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Application Note

December 01, 2022

Keywords or phrases:
Neuroscience, Neuronal activity, Neuronal Function, GECI, Calcium flux, iPSC, Cell Analysis Systems

Long-Term Live-Cell Visualization and Quantification of Neuronal Activity

S. L. Alcantara[1], J. Trigg[1], J. Rauch[2], L. Oupicka[2], N. Holtz[2], E. Endsley[2], T. Dale[1]*

[1] Sartorius UK Ltd., Royston, Hertfordshire, UK
[2] Sartorius Corporation, 300 West Morgan Road, Ann Arbor, MI 48108 USA

* With thanks to T. Campbell**, T. Jackson, A. Overland, J. Brown, T. G. Garay
** Talisman-therapeutics.com

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® Live-Cell Analysis System 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.

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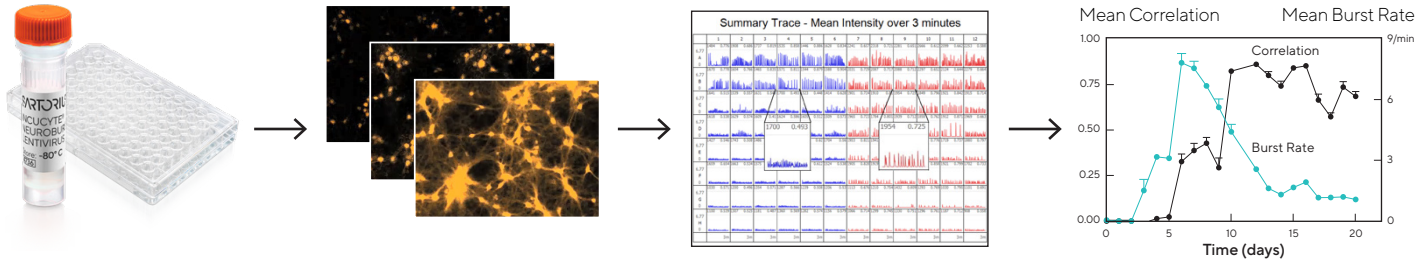
Assay Principle

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 Lentivirus, a neuronal specific, live-cell genetically-encoded calcium indicator (GECI) and the Incucyte® Live-Cell Analysis Systems configured with either an Orange/NIR or a Green/Orange/NIR Optical Module. Along with integrated analysis tools provided by the Incucyte® Neuronal Activity Analysis Software Module, this 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.



1. **Prepare assay plate**
   Infect neurons in mono- or co-culture with Incucyte® Neuroburst Orange Lentivirus.

2. **Acquire movies**
   Capture short-term, calcium-flux kinetics using Incucyte® Stare Mode acquisition.

3. **Analyze active cells**
   Quantify burst rate of active neurons in every movie and visually assess morphology with phase-contrast images.

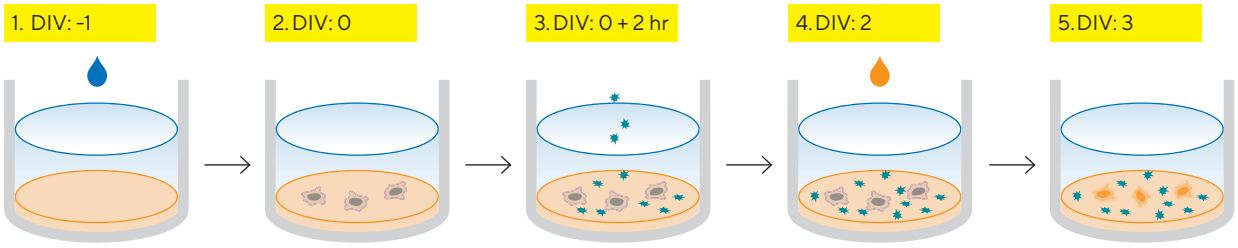
4. **Conduct chronic studies**
   Continuously interrogate dynamic changes in the same population of cells in individual wells over weeks or months.

Figure 1: *Incucyte® Neuronal Activity Assay Workflow*

The Incucyte® Neuronal Activity Assay allows for measurements of long-term synaptic activity from neuronal cell models in physiologically relevant conditions. The assay provides an end-to-end solution consisting of reagents, protocols, instrumentation, and software for a user-friendly workflow.

The Incucyte® Live-Cell Analysis System 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 hours. As shown in these sample traces of iPSC-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 Lentivirus



Coat plate with matrix of choice and incubate at ambient temperature overnight.

