

Quantitative live-cell analysis for optimization of culture conditions and evaluation of cell health in human induced pluripotent stem cell-derived neurons

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Introduction

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 new approach aimed at modeling neurological diseases. Monitoring neuronal morphology and cell health in long-term culture is critical for the characterization and evaluation of these novel model systems. Traditional approaches rely on endpoint assays and imaging techniques that require immunochemical staining, which lacks real-time kinetic information.

This presents a liability when studying models of the nervous system where the integrity of neural networks is compromised and the dynamic complexity of neurological responses and features are lost when utilizing these types of approaches. For these model systems, continual real-time monitoring offers a significant advantage in that 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 capturing of rare or transient events that are often missed with end-point assays.

In this application note, we describe methods and present validation data highlighting optimal culture conditions for evaluation of cell viability and neurite outgrowth in hiPSC-derived neurons from Cellular Dynamic International (CDI, iCell Neurons). We also monitor neurite outgrowth and cellular viability in iCell Gluta Neurons from CDI using a quantitative, live-cell imaging and analysis approach with the IncuCyte® S3 over days/weeks in 96-well microplate culture. To exemplify a real-time imaging and analysis approach using hiPSC-derived neurons, we assess neuronal excitotoxicity using the IncuCyte® S3 Phase/Fluorescent NeuroTrack applications multiplexed with Annexin V reagents. These assays outline optimal culture conditions for an example iPSC-derived neuronal system and demonstrate the ability of the IncuCyte approach for real-time, long-term quantitative analysis of iPSC-derived neuronal cell health.

Materials & Methods

Optimization of culture conditions

96-well cell culture plates (MidSci, #TPP92096) were coated with 100 µg/mL poly-D-lysine (PDL, Millipore, A-003-E), 0.01% poly-L-ornithine (PLO, Sigma, P6355) or 0.07% polyethylenimine (PEI, Sigma, 408727) and incubated overnight at room temperature in a tissue culture hood. Following incubation, surface coatings were completely aspirated, and each well was rinsed twice with 150 µL WFI water. Plates were left to dry for at least one hour with lid removed in a tissue culture hood. For wells with additional laminin/Matrigel coating, 3.3 µg/mL laminin or 0.028 mg/mL Matrigel was added and incubated at 37°C for at least 1 hour. Laminin and Matrigel solutions were aspirated immediately prior to addition of cell suspension to avoid drying.

With the exception of culture surface coatings, iCell Neurons were stored, handled and maintained according to manufacturer's instructions in the iCell Neurons User's Guide from CDI. iCell Neurons were seeded at 50,000 or 10,000 cells/well for evaluating coatings or excitotoxicity, respectively. Morphology and neurite outgrowth was monitored for 14 days using the IncuCyte S3® live cell imaging and analysis system. For co-culture experiments, iCell Neurons were infected with IncuCyte® NeuroLight Red (MOI 1) 2-4 hrs post-plating. Following incubation for 16-24 h, virus was removed, media replaced, and primary rat astrocytes were seeded at 15,000 cells/well. On day 3 post-plating, 50% of the media was replaced with media containing 5-fluoro-2'-deoxyuridine and uridine (8 µg/mL and 28 µg/mL, respectively) to inhibit further astrocyte proliferation.

Evaluation of neuronal excitotoxicity

96-well cell culture plates were coated with 0.07% PEI + 3.3 µg/mL laminin as described above. iGluta Neurons were stored, handled and maintained according to manufacturer's instructions in the iCell GlutaNeurons User's Guide from CDI, except laminin and penicillin/streptomycin were excluded from the media. Cells were seeded at 10,000 cells/well, and morphology and neurite outgrowth was monitored for 14 days using the IncuCyte S3 live-cell imaging and analysis system. For co-culture experiments, neurons were infected with

IncuCyte NeuroLight Red, astrocytes were seeded, and cells were treated as described above. On DIV14, cells were exposed to glutamate or kainate +/- glutamate receptor antagonists in the presence of red or green IncuCyte® Annexin V reagent, a fluorescent marker of cell death/apoptosis. Neurite length and apoptosis was monitored and quantified for 72 hrs using the IncuCyte S3 live-cell analysis platform in conjunction with IncuCyte® NeuroTrack analysis software.

