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Quantification of Neurite Dynamics in Mono-cultures and Co-cultures Using the Incucyte® Live-Cell Analysis System

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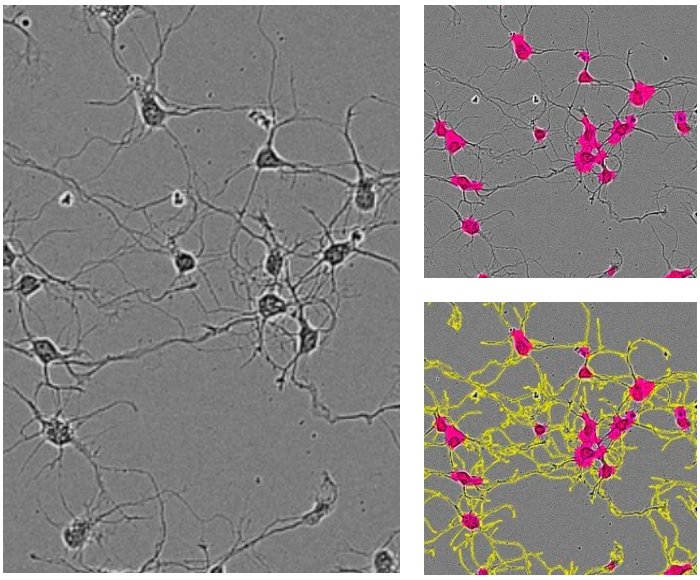
Introduction

Neurite dynamics play a fundamental role in the development and function of the nervous system. Formation and maintenance of synaptic networks are necessary for healthy brain function and plasticity and occurs through continuous changes in the fine structure of neurons. Neurite dynamics can be altered in disease states, injury, or following exposure to neurotoxic agents. Monitoring neuronal morphology in long-term *in vitro* cell cultures is critical for the characterization and evaluation of disease models and to understand neuronal development. Ideally, approaches to track neurite dynamics would allow continuous automated measurements of structural parameters, including neurite length and number of branch points. These methods should be non-perturbing and enable quantification of neurons in mono- or co-culture with glia.

This application note describes the use of live-cell imaging to kinetically quantify neuronal outgrowth using the Incucyte® Live-Cell Analysis System in conjunction with the Incucyte® Neurotrack Analysis Software Module. This assay permits the analysis of neurons in mono-culture (label-free) or in co-culture with astrocytes (Figure 1), using a non-perturbing Incucyte® Neurolight Red or Orange Lentivirus for continuous analysis of neurite length and branch points. Furthermore, the neurite analysis assay can be multiplexed with cell health reagents, Incucyte® Annexin V Red, Orange, or NIR Dye, to determine the onset of apoptosis in real time.

Find out more: www.sartorius.com/incucyte-neurite-outgrowth

Mono-culture: Primary Rat Cortical Neurons



Co-culture: Primary Rat Cortical Neurons + Rat Astrocytes

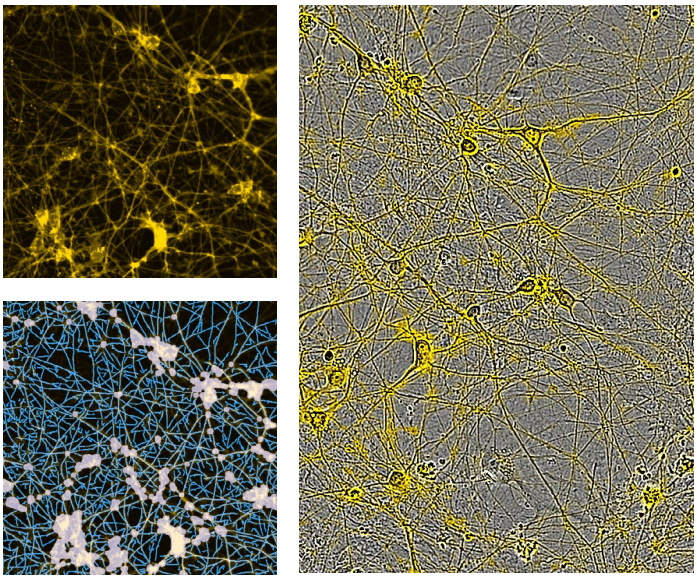


Figure 1

Automated Analysis and Visualization of Neuronal Mono-cultures and Co-cultures

Analysis of primary rat cortical neuron structures using label-free segmentation masking (top panel) showing phase image of neurons at Day 5 post-seeding, cell body cluster (pink), and neurite masks (yellow). Assessment of neurite structures in co-culture with astrocytes using Incucyte® Neurolight Orange Lentivirus (bottom panel) at Day 7 post-infection, cell body cluster (white), and neurite masks (blue).