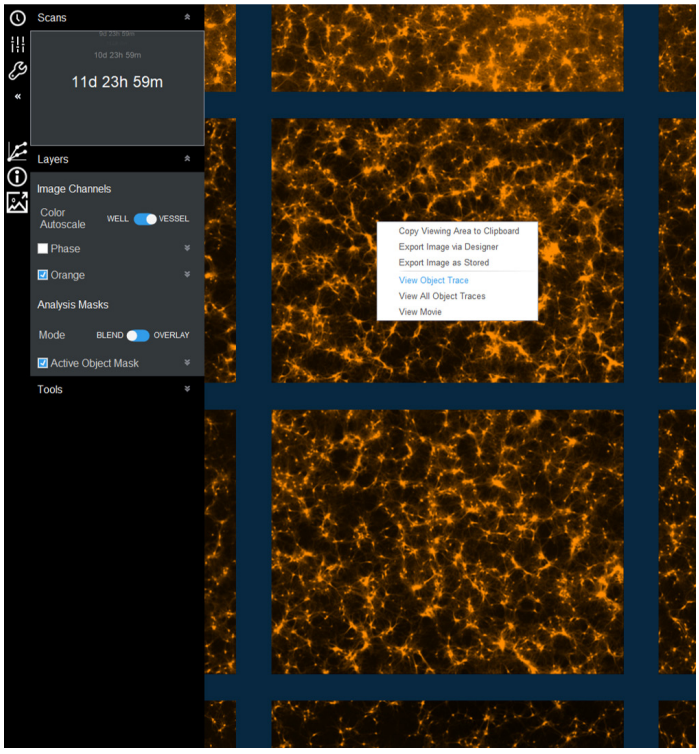
Plate Incucyte® rCortical Neurons.

Plate Incucyte® rAstrocytes.

Add Incucyte® Neuroburst Orange Lentivirus.

1. Remove Incucyte® Neuroburst Orange Lentivirus.
2. Add Uridine + 5- Fluoro-2′-deoxyuridine.
3. Start Neuronal Activity scanning.

B.



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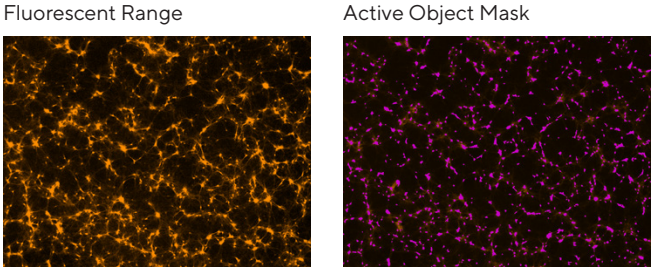

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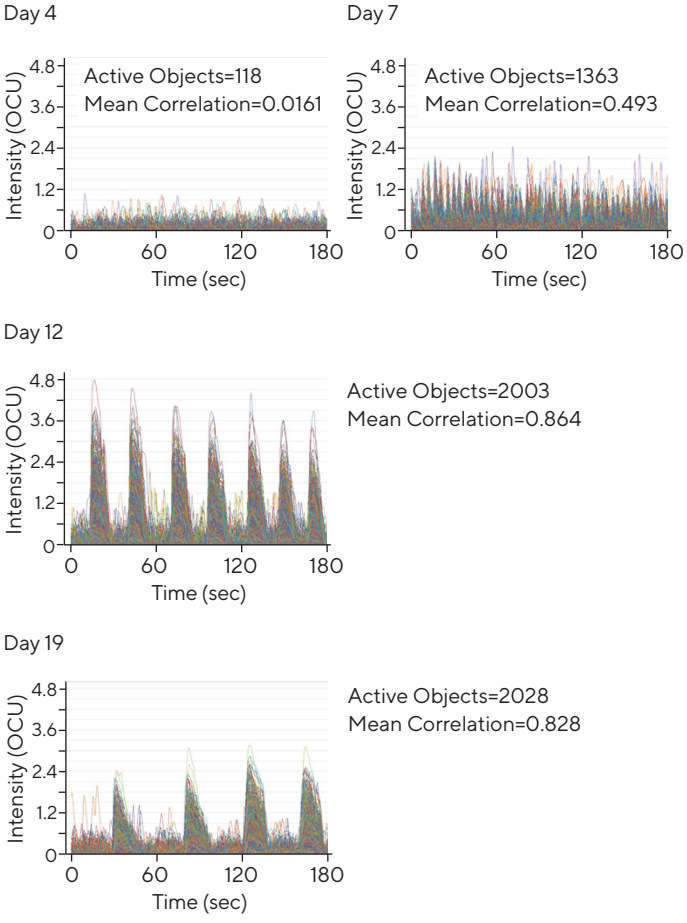


Figure 2: *Incucyte® Neuronal Activity Assay Protocol and Purpose-built Software*

Quick guide workflow of Incucyte® Neuroburst Orange Lentivirus infection protocol (A). The Incucyte® Neuronal Activity Analysis Software Module user interface is capable of displaying object traces, viewing 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 minute scan indicate changing neuronal activity (fluorescence intensity) over 17 days in culture (D).