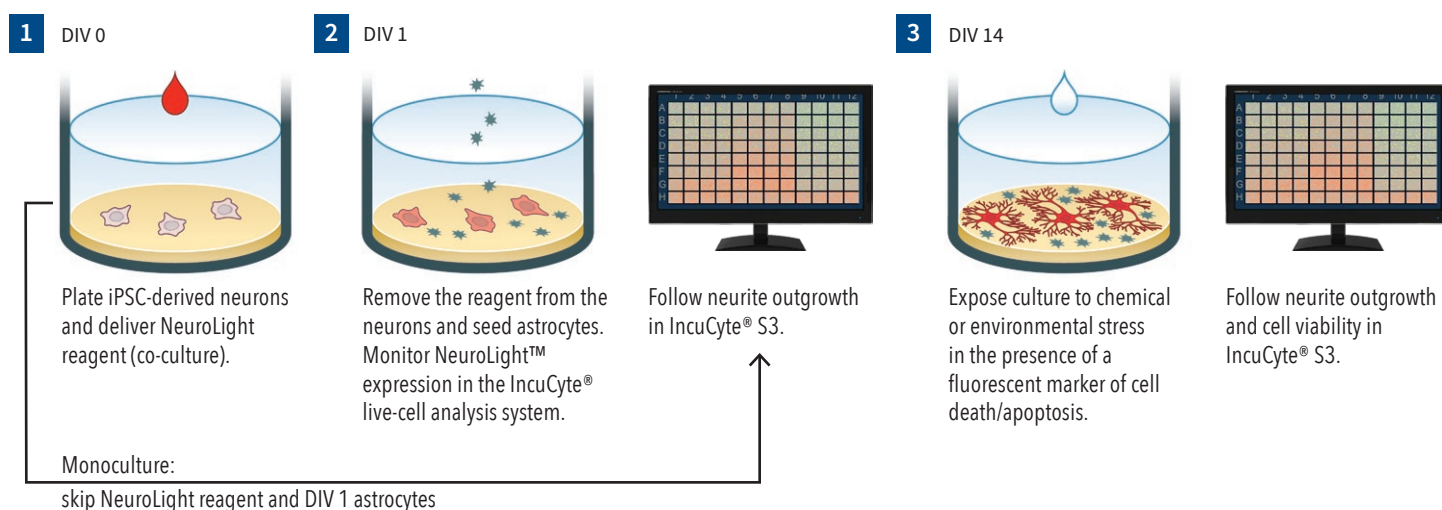


Figure 1. Assay Workflow (Monoculture)

1. DIV0 - Coat wells and plate iPSC-derived neurons.
2. Follow neurite outgrowth using Phase NeuroTrack.
3. DIV14 - Treat cells with neurite/cell health modulating test agents in the presence of Annexin V red reagent (marker of apoptosis).
4. Follow neurite outgrowth and cell viability using the IncuCyte S3 for 72 hrs.
5. Assay Workflow (Co-culture).
6. DIV0 - Coat wells, plate iPSC-derived neurons and deliver IncuCyte NeuroLight reagent.
7. DIV1 - Remove virus and plate primary rat astrocytes 15K cells/well.
8. DIV3 - Remove 50% media and replace with media containing 5-fluoro-2'-deoxyuridine and uridine.
9. Follow neurite outgrowth using IncuCyte Fluorescent NeuroTrack.
10. DIV14 - Treat cells with neurite/cell health modulating test agents in the presence of IncuCyte Annexin V green reagent (marker of apoptosis).
11. Follow neurite outgrowth and cell viability using the IncuCyte S3 for 72 hrs.

Validation Data

Culture plate coating conditions

hiPSC technology is a growing model for studying human diseases of the nervous system, yet the basic culture conditions for plating and maintaining these cells has not been thoroughly characterized. Here we tested three commonly used culture substrates +/- secondary laminin or Matrigel coating to determine optimal conditions for in vitro cellular morphology and neurite outgrowth in hiPSC-derived neurons. Figure 2 displays 10x HD phase images captured on DIV14 of iCell Neurons seeded at 50,000 cells/well in culture plates coated with PDL, PLO or PEI +/- laminin or Matrigel. Plates coated with PDL or PLO exhibited dramatic cell clustering and radial neurite cabling to the plate. This clustering morphology occurred on plates coated with PDL or PLO regardless of secondary coating with either laminin or Matrigel. Conversely, iCell Neurons seeded on plates coated with PEI displayed a more homogenous cell monolayer and classical neurite outgrowth. Secondary coating with either laminin or Matrigel showed a similar neuronal monolayer as PEI alone, although neurite outgrowth appeared more robust.

As secondary coating with laminin or Matrigel following PEI coating appeared to show similar cell morphology and neurite outgrowth, we chose to use laminin for further experimentation

due to its relative ease of use. In order to determine if secondary coating with laminin led to a statistically significant increase in neurite outgrowth compared to PEI alone, we used Phase NeuroTrack software on the IncuCyte S3 to quantify neurite length over time for both culture conditions. Figure 3 shows 20x phase images of iCell Neurons seeded in PEI alone or PEI + laminin at DIV3 and DIV14. At both DIV3 and DIV14, cells grown on PEI + laminin exhibited markedly improved neurite outgrowth to cells grown on PEI alone. This increase was quantified and graphed over time using the Phase NeuroTrack analysis software on the IncuCyte S3.

We tested whether the optimized seeding conditions of iCell Neurons in monoculture on PEI + laminin translated to iCell Neurons plated in co-culture with primary rat astrocytes. Figure 4 shows both phase and fluorescent images of iCell Neurons plated on PEI + laminin in co-culture with astrocytes at DIV6 and DIV14. We observed a robust increase in neurite length over time with no clustering of neurons or astrocytes at any point in the experiment. Although iCell Neurons in co-culture with astrocytes did not display a dramatic difference in morphology or neurite length with other coating conditions (data not shown), we recommend seeding on PEI + laminin to maintain consistency with monoculture experiments.