Assay Principle and Quantification

An overview of the workflow to enable quantification of neurite outgrowth is represented in Figure 2. For mono-cultures, label-free measurements are enabled by direct phase imaging post cell seeding. When co-cultures are preferred, an initial infection with Incucyte® Neurolight Lentivirus is required to visualize the neurons. Optimization of cell density, well coatings, multiplicity of infection (MOI), and viral exposure time are required based on the research model used.

The Incucyte® Neurolight Lentivirus is a live-cell neuronal labeling reagent driven by a synapsin promoter, resulting in the long-term expression of red (mKate2) or orange (TagRFP) fluorescent protein in neuronal cell bodies and neurites. The Incucyte® Neurolight Lentivirus ensures highly efficient, yet non-disruptive labeling of primary or induced pluripotent stem cells (iPSC)-derived neurons over weeks enabling the kinetic quantification of neurite length and branching in the presence of astrocytes and other non-neuronal cell types. Purpose-built integrated Incucyte® Neurotrack Analysis Software automatically segments phase or fluorescent images and generates full time-course plots for each well in 96- and 384-well plates. Metrics such as Total Neurite Length (mm/mm^2), Branch Points (count/mm^2), Cell Body Clusters (count or area/mm^2), or Nuclear Count (for cells with a nuclear fluorescent label), and corresponding normalization values are generated in real time.

Finally, combining novel, non-perturbing reagents that deploy longer wavelength fluorophores designed for neuronal-specific measurements along with lab-tested protocols ensures reproducible and unprecedented access to phenotypic information.

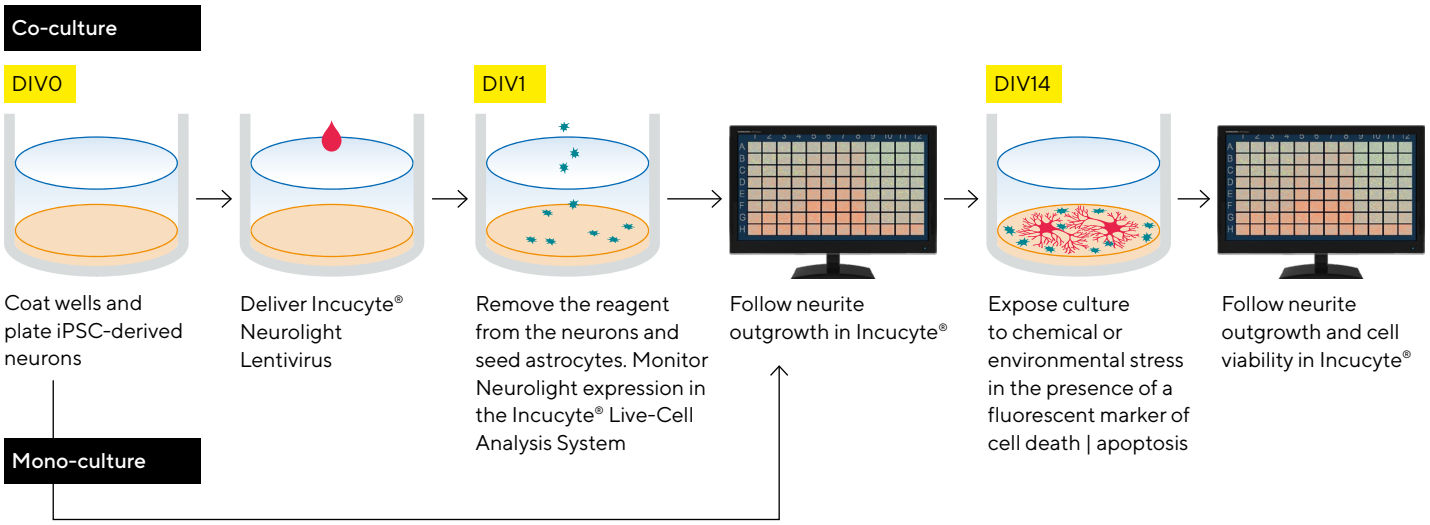


Figure 2

Quick Guide of Neurite Outgrowth Assay Workflow for Both Mono-culture and Co-culture Models