Table 1: *Neuronal Activity Analysis Metrics*

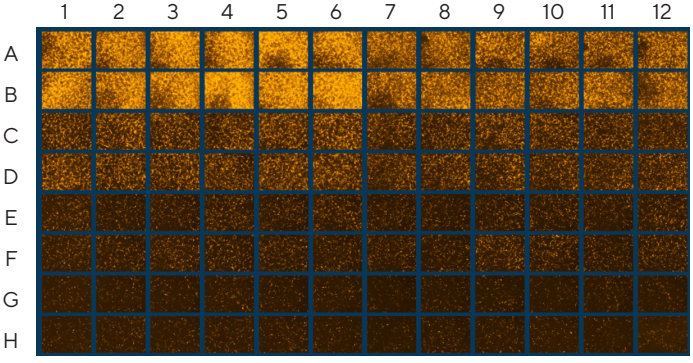
Metric	Description
Active Object Count (1/image)	The number of objects (cells/cell clusters) that burst at least once above the Minimum Burst threshold over the total scan time.
Mean Intensity (OCU)	The mean intensity of an object over the total scan time. All objects within the image are calculated individually, then values are averaged.
Mean Correlation	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.
Mean Burst Duration (sec)	The duration of each calcium burst over the total scan time is calculated individually, then values are averaged.
Mean Burst Rate (1/min)	The number of calcium bursts over the total scan time divided by the scan time in min.
Mean Burst Strength (OCU)	The area under each calcium burst divided by the duration of that burst is calculated individually, then values are averaged.

Results

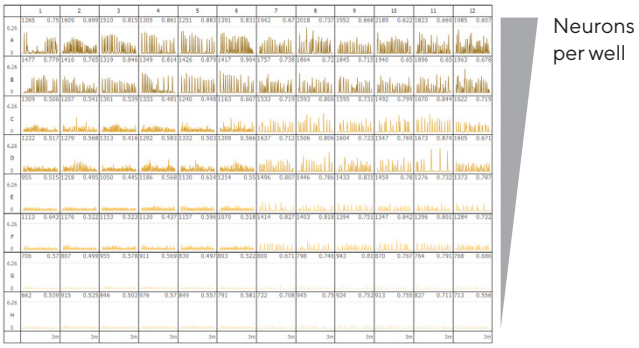
Optimization of Incucyte® Neuroburst Orange Lentivirus

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 3A, fluorescence intensity within the range image strongly correlates with cell density, with the highest amount of activity observed at 40K neurons/well. The range image also provides the researcher with a qualitative assessment of morphology, toxicity, and transduction efficiency. Summary Ca^{2+} traces of neuronal activity provide a quantitative assessment of activity within each well, and density of neurons tested was optimal for visualization of neuronal activity within each scan (Figure 3B) and detection of active objects over the full 12-day time course (Figure 3C).

A.



B. Summary Trace Activity at Day 12



C. Number of Active Objects over 12 Days

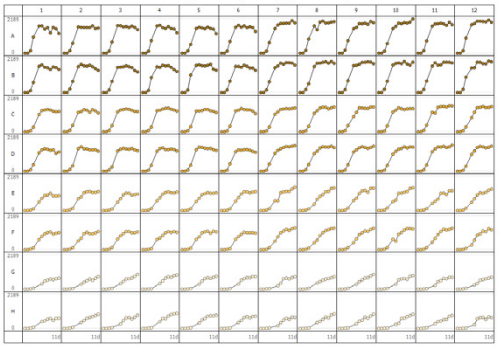


Figure 3: *Functional Activity of Primary Neurons.*

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 Incucyte® Neuroburst Orange Lentivirus. 96-well vessel 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).