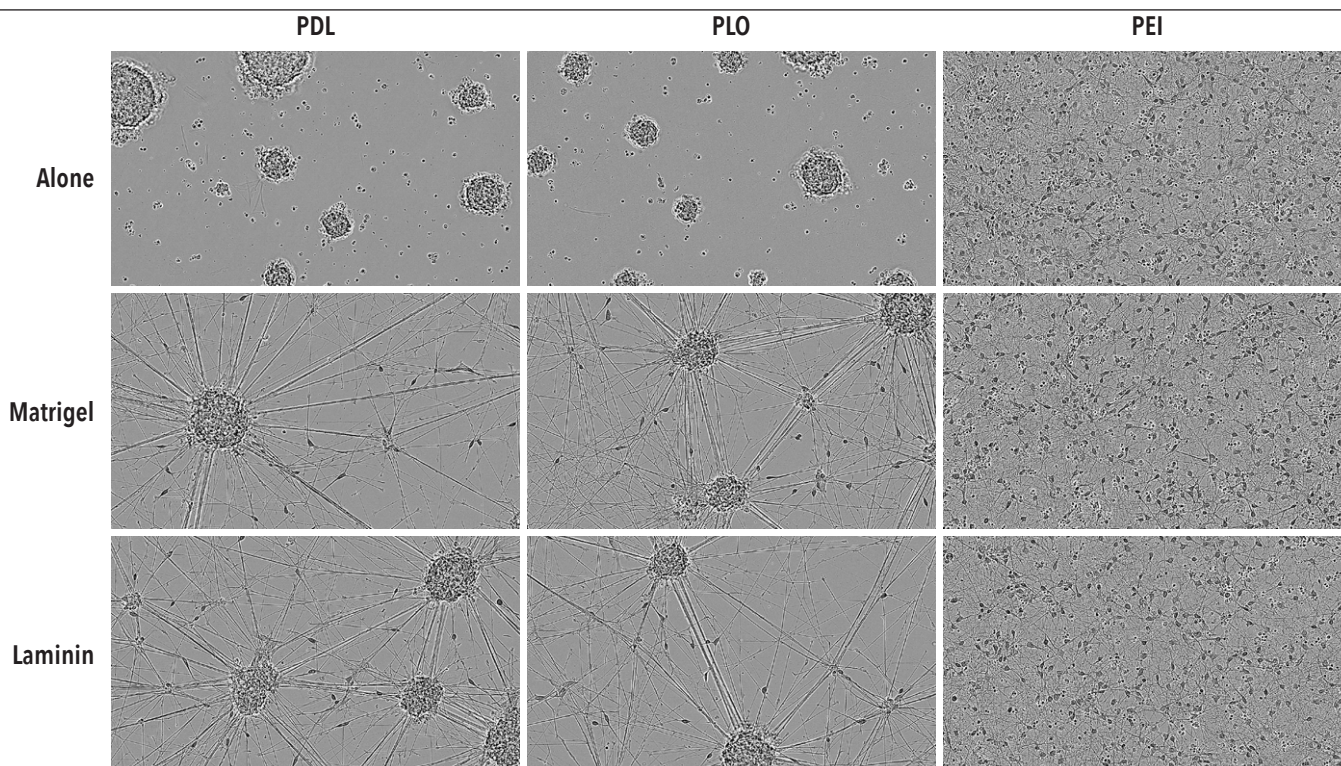


Figure 2. Optimal adherence and morphology of iCell Neuron monocultures seeded on PEI + laminin.

iCell Neurons were seeded at 50,000 cells/well on PDL, PLO or PEI +/- Matrigel or +/- laminin. Cells plated on PDL or PLO formed large neurospheres by DIV14 in the presence or absence of additional laminin or Matrigel coating. Cells plated on PEI +/- laminin or Matrigel displayed a more homogenous monolayer. All images captured at 10x magnification.

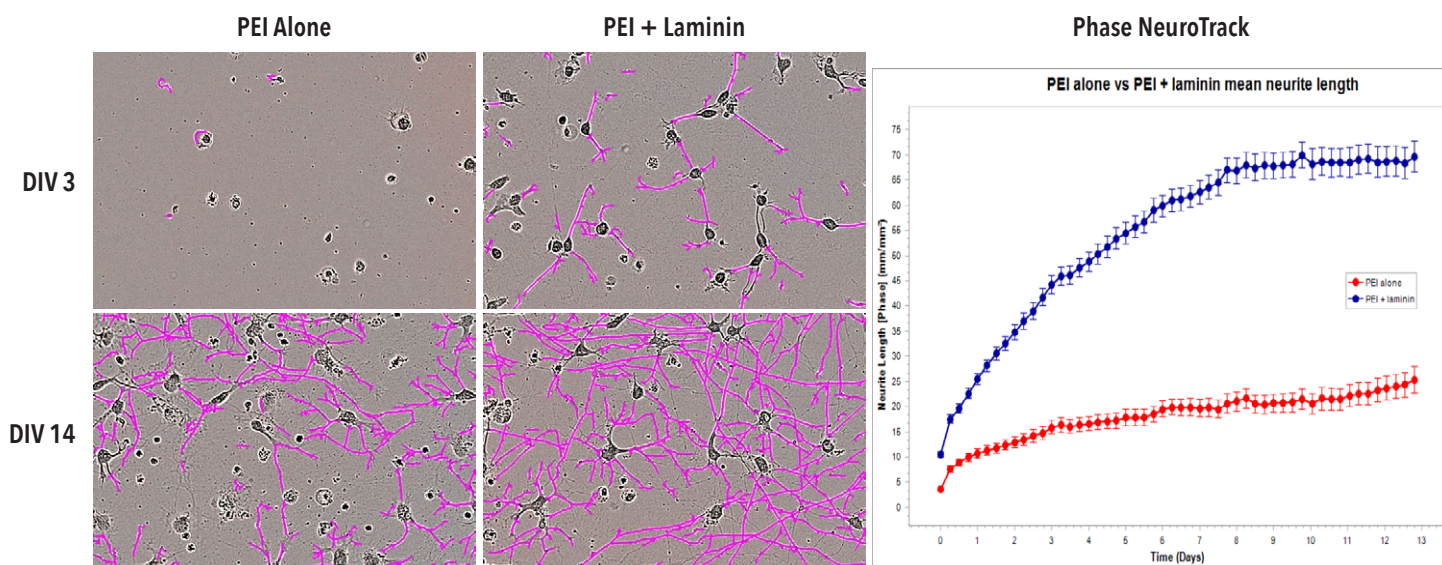


Figure 3. Improved neurite outgrowth in iCell Neuron monoculture seeded on PEI + laminin compared to PEI alone.

