisher Cat. No. 2834) for 20 hrs at room temperature. The following day, PEI was removed and wells were washed 2X with sterile water (cell culture grade). 100 μL of 3.3 μg/mL laminin was then added to each well and incubated for at least 1 hr at 37°C. Laminin (Sigma Cat. No. L2020) was removed immediately prior to cell seeding.

Primary rat forebrain neurons: 20K forebrain or cortical neurons (Essen BioScience Cat. No. 4736) were plated in Complete BrainPhys™ Neuronal Medium (Stemcell Technologies, Cat. No. 05711) including 5-FdU/U. 50% of the assay media was replaced every 3 days for the duration of the experiment.

IncuCyte S3 for Neuroscience User Interface

The IncuCyte S3for Neuroscience user interface is designed to visualize neuronal activity within each well of a 96-well plate. Each scan consists of a 30-180 second “Stare Mode” capture of cellular activity at a rate of three frames per second. Each “Stare Mode” acquired movie is distilled into a single range image to allow for simple viewing (Figure 2b). This image represents the range of intensities that are detected from each cell within the culture over the specified scan time. Using this image, automated image segmentation tools are used to identify active objects (cells) within each well (Figure 2c). Based on the changing fluorescent intensity of each individual cell, intensity traces are displayed for every active cell in the culture (Figure 2d). Scanning is typically completed once every 24 hrs. As shown in these sample traces of IPS-derived iCell® GlutaNeurons (Cellular Dynamics), the activity within these cultures can significantly change from day-to-day as the network matures, in this case with minimal activity at day 4, a gradual increase at day 7, and highly synchronous activity visualized at day 12 and day 17. Once these data are collected, several automated metrics are calculated for each well and at each scan time, allowing for simple visualization of changing metrics over the full time-course of the experiment (Table 1).

A. Quick Guide: IncuCyte® NeuroBurst Orange Reagent

1. DIV: -1
2. DIV: 0
3. DIV: 0 + 2 hours
4. DIV: 2
5. DIV: 3

B. Prepare assay plate

1. Inoculate 20K rForebrain or rCortical neurons
2. Plate rAstrocytes
3. Conduct chronic studies

C. Acquire movies

1. Capture short-term, calcium-flux kinetics using IncuCyte® Stare mode acquisition
2. Visually assess morphology with phase contrast images

D. Analyze active cells

1. Quantify burst rate of active neurons in every movie
2. Visually assess morphology with phase contrast images

E. Conduct chronic studies

1. Continuously interrogate dynamic changes in the same population of cells in individual wells over weeks or months

Figure 2. IncuCyte S3 for Neuroscience and Neuronal Activity Application. Quick guide workflow of NeuroBurst Orange infection protocol (A). The Neuronal Activity user interface is capable of displaying object traces, scanning movies, and longitudinal data of neuronal activity from each well (B). Fluorescent range image and automated segmentation mask of each active object represents a snapshot of activity over the complete scan (C). An example of iCell GlutaNeuron calcium traces from each 3 min scan indicate changing neuronal activity (fluorescence intensity) over 17 days in culture (D).
Every object is compared to every other object in the image to generate a value between -1 and 1, with 0 being completely random and 1 being highly synchronized. This is a measure of network connectivity.

<table>
<thead>
<tr>
<th>Metric</th>
<th>Description</th>
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<tbody>
<tr>
<td>Active Object Count (1/image)</td>
<td>The number of objects (cells/cell clusters) that burst at least once above the Minimum Burst threshold over the total scan time.</td>
</tr>
<tr>
<td>Mean Intensity (OUC)</td>
<td>The mean intensity of an object over the total scan time. All objects within the image are calculated individually, then values are averaged.</td>
</tr>
<tr>
<td>Mean Correlation</td>
<td>Every object is compared to every other object in the image to generate a value between -1 and 1, with 0 being completely random and 1 being highly synchronized. This is a measure of network connectivity.</td>
</tr>
<tr>
<td>Mean Burst Duration (sec)</td>
<td>The duration of each calcium burst over the total scan time is calculated individually, then values are averaged.</td>
</tr>
<tr>
<td>Mean Burst Rate (1/min)</td>
<td>The number of calcium bursts over the total scan time divided by the scan time in min.</td>
</tr>
<tr>
<td>Mean Burst Strength (OUC)</td>
<td>The area under each calcium burst divided by the duration of that burst is calculated individually. then values are averaged.</td>
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Table 1: Neuronal Activity Analysis Metrics.

Results

Optimization of IncuCyte NeuroBurst Orange

Primary rat neurons (E18) in co-culture with primary rat astrocytes represent a well-tested model for studying neuronal activity. In this experiment, E18 rat forebrain neurons were plated at decreasing cell densities (5–40K/well) in co-culture with a fixed number of rat astrocytes (15K per well). As visualized in Figure 2a, fluorescence intensity within the range image strongly correlates with cell density, with the highest amount of activity observed at 40K neuron/well.