Functional Profiling of Different iPSC-derived Neurons

Using the Incucyte® Live-Cell Analysis System and the Incucyte® Neuroburst Orange Lentivirus, we evaluated four different types of iPSC-derived neurons over 30–50 days in culture. These included iCell® GlutaNeurons (Figure 4A), iCell® GABANeurons (Figure 4B), iCell® DopaNeurons (Figure 4C) co-cultured with primary rat astrocytes, as well as CNS.4U neurons (Figure 4D). 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 minute scan (Figure 4A and 4B), 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® CNS.4U cells represent an *in vitro* co-culture model of hiPSC-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.

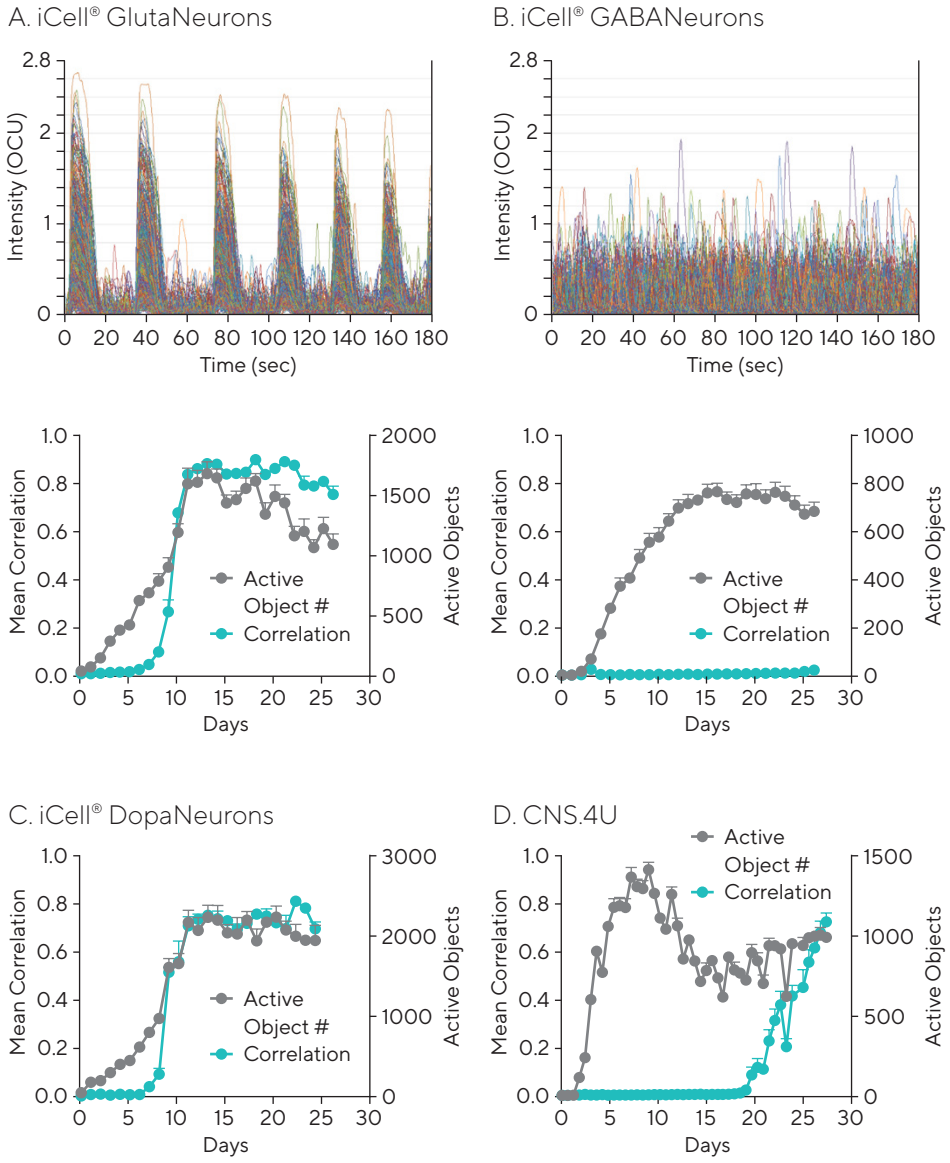


Figure 4: *Functional Activity of Different iPSC-derived Neurons*

iCell® GlutaNeurons, iCell® GABANeurons, iCell® DopaNeurons (Cellular Dynamics International) and CNS.4U neurons (Ncardia®) were all seeded at 20K cells/well. iCell® GlutaNeurons, iCell® GABANeurons and iCell® DopaNeurons were also plated with a co-culture of rat astrocytes seeded at 15K cells/well. Neurons were infected with Incucyte® Neuroburst Orange Lentivirus, 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® GABANeurons (B). Mean Correlation and Active Object Count were quantified for iCell® DopaNeurons (25 days) (C) and CNS.4U neurons (45 days) (D). Data points represent Mean ± SEM.