Cell culture plates were coated with PEI or PEI + laminin. iCell Neurons were seeded at 10,000 cells/well. Phase images show neurite length at DIV3 and DIV14. Neurite length was quantified using Phase NeuroTrack software and plotted over time. All images captured at 20x magnification. Each data point represents mean \pm SEM, n=4.

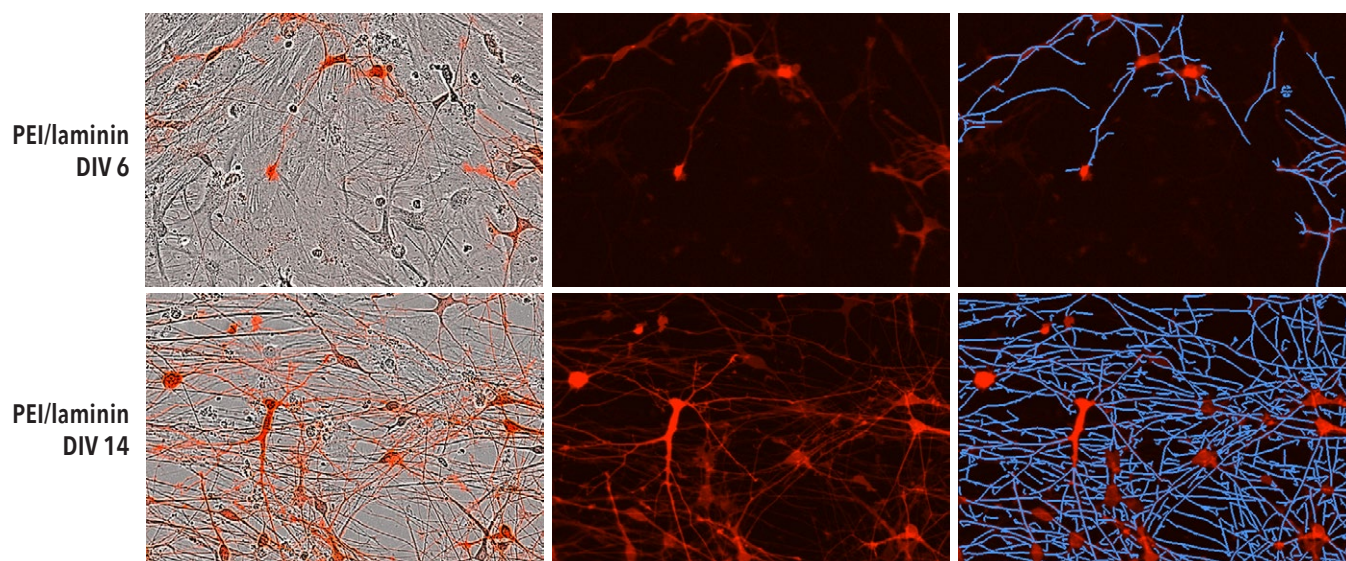
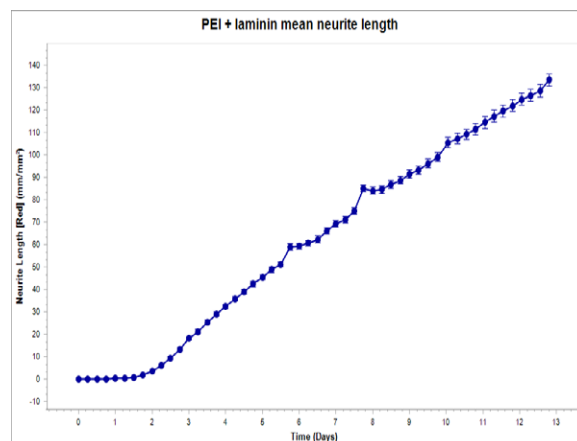


Figure 4. PEI + laminin coating enables robust neurite outgrowth in iCell Neuron co-culture with primary rat astrocytes.

Cell culture plates were coated with PEI + laminin. iCell Neurons were seeded at 10,000 cells/well and primary rat astrocytes were seeded at 15,000 cells/well. iCell Neurons were infected with IncuCyte NeuroLight Red and neurite length was quantified using Fluorescent NeuroTrack software and plotted over time. All images captured at 20x magnification. Each data point represents mean \pm SEM, n=4.



Analysis and quantification of neuronal excitotoxicity

In vitro models of human neurophysiology are currently needed for investigation into the cellular mechanisms underlying neurological disorders and neurotoxicity. Neurite length can be used as a sensitive marker of neurodegenerative disease and neurotoxicity (Conforti et al., 2007), and it has been shown that excess glutamate and other neurotransmitter-induced toxicity can play a role in some forms of disease progression (Choi, 1988; Mattson, 2008). Current investigations of glutamate and other neurotransmitter-mediated neurotoxicity are often pursued using non-human cells/subjects (both in vitro and in vivo). However, the high degree of interspecies differences in attempting to model human disease has made these efforts challenging. In addition, in vivo models afford limited throughput while many in vitro models use short term readouts that may not easily detect chronic toxicity. Here we use both HD phase (monoculture) and fluorescent (co-culture) NeuroTrack analysis software multiplexed with IncuCyte Annexin V cell viability reagents to quantitatively characterize potential long term neurotoxic effects of glutamate and kainate in hiPSC-derived neurons.