This simplified workflow enables label-free quantification of neurite dynamics in mono-culture or utilizes the Incucyte® Neurolight Orange Lentivirus for non-perturbing fluorescence analysis of neurons in co-culture. Using the Incucyte® Live-Cell Analysis System, visualize and quantify long-term changes in neurite outgrowth alongside optional readouts of cell viability using fluorescence cell death reporters.

Measuring Neuronal Parameters in Mono-cultures

A major limitation in studying human diseases affecting the nervous system is the ability to culture, monitor, and analyze neuronal cells that accurately represent human phenotypes of these disorders. The use of human-induced pluripotent stem cell (hiPSC)-derived neurons has provided a valuable approach aimed at modeling neurological diseases in humanized models. Monitoring neuronal morphology in long-term cultures is critical for the characterization and evaluation of these advanced model systems. Continuous real-time monitoring offers a significant advantage as it provides a more physiologically relevant picture of neuronal cell behavior, allows for non-invasive, repeated measurements of the same neuronal networks over time, and enables the capture of rare or transient events that are often missed with end-point assays.

When external modification of precious cellular models is not an option, either to minimize handling and perturbance (stem cells) or due to difficulties of genetic modification (primary cells), label-free phase imaging of seeded cultures provides an optimal solution. Figure 3 exemplifies the value of quantifying neuronal outgrowth in such environments and provides an example of the power of automatic segmentation and quantification. These metrics quantify biologically relevant processes such as neurite extension, branching, and loss of neurite length due to retraction or fragmentation. Statistical data, such as standard deviation and standard error of the mean, are automatically produced for user-defined replicates.