Insights Into Structure-Function Quantification

Peripheral neuropathies are a common side effect of chemotherapeutic drugs such as paclitaxel (Taxol®) and are associated with numbness and loss of sensory function. To study potential neurotoxic effects, primary rat cortical neurons were first co-cultured with primary rat astrocytes for 11 days, allowing the cultures to mature and stabilize. Baseline measurements of activity and morphology were made each day using Incucyte® Neuroburst Orange and Incucyte® Neurolight Orange Lentivirus respectively (Figure 5). At Day 11, cultures were treated with a range of concentrations of Taxol®. Activity and morphology were monitored for a further 11 days in culture. Figure 5 illustrates that by Day 21, at sub-nanomolar (<10^{-9} M) concentrations of Taxol® only small changes in neurite length were observed, while a reduction in neuronal activity occurred (Figure 5C). Individual well traces indicated both concentration- and time-dependent responses of neuronal activity following Taxol® treatment as shown in Figure 5D.

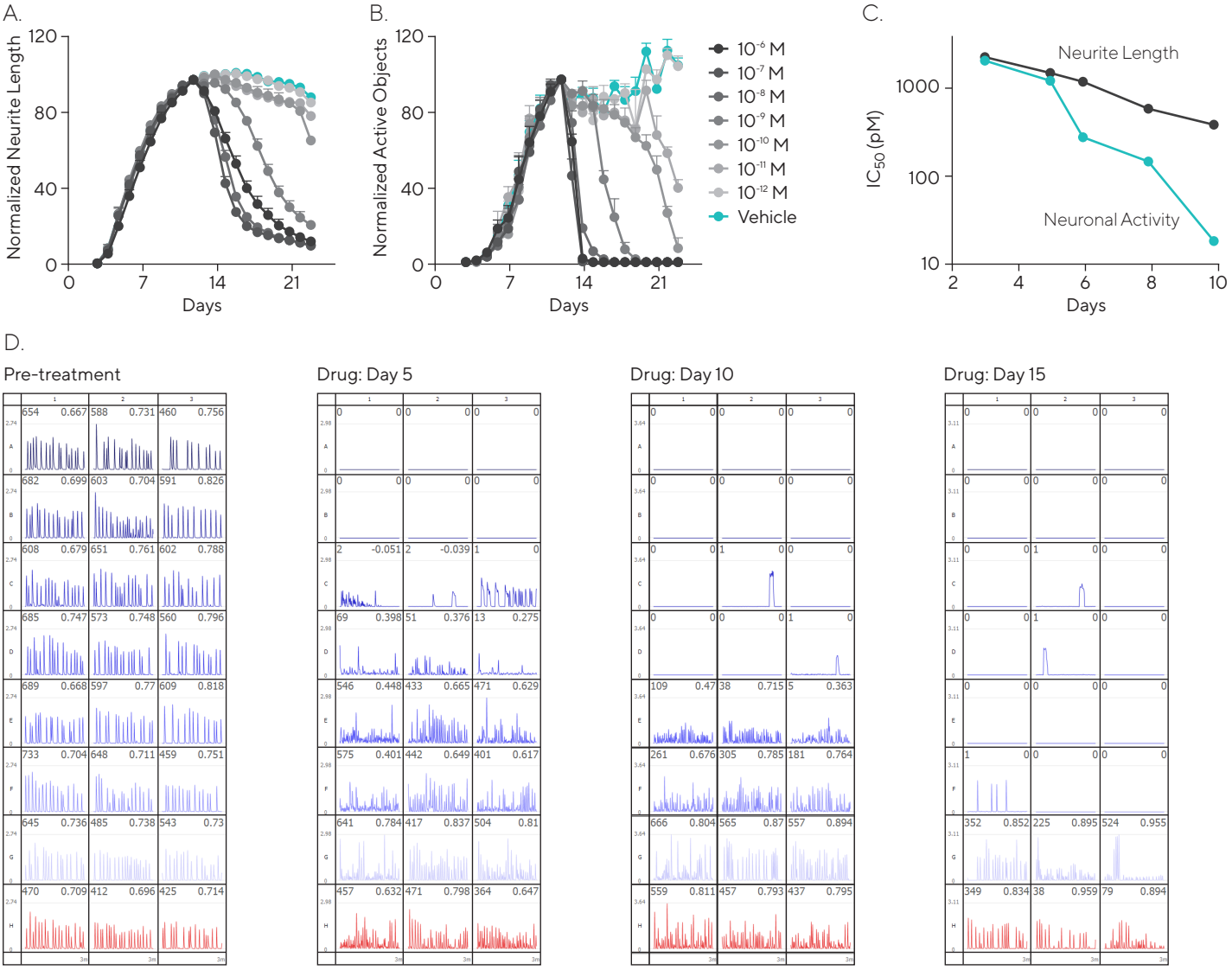


Figure 5: *Quantifying Pharmacological Neurotoxic Effects of Chemotherapeutic Taxol®*