Figure 5 shows example phase images of iGluta Neurons in monoculture at DIV2, DIV14 and DIV17. Glutamate (1 mM) was added on DIV14 following image acquisition, and its effect on neurite length and cell viability was monitored for 72 hours. Neurite length at DIV17 was dramatically reduced following glutamate addition.

Addition of both glutamate and kainate at DIV14 produced a concentration- and time-dependent decrease in neurite length with concomitant increase in cell death in iGluta Neurons, both in monoculture & co-culture with primary rat astrocytes (figures 6-9). To investigate a potential mechanism mediating this toxicity, NBQX and MK801 (AMPA receptor and NMDA receptor antagonists, respectively) were added at DIV14 along with glutamate or kainate. NBQX and MK801 reduced the neurotoxic effects of both glutamate and kainate, suggesting that these effects are, at least in part, glutamate receptor-mediated.

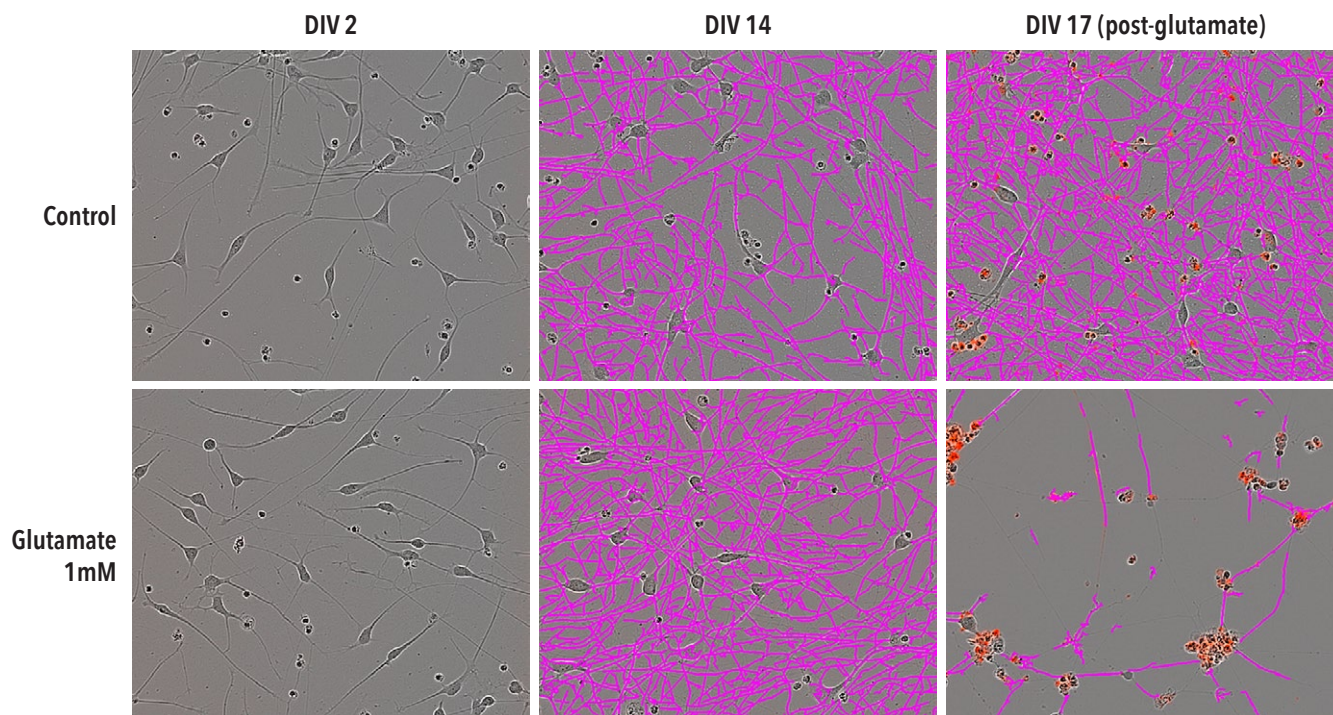


Figure 5. Glutamate-induced excitotoxicity reduces neurite length and increases cell death.

Example Phase NeuroTrack images for quantifying neurite length following excitotoxic agent administration in iGluta neurons imaged at DIV2, DIV14 and DIV17. DIV17 corresponds to 72 hrs post-glutamate (1mM) addition. Neurite length is significantly reduced following glutamate treatment. A corresponding increase in IncuCyte Annexin V (red) fluorescence indicates an increase in cell death. All images captured at 20x magnification.