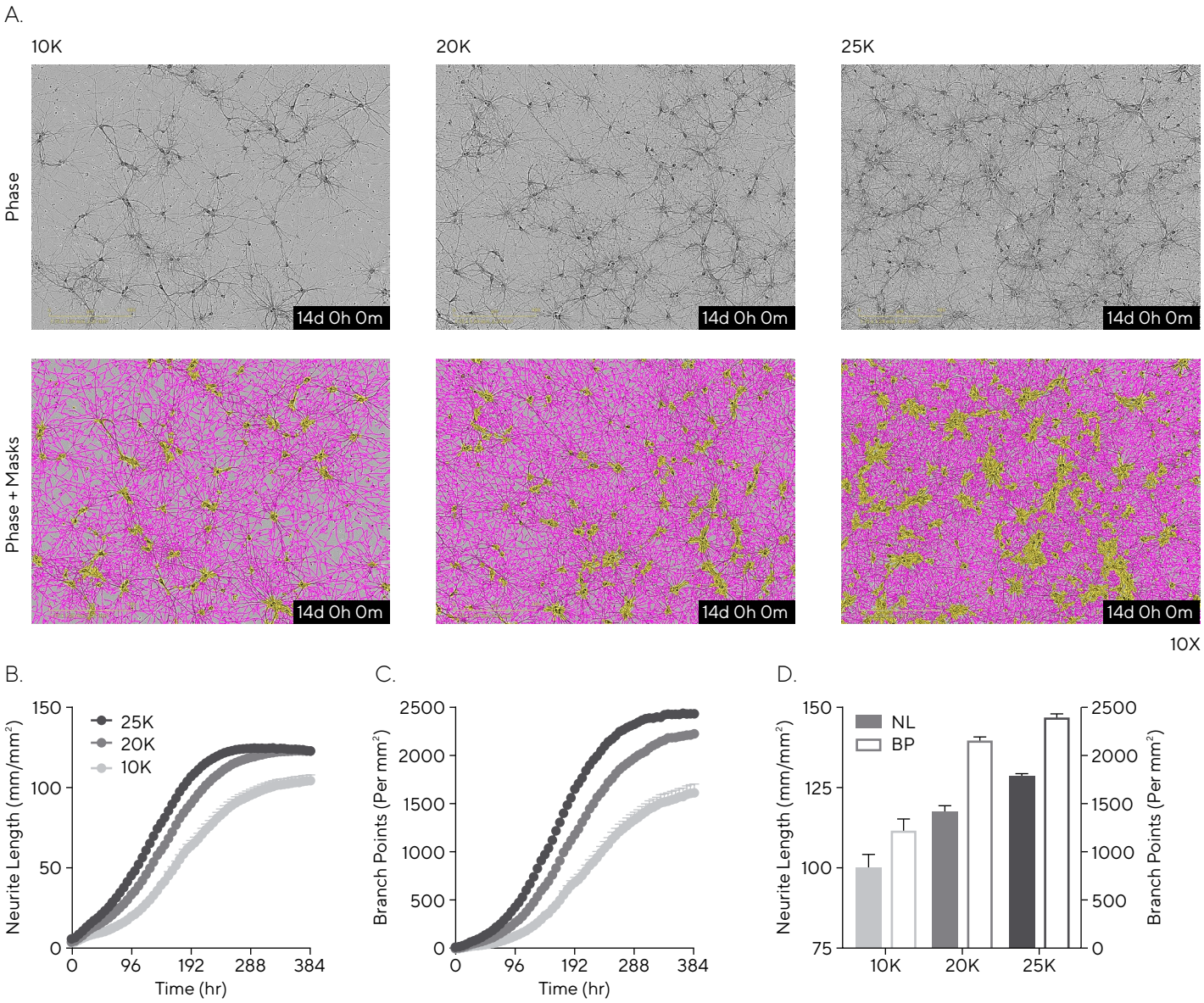


Figure 3

Label-Free Quantification of Neurite Outgrowth in Neuronal Mono-cultures

Rat primary cortical neurons were seeded into poly-D-lysine (PDL)-coated 96-well plates at a range of cell densities (10–25K cells/well). High-definition phase contrast images were acquired using the Incucyte® Live-Cell Analysis System over 16 days and automatically quantified using integrated Incucyte® Neurotrack Analysis Software. Representative images shown for phase and segmentation masks (cell body cluster in orange, neurites in pink) for each seeding density at Day 14 (A). Kinetic quantification reveals density-dependent differences in neurite length (B) and branch points (C), which both increase with an increase in seeding density (D). Data shown as mean ± SEM, n = 24 replicates.

Quantifying Neuronal Outgrowth in Co-cultures

Incucyte® Neurolight Lentivirus is a lentiviral reagent designed to transduce multiple neuronal cell types specifically and efficiently with low toxicity. Cell-handling protocols and assay conditions were optimized to produce a robust 96-well plate format capable of supporting medium throughput screening activities and mechanistic studies.

When developing an *in vitro* assay using a fluorescent protein, it is important to show that the expressed label does not affect the global biology of the system. We examined the effects of the Incucyte® Neurolight Lentivirus on neurite dynamics in rat primary neurons and astrocytes using an antibody against βIII-tubulin to assess total neurite length in cultures exposed to

varying Incucyte® Neurolight Red Lentivirus MOIs. Quantification of βIII-tubulin antibody-stained fluorescent images using Incucyte® Neurotrack Analysis Software provides an independent measure of neurite length and can expose possible adverse effects of the reagent (Figure 4). The MOI graph represents the neurite length calculated at 7 days post-infection following exposure to control solution (no Incucyte® Neurolight Red Lentivirus). Incucyte® Neurolight Red Lentivirus produced no significant changes in total neurite length up to MOI 3.3 (pink circles) indicating that Neurolight infection does not significantly alter neurite length (Figure 4A).