Rat cortical neurons seeded at 30K cells/well were co-cultured with rat astrocytes seeded at 15K cells/well and transduced with Incucyte® Neuroburst Orange or Neurolight Orange Lentivirus at Day 3 in culture. Live-cell analysis measurements were made each day using the Incucyte® Live-Cell Analysis System. 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 (IC_{50} 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 (D).

Stem Cell Development Workflow

The development of advanced human cell-based models, such as human iPSC-derived neurons and glia in mono-culture and co-culture, that are species- and -disease relevant, have increased in recent years. Live-cell analysis provides insights into these translational models and enables the opportunity to identify novel pharmacological treatments for neurodegenerative diseases.

Robust methods are needed when developing human iPSC-derived models. This includes the characterization of the cells and quality control (QC) of the final model.

Figure 6 illustrates the differentiation and characterisation workflow developed at Talisman Therapeutics for use with human iPSC-derived neurons. This workflow describes where the use of both neurite outgrowth and neuronal activity, as quantified on the Incucyte® Live-Cell Analysis System can be integrated into the workflow alongside other techniques. Once matured, these neurons are shown to form active networks suitable to test novel treatments for disease.

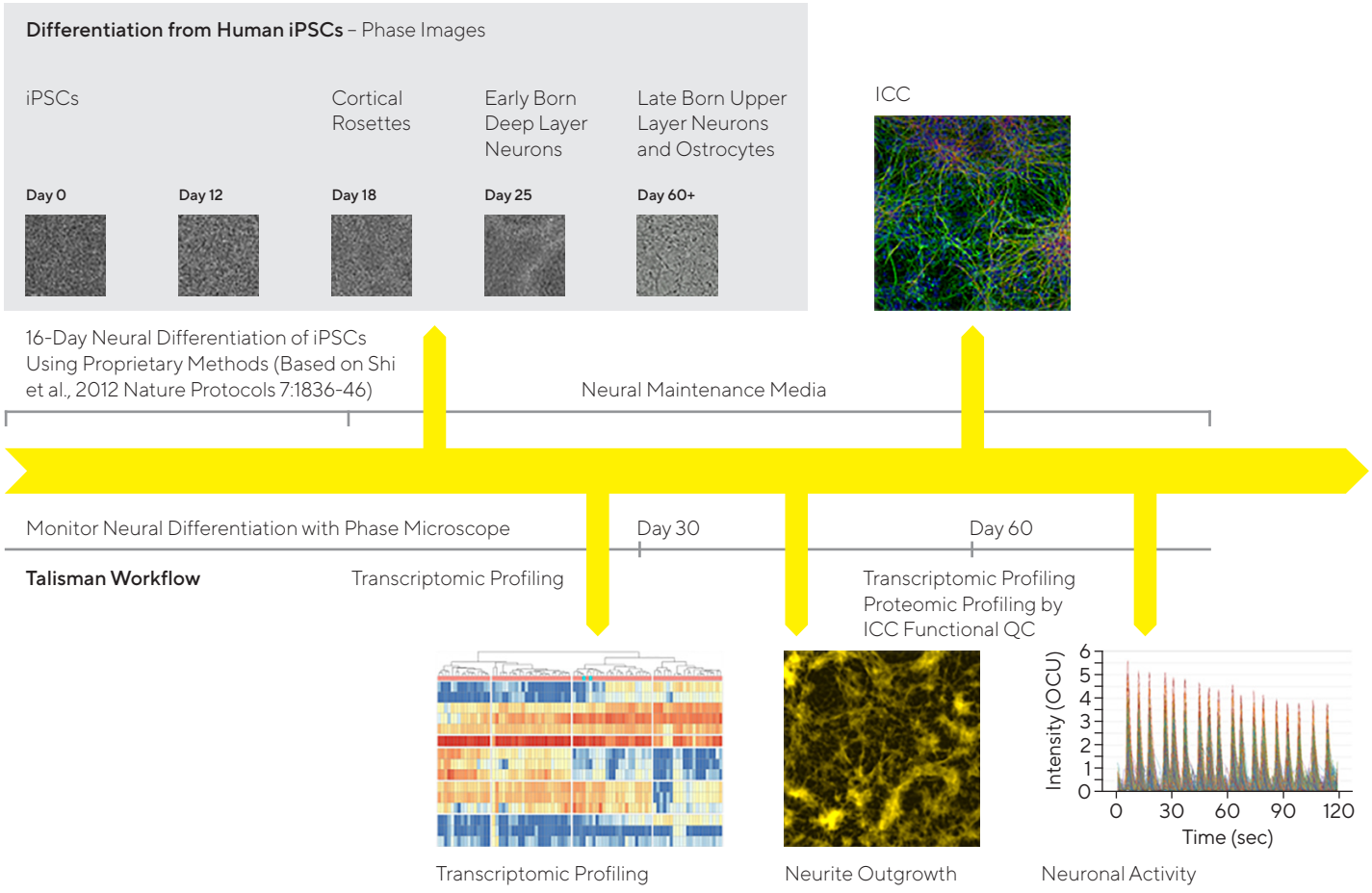


Figure 6: *Differentiation of Human iPSC-Derived Neurons Workflow*

Glia Modulation