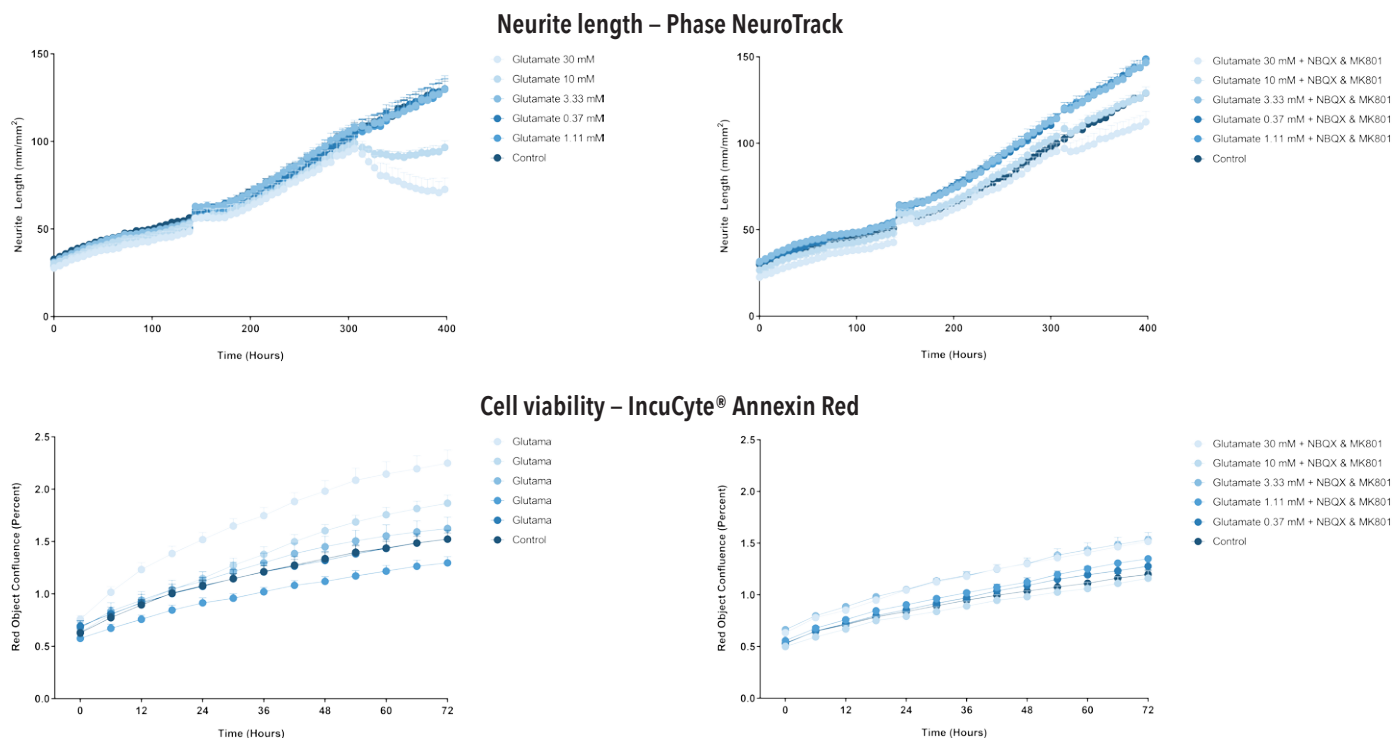


Figure 6. Glutamate exposure decreases neurite length and increases cell death in a concentration- and time-dependent manner in iGluTA Neuron monoculture. Time course of the effects of glutamate addition at day 14 (arrow) on neurite length (top panels) and cell viability (bottom panels). Glutamate causes a concentration-dependent decrease in neurite length with concomitant decrease in cell viability (increased IncuCyte Annexin Red fluorescence). Addition of NBQX (20 μ M) & MK801 (10 μ M) protects iGluTA Neurons from glutamate toxicity. Each data point represents mean \pm SEM, n=6.