Figure 4 displays a 96-well microplate view measuring neurite length over 12 days post-infection. Examination of the assay time course shows consistent responses across all wells. Low intra-plate variation was observed with a standard deviation of less than 9.2 mm/mm^2 at each time point (Figure 4B). The coefficient of variation (%) measured in individual experiments at 12 days post-infection ranged from 1.4% –12.6% with an overall mean value of 4.4%. Further, inter-plate variation in maximum neurite length was assessed for control wells from 13 plates run over a 5-month period. The cross-plate variability of all experiments was within 20% of the overall mean neurite length (Figure 4C). Heat maps of individual experiments revealed no significant position effects (data not shown). The Incucyte® Neurotrack co-culture assay provides a robust, medium throughput measurement of neurite dynamics over a minimum of a 12-day period.

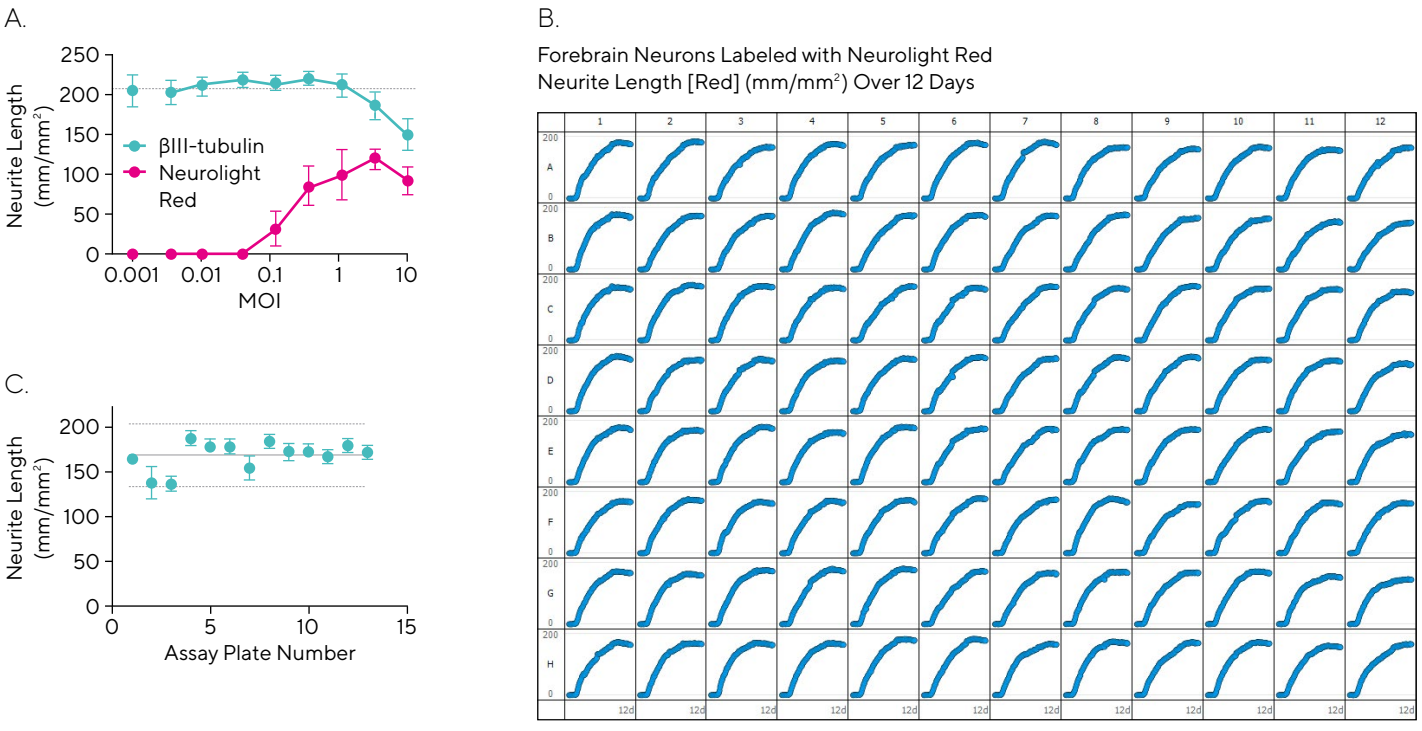


Figure 4

Validation of Incucyte® Neurolight Lentivirus Infection

Fluorescent images of βIII-tubulin-stained (teal) or Incucyte® Neurolight Red (pink) infected rat forebrain neurons were quantified using the Incucyte® Neurotrack Analysis Software Module. Neurite length values (mm/mm^2, mean ± SD, n = 4) were obtained and MOIs (0.001–10) compared from the same wells at 7 days following infection (A). The 96-well plate view displays neurite lengths over 12-day assay and data combined in time course (mean ± SD) to understand intra-assay variability (B).Neurite length was calculated in untreated control wells at 12 days post infection (mean ± SD, n = 8) for 13 separate plates run over 5 months to obtain inter-assay variability. Solid line represents overall mean neurite length (167.0 ± 16.0 mm/mm^2) and dashed lines represent the ± 20% limits from the overall mean (C).

Importance of Astroglia in iPSC Differentiation

It is widely accepted that the nervous system presents a coordinated involvement of neurons and glia. Glial cells are active partners to neurons in brain development and activity via bidirectional communication. This is orchestrated at the tripartite synapse, which is composed of the neuronal pre- and post-synapses and their close interaction with the surrounding astroglia.

To investigate the effect of astrocytes in neurite development, a humanized live-cell model of neuronal activity was developed in collaboration with Talisman Therapeutics. Recent advances in hiPSCs 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 performed in mono-cultures or co-cultures via automatic segmentation of time-lapse imaging using the Incucyte® Neurotrack Analysis Software Module were performed and are shown in Figure 5. When co-cultured with astrocytes, neurons developed a greater number of neurites with increased branching compared to mono-cultures, demonstrating the importance of astroglia in iPSC differentiation.