As opposed to the traditional view that brain function results exclusively from neuronal activity, it is now widely accepted as a more coordinated perspective involving both neurons and glia. Astrocytes are regarded as active partners in brain activity via bidirectional communication, orchestrated at the tripartite synapse, composed of the neuronal pre- and post-synapses and their close interaction with the surrounded astroglia.

To investigate the effect of astrocytes in the signalling response of neurons via measurements of calcium oscillations, a humanized live-cell model of neuronal activity was developed in collaboration with Talisman Therapeutics. Recent advances in hiPSC offer a powerful in vitro model strategy for the study of both healthy and disease stages of the human nervous system. Non-perturbing neurite outgrowth measurements can be performed in mono- or co-culture (post-infection with Incucyte® Neurolight Orange Lentivirus) via automatic segmentation of time-lapse imaging using the Incucyte® Neurotrack Analysis Software Module. Cell bodies and neurites are discriminated and kinetically quantified. Co-cultures include interactions observed in more complex systems that monocultures are unable to capture. When co-cultured with astrocytes, neurons developed greater and more branched neurites compared to monocultures (Figure 7). The functional profile of co-cultures also differed of that of monocultures, the former showing greater active objects (1/image), burst duration (sec), and lower burst rate (1/min), at a similar correlation, indicating greater network stability in the presence of glial cells. The presence of astrocytes impacts neuronal architecture and network activity. Network activity in neuron-astrocyte co-cultures differs from that seen in neuronal monocultures, displaying a reduced frequency of longer-lasting calcium events, characteristic of a more mature neuronal network through the development of a network's ability to fire trains of action potentials.