Long term expression of NeuroBurst GECl does not impact functional measurements of neuronal activity

In a long-term, live-cell experiment, it is imperative that reagents used for cell analysis are non-perturbing to the functional biology of the culture. This ensures that any changes that are recorded are a result of the experimental conditions and not the reagent used to detect them. To verify that long term expression of the GECl does not negatively impact activity or synchronicity of IPC-derived neurons, co-cultures of Cell Glutamate neurons were infected 3 or 21 days after culture initiation to determine if differences in activity could be observed as a function of length of GECl expression. No measurable difference in neuronal activity was found (Figure 4). Furthermore, we did not observe any acute toxicities associated with viral transduction. Provided a careful exploration of optimal viral concentration was completed (data not shown). These data indicate that the presence of the GECl does not buffer calcium to the extent that an effect on the biology is observed, and that acute toxicities associated with viral infection can be avoided with proper experimental optimization.

Figure 4. Long term expression of NeuroBurst Orange GECl does not alter neuronal activity.

Figure 5. Culture conditions impact neuronal activity

Cell culture conditions can have an impact on neuronal function. Complete BrainPhys Neuronal Medium is a serum-free, neurophysiological basal medium specifically developed for improved neuronal function. To test whether the media could affect the function of IPC-derived neurons, we cultured Peri.4U cells, IPC-derived peripheral neurons provided by Ncardia, in either BrainPhys medium or the media provided by the manufacturer (Neuro.4U). Figure 5). Qualitative inspection of cell morphology did not reveal obvious differences in cell health or neuronal network structure. Quantitative measurements of neuronal function indicate that both media types support neuronal activity with noticeably increased activity observed within the first three days of culture. However, the number of active objects observed in the Ncardia medium were higher than in complete BrainPhys medium. Interestingly, although the synchronicity of cells in both culture conditions remained low for the extent of the 25 day experiment, we did observe higher synchronicity in co-cultures grown in BrainPhys medium compared to Ncardia medium. Although changes in environmental conditions (media and supplements) do not appear to affect qualitative observations of neuronal morphology and network complexity, these data illustrate that there were significant alterations in neuronal function and connectivity.

Figure 5. Culture conditions impact neuronal activity.

Figure 3. Functional activity of primary neurons. Primary rat forebrain neurons were seeded at 40K (rows A and B), 20K (rows C and D), 10K (rows E and F), and 5K (rows G and H) cells / well. All densities of neurons were plated in a co-culture with primary rat astrocytes seeded at 15K cells/well and transduced with the NeuroBurst Orange Reagent. 96-well view of the range image over the course of the scan provides a snapshot of active wells at each time point (A). Summary traces of fluorescence intensity across all active objects for the 96-well plate at day 12 provide an overview of activity and display metrics of bursting intensity, active object number and mean correlation (B). 96-well throughput with high kinetic reproducibility over 12 days in culture (C).
Kinetic Profile of different iPSC-derived Neurons

Using the IncuCyte S3 for Neuroscience and the NeuroBurst Orange Reagent, we evaluated four different types of iPSC-derived neurons over 30-50 days in culture. These included iCell GlutaNeurons (Figure 6a), iCell GABANeurons (Figure 6b), iCell DopaNeurons (Figure 6c) co-cultured with primary rat astrocytes, as well as CNS.4U neurons (Figure 6d). iCell GlutaNeurons, described as human glutamatergic-enriched cortical neurons derived from iPSCs, displayed a rapid induction of calcium burst activity in >1500 cells that became highly correlated within 10 days of co-culture. iCell GABANeurons, characterized as a culture of >95% pure population of GABAergic (inhibitory) neurons, also displayed a rapid increase in the number of cells with calcium burst activity within the first week of co-culture. However, iCell GABANeurons did not display significant correlation at any time-point tested, in line with their inhibitory phenotype. A closer examination of cellular activity at day 14, displayed as object traces over the full 3 min scan (Figure 6a and b), supports the observation of a significant number of active cells in both the iCell Gluta- and GABAneurons; the former displaying higher calcium burst intensity and synchronicity when compared to the latter.

Interestingly, the kinetics of iCell DopaNeuron activity was strikingly similar to iCell GlutaNeurons, illustrating a very rapid induction of highly active, highly correlated networks within the first 10 days of culture. Ncardia’s CNS.4U cells represent an in vitro co-culture model of iPSC-derived neurons and astrocytes. These cells showed significant activity from nearly 1200 cells within the first week of culture and an increase in correlated activity (network connectivity) at approximately day 34 in culture, reaching a correlation of 0.7 at day 45 when the experiment was terminated.

**Figure 6.** Functional activity of different iPSC-derived neurons. iCell GlutaNeurons, iCell GABA Neurons, iCell DopaNeurons (Cellular Dynamics International) and CNS.4U neurons (Ncardia) were all seeded at 20K cells/well. iCell GlutaNeurons, iCell GABA Neurons and iCell DopaNeurons were also plated with a co-culture of rat astrocytes seeded at 15K cells/well. Neurons were infected with NeuroBurst Orange reagent, and active object number and mean correlation were quantified for up to 45 days. Example calcium oscillation traces and kinetic graphs of activity metrics over time for iCell GlutaNeurons (A) and iCell GABA Neurons (B). Mean correlation and active object number were quantified for iCell DopaNeurons (DS day 14) (C) and CNS.4U neurons (14 days) (D). Data points represent Mean ± SEM.