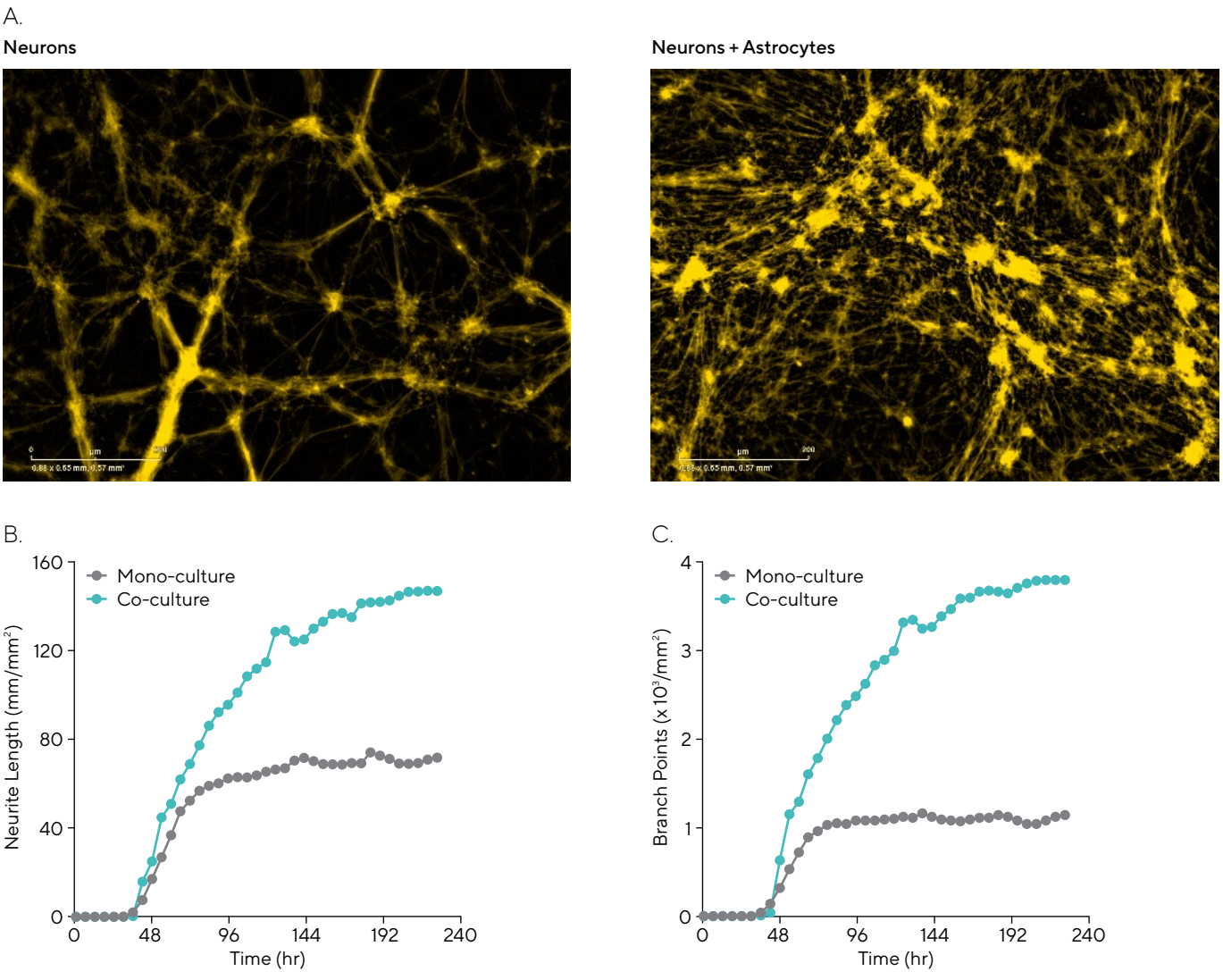


Figure 5

iPSC-Derived Neurons Co-cultured with Astrocytes Show Increased Neurite Outgrowth

iPSC-derived neurons developed by Talisman Therapeutics were infected with Incucyte® Neurolight Orange Lentivirus, co-cultured with mature astrocytes and monitored and quantified using the Incucyte® Live-Cell Analysis System. Representative fluorescence images shown for both mono-cultures and co-cultures (A). Time-course plots revealed that neurons in co-cultures yield greater neurite outgrowth (B, neurite length) and branching (C, branch points) when compared to neurons cultured in isolation (mean ± SEM).

Quantitative Pharmacology in a Parkinson's Disease Model

Neurodegenerative diseases, such as Parkinson's disease (PD), are chronic and debilitating disorders that progressively cause degeneration and/or death of neuronal cells. Neurite length can be a sensitive marker of neuro-degenerative disease and neurotoxicity. To develop a model of Parkinson's disease, we created a co-culture system of rat primary striatal neurons and astrocytes (Figure 6). Following a period of neurite development (10 days), the dopaminergic-specific neurotoxin 6-hydroxydopamine (6-OHDA) was applied to induce disease-relevant neuronal damage. A time- and concentration-dependent effect on neurite disruption was observed, yielding an IC_{50} value for 6-OHDA of 70.5 µM for neurite length. 6-OHDA at the maximum concentration tested (500 µM) caused 95% of neurites to be abolished. This model system may provide a quantitative phenotypic assay for agents designed to reverse or minimize the neurotoxic effect of 6-OHDA and aid in the development of improved therapeutics for Parkinson's disease.