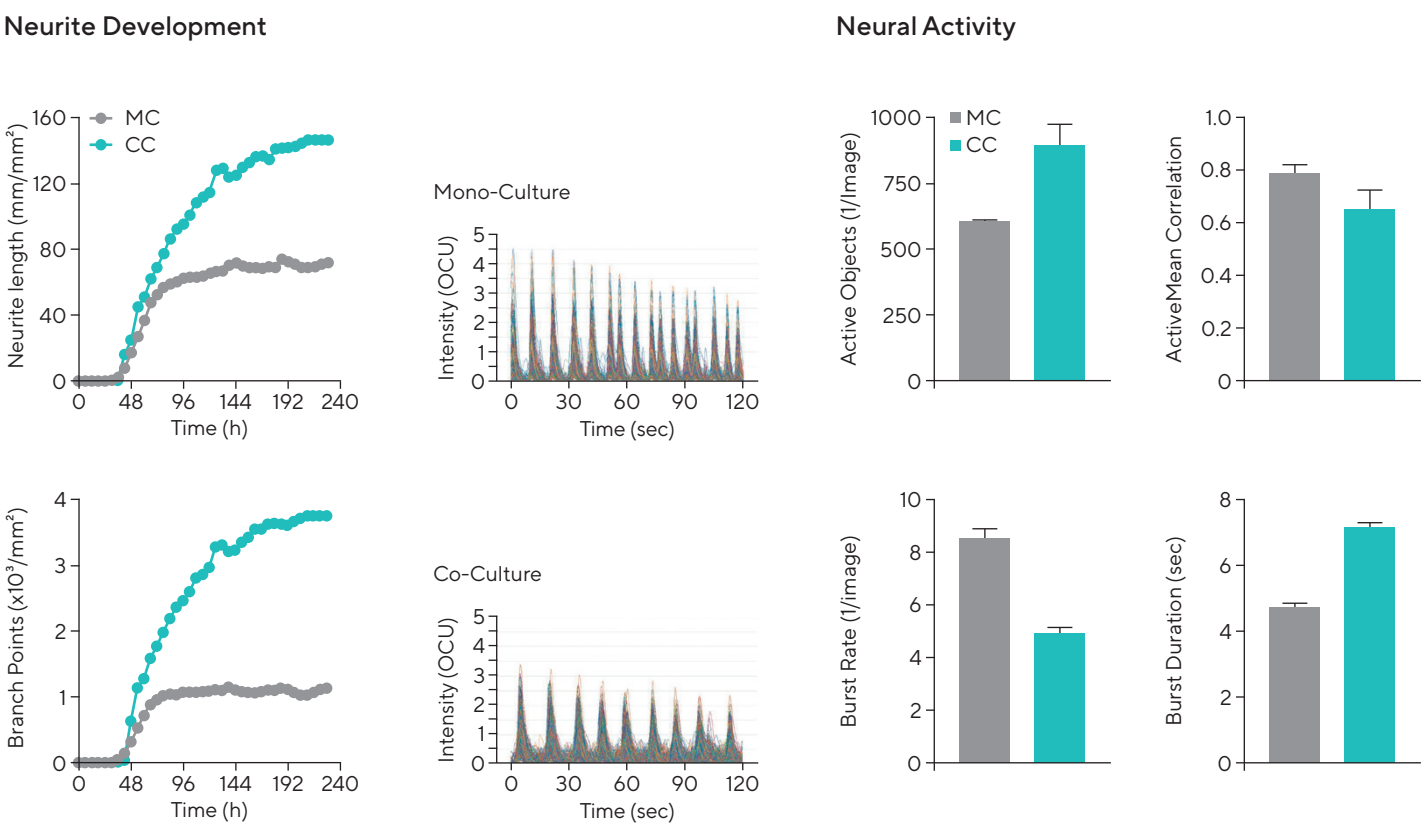


Figure 7: *iPSC Neurons Co-cultured With Mature Astrocytes Yield Greater Outgrowth and Branching and Neuronal Network Activity Is Modified.*

Network activity in neuron-astrocyte co-culture differs from monocultures, displaying a reduced frequency of longer-lasting burst rates (characteristic of a more mature neuronal network through the development of a network's ability to fire trains of APs). n = 12. Traces and bar charts are 23 days post-infection.

Conclusions

In this application note, we present data to support the use of the Incucyte® Live-Cell Analysis System configured with an Orange/NIR or Green/Orange/NIR Optical Module to characterize and refine different neuronal phenotypes and their maturation for modeling their function *in vitro*. This single live-cell imaging platform, in combination with the Incucyte® Neuroburst Orange Lentivirus, 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-sparing, and a built-in, guided interface for non-experts provided by the Incucyte® Neuronal Activity Analysis Software Module. The system can be used within the operator's own incubator under physiological conditions and may be used with a variety of neuronal cell types (such as primary neurons and iPSC-derived models).

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.

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 GABA and Gluta iPSC-derived neuronal models, we have shown how the Incucyte® Live-Cell Analysis System provides a means to study a variety of cell models using relevant quantitative metrics (e.g. neurite length and cellular activity). Additionally, these disease-relevant humanized models allow us to investigate pharmacological modulation. Lastly, the robustness and throughput provided by the Incucyte® Live-Cell Analysis System enables researchers to focus on isolating variables in order to improve iPSC-derived neuronal model development.

The Incucyte® Live-Cell Analysis System 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.

North America
Sartorius Corporation
300 West Morgan Road
Ann Arbor, Michigan 48108
USA
Phone +1 734 769 1600
Email: orders.US07@sartorius.com

Europe
Sartorius UK Ltd.
Longmead Business Centre
Blenheim Road
Epsom
Surrey, KT19 9QQ
United Kingdom
Phone +44 1763 227400
Email: euorders.UK03@sartorius.com

Asia Pacific
Sartorius Japan K.K.
4th Floor, Daiwa Shinagawa North Bldg.
1-8-11, Kita-Shinagawa 1-chome
Shinagawa-Ku
Tokyo 140-0001
Japan
Phone +81 3 6478 5202
Email: orders.US07@sartorius.com

Find out more: www.sartorius.com/incucyte

For questions, email: AskAScientist@sartorius.com