**Figure 7.** Measurements of neuronal structure vs. function. Rat cortical neurons seeded at 30K cells/well were co-cultured with rat astrocytes seeded at 15K cells/well and transduced with NeuroBurst Orange or NeuroLight Orange at day 3 in culture. Live-cell analysis measurements were made each day using IncuCyte S3 for Neuroscience. After 11 days, neural networks had fully formed and stabilized. Taxol or vehicle control was then added and cultures were monitored for an additional 11 days. Time-courses of neurite development (A) and neuronal activity (B) prior to, and after the addition of control or increasing concentrations of Taxol are shown. Potency (IC50) values plotted against time post-treatment for neuronal activity (grey) and neurite length (orange) (C). Data is expressed as % neurite length or active object count, normalized to the pre-treatment value. Data points represent Mean ± SEM. Neuronal activity summary traces at pre-treatment and at 5, 10 and 15 days post-treatment display decreased activity levels over the course of the experiment (B).
Conclusions
In this application note, we present data to support the use of the IncuCyte S3 for Neuroscience to characterize and refine different neuronal phenotypes and their maturation for modeling their function in vitro. This single live-cell imaging platform allows users to assess calcium flux kinetics and continuously monitor morphology of neuronal populations long-term using non-perturbing reagents, validated protocols that are cell-spacing, and a built-in, guided interface for non-experts provided by the IncuCyte S3 Neuronal Activity Analysis Software Module. The system can be used within the operator’s own incubator under physiological conditions.

We also introduce the IncuCyte Neuroburst Orange reagent, a genetically-encoded fluorescent calcium sensor that is non-perturbing, long-lasting, and may be used with a variety of neuronal cell types (such as primary neurons and iPSC-derived models). This reagent enables users to determine when neuronal activity starts and how such activity changes continually over time with minimal disturbance along with quantitative monitoring of morphological changes. Spontaneous Ca2+ oscillations can be captured and recorded directly from thousands of neurons over weeks to months. Analysis is amply supported with cell-by-cell information through segmentation, and turnkey ‘real time’ visualization of both raw and compressed data.

As described above, this system and reagents can provide valuable, “real world” kinetic insights into neuronal network activity and connectivity in neurological models that might be missed by traditional end-point analysis. Here, we utilize the IncuCyte S3 for Neuroscience to assess media and supplement influences on the morphology and function of iPSC-derived neurons in the context of calcium bursting. As described, we detected increased numbers of active cells within Peri-AU cultures with use of NcAria Medium compared to Complete BrainPhys Medium, but higher synchronicity in co-cultures with BrainPhys Medium. This illustrates how the measurement of calcium bursting with real-time imaging can detect subtle functional changes in response to media and supplement differences.

Knowing when iPSC-derived neurons become functionally active, how to optimize their activity, and gaining insight into the synaptic connectivity of cultured neurons has eluded neuroscience researchers. Using predominantly QABA and Gluta iPSC-derived neuronal models, we have shown how the IncuCyte S3 for Neuroscience provides a means to study a variety of cell models using relevant quantitative metrics (e.g. neurite length and cellular activity.) Interestingly, our work in CNS-AU neurons, which develop spontaneous activity within days but take >30 days to exhibit synaptic connectivity, clearly illustrates how these models can dramatically differ from one another. Lastly, the robustness and throughput provided by the IncuCyte system enables researchers to focus on isolating variables in order to improve iPSC-derived neuronal model development.

The IncuCyte S3 for Neuroscience provides a complete end-to-end solution for the characterization of neuronal phenotypes and their maturation, not only for neuronal cell function, but also provides important information for a wide variety of neurological questions that may be missed by other methods.

Resources
Find more information on our Neuroscience Literature and Resource page.

Additional information available online from www.sartorius.com/incucyte

Related Protocols IncuCyte® S3 Neuronal Activity Assay

Reagents IncuCyte® Neuroburst Orange

Instrumentation IncuCyte® S3 Live-Cell Analysis System for Neuroscience

Information on Instruments, Reagents, and Software

Reagents, Software and Consumables for Neuroscience Research

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<tr>
<th>Product</th>
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| IncuCyte® S3 Live-Cell Analysis System for Neuroscience | Includes image acquisition and analysis system with
  • 4x, 10x, and 20x objectives
  • Controller with 16.4 TB storage
  • HD Dual Color Orange/NIR Optical Module | 4763     |
| IncuCyte® S3 Neuronal Activity Analysis Software Module | Enables microplate analysis of neuronal activity via fluorescent calcium detection and movie mode acquisition | 9600-0022 |
| IncuCyte® Neuroburst Orange Lentiviral Reagent | Live-cell neuronal labeling reagent for long-term expression of a fluorescent genetically-encoded calcium indicator | 4766     |
| IncuCyte® NeuroActive Orange Cell Kit         | One kit
  • IncuCyte® Neuroburst Orange Lentiviral Reagent
  • IncuCyte® Actinomycin
  • IncuCyte® rCortical Neurons                 | 4761     |