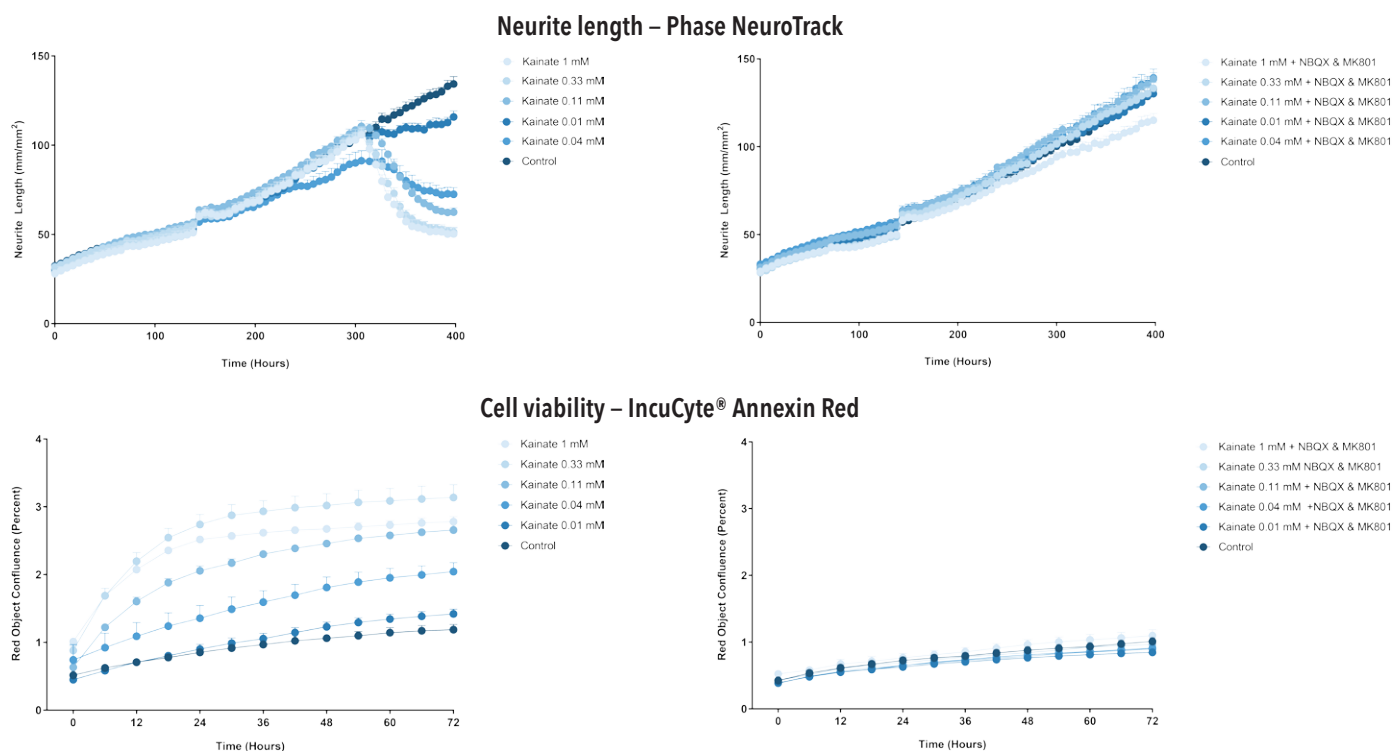


Figure 7. Kainate exposure decreases neurite length and increases cell death in a concentration- and time-dependent manner in iGluTA Neuron monoculture. Time course of the effects of kainate addition at day 14 (arrow) on neurite length (top panels) and cell viability (bottom panels). Kainate causes a concentration-dependent decrease in neurite length with concomitant decrease in cell viability (increased Annexin Red fluorescence). Addition of NBQX (20 μ M) & MK801 (10 μ M) protects iGluTA Neurons from kainate toxicity. Each data point represents mean \pm SEM, n=6.

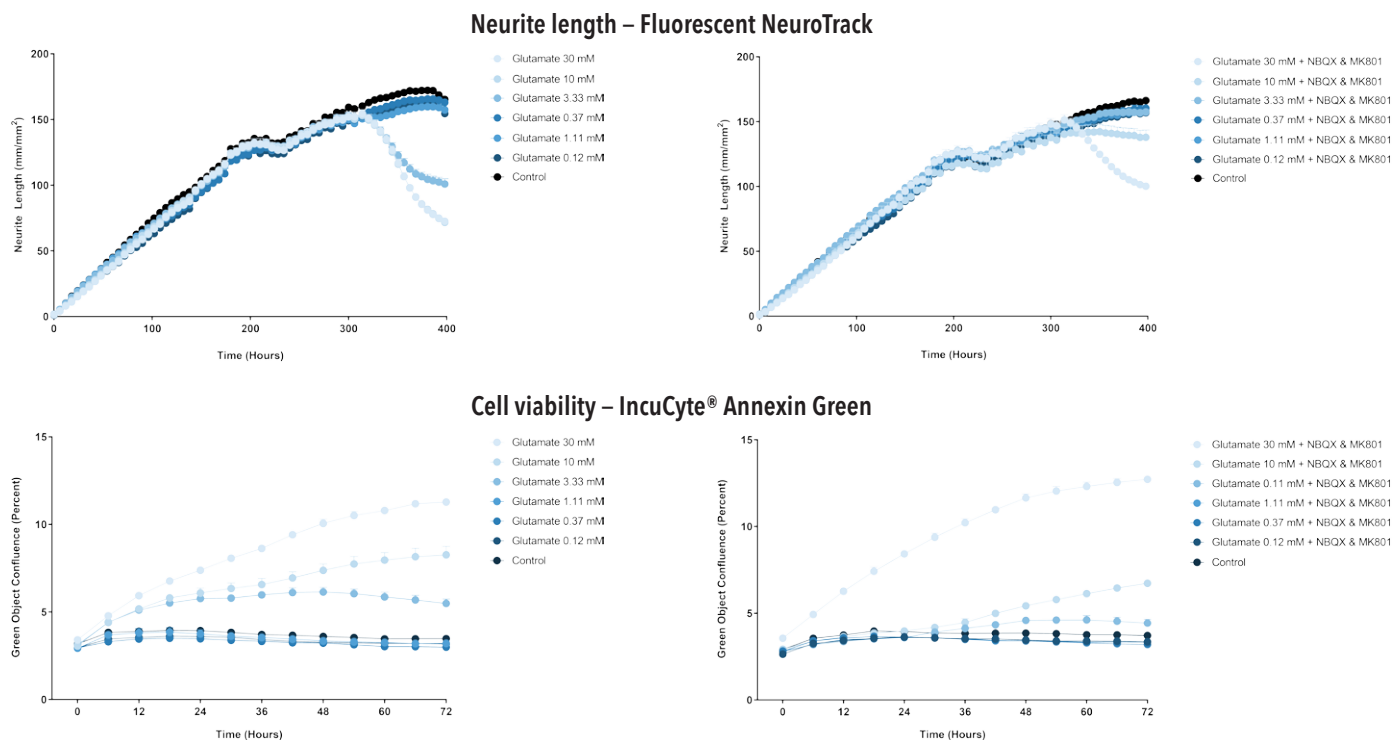


Figure 8. Glutamate exposure concentration- and time-dependently decreases neurite length and increases cell death in iGluta Neuron co-culture.

Time course of the effects of glutamate addition at day 14 (arrow) on neurite length (top panels) and cell viability (bottom panels). Glutamate causes a concentration-dependent decrease in neurite length with concomitant decrease in cell viability (increased Annexin Green fluorescence). Addition of NBQX (20 μ M) & MK801 (10 μ M) protects iGluta Neurons from glutamate toxicity. Each data point represents mean \pm SEM, n=6.