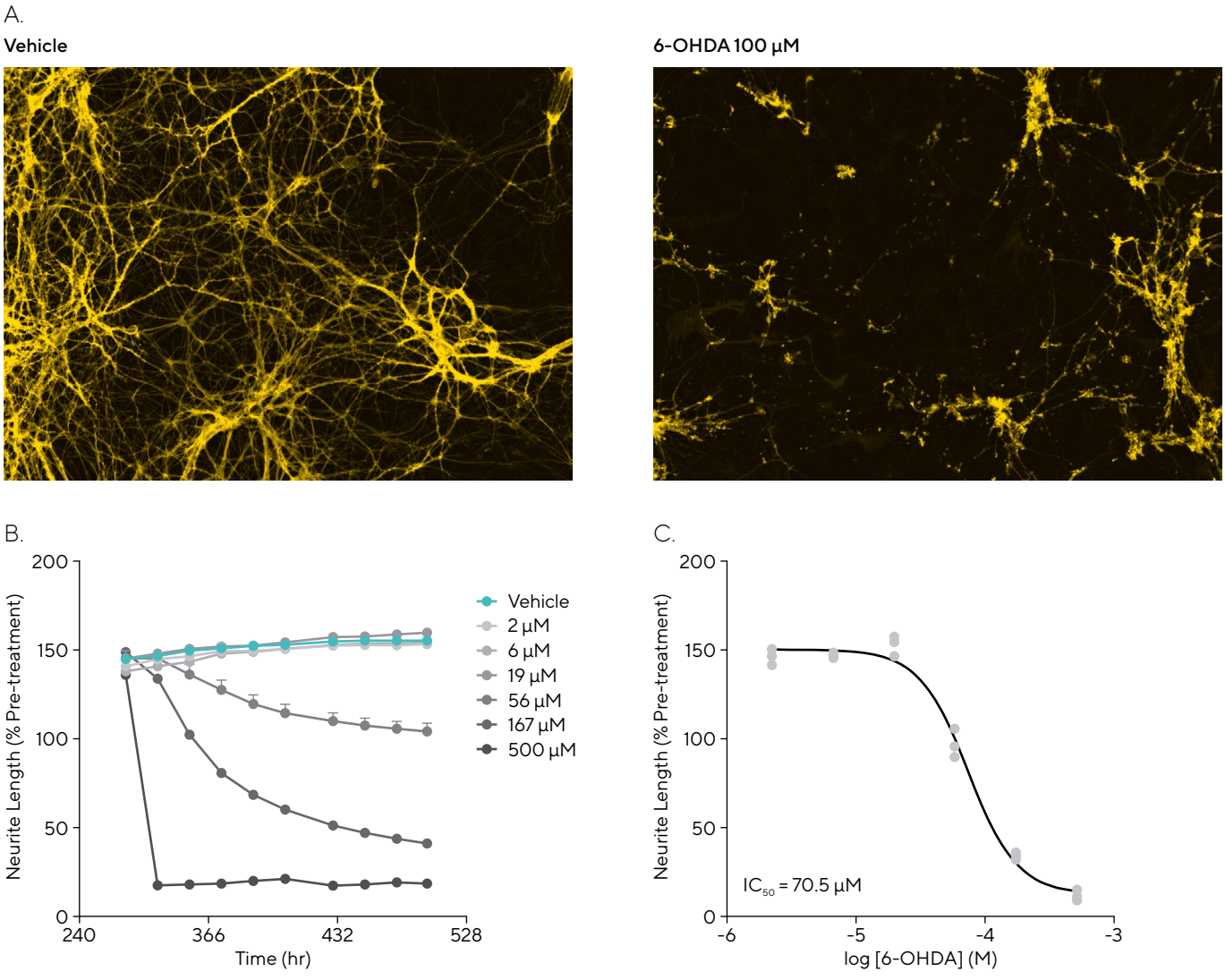


Figure 6

6-OHDA-Induced Neurite Disruption in a Parkinson's Disease Model

Rat primary cortical neurons were labeled with Incucyte® Neurolight Lentivirus and co-cultured with astrocytes. 10 days post-seeding, once the neurite network had stabilized, co-cultures were treated with varying concentrations of 6-OHDA and monitored for 12 days (A). Representative fluorescence images are shown for vehicle and 100 µM 6-OHDA (10 days). (B) Time-course graphs represent the change in neuronal length normalized to pre-treatment value at the different drug concentrations (%, mean ± SEM, 3 replicates). (C) Concentration response curve derived from temporal profiles shows individual neurite length values at 12 days post-treatment.

Conclusions

The Incucyte® Live-Cell Analysis System, combined with Incucyte® Neurotrack Analysis Software Module and when necessary, the Incucyte® Neurolight Lentivirus, provides a robust method to image neurons in mono-cultures and co-cultures and quantify changes in neurite length of living cultures from days to weeks. The image analysis software is flexible for quantification of many cell types, from immortalized cell lines to primary neurons and iPSCs. An intuitive user interface allows rapid assay optimization and automated quantification of neurite dynamics. All data and time points can be verified by inspecting individual images and/or time-lapse movies. Observation of cell morphology provides additional validation and insight into the biological effect of treatment groups. This approach provides a sensitive method to detect pharmacological manipulations that alter neurite dynamics, including processes such as elongation and retraction.

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