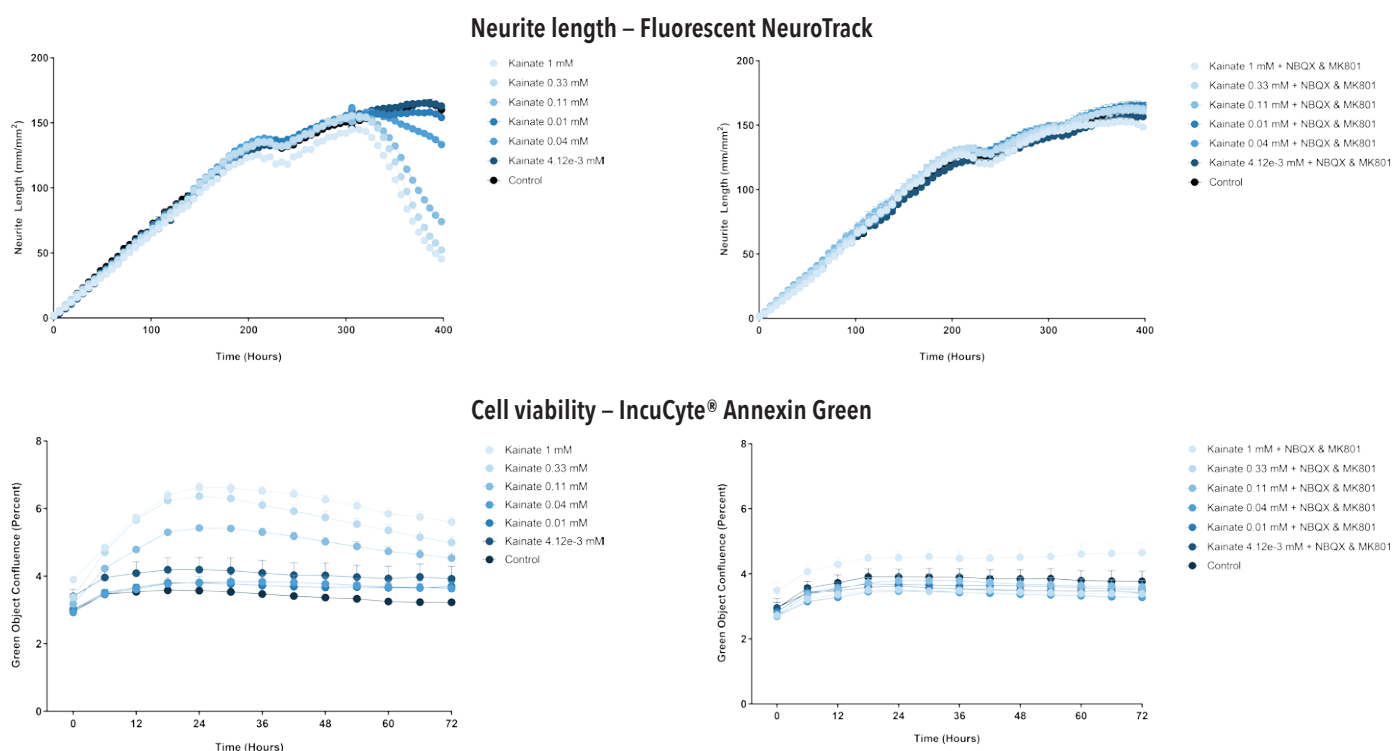


Figure 9. Kainate exposure concentration- and time-dependently decreases neurite length and increases cell death in iGluta Neuron co-culture.

Time course of the effects of kainate addition at day 14 (arrow) on neurite length (top panels) and cell viability (bottom panels). Kainate causes a concentration-dependent decrease in neurite length with concomitant decrease in cell viability (increased Annexin Green fluorescence). Addition of NBQX (20 μ M) & MK801 (10 μ M) protects iGluta Neurons from kainate toxicity. Each data point represents mean \pm SEM, n=6.

Conclusions

Testing multiple culture and plate coating conditions, we find that iCell Neurons adhere optimally with a plate coating of PEI and laminin. To exemplify a real-time imaging approach using hiPSC-derived neurons, we evaluated glutamate- and kainate-induced excitotoxicity using the IncuCyte S3 phase/fluorescent NeuroTrack applications multiplexed with Annexin V reagents in iCell Gluta Neurons. Glutamate and kainate produced a concentration- and time-dependent decrease in neurite length with a concomitant increase in red or green object count (indicating cell death) over 72 hours. Treatment with the NMDA receptor antagonist MK-801 and the AMPA receptor antagonist NBQX reduced the glutamate- and kainate-induced effects on neurite length and cell death.

These results exemplify that the IncuCyte S3 Live-Cell Analysis system enables the study of neurotoxicity in a model of hiPSC-derived neurons over days/weeks in culture. This system also allows for users to optimize culture conditions and maintenance of their own iPSC-derived neuronal models by providing automated analysis of microplate assays. The IncuCyte NeuroTrack software offers a means to image iPSC-derived neurons in monoculture or in co-culture with astrocytes and quantitate changes in neurite length and cell viability in real time. This approach also provides a sensitive method to detect pharmacological manipulations that alter neurite dynamics and induce toxicity. The IncuCyte approach for real-time, long-term quantitative analysis of neuronal cell health fills a critical need in the study of human neurophysiological disorders and iPSC-derived neurons.

References

1. Conforti L, Adalbert R and Coleman MP: **Neuronal death: where does the end begin?** *Trends Neurosci* 2007, 30:159-66.
2. Choi DW: **Glutamate neurotoxicity and diseases of the nervous system.** *Neuron* 1988, 1:623-34.
3. Mattson MP: **Glutamate and neurotrophic factors in neuronal plasticity and disease.** *Ann N Y Acad Sci* 2008, 1144:97-112.