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White Paper

November 12, 2018

Keywords or phrases:
Neuroscience, Neuronal Activity, Neurite Dynamics, Neuroimmune Assays, Phagocytosis, Chemotaxis, Live-Cell Assay, Neuronal Cell Health, GECI

Live-Cell Analysis for Neuroscientists

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Introduction

The last few decades have witnessed remarkable developments in the field of neuroscience. Deep sequencing and epidemiological work, for example, has provided stunning insight to the genetic basis for neurological disease. High-resolution techniques have also resolved numerous signaling pathways for learning and memory at both the cellular and molecular level. Despite this, identifying novel, truly effective treatments for patients has proved challenging, and most human brain and nervous system functions remain an enigma.

Several fundamental challenges exist. First, the nervous system is extraordinarily complex—there are > 100 billion interconnected neurons in the average human brain, and many more support cells. These cells are highly plastic and constantly change throughout development, adult life, and the almost inevitable decline of age and disease. Second, accessing living healthy and diseased human neural tissue for research with ethical consent is extremely difficult. Small biopsy samples may be obtained but are rarely sufficient for in-depth *in vitro* analyses and functional studies. Of all cells, neurons are extremely sensitive to damage or environmental change (e.g., hypoxia) which adds further technical complexity. Finally, in many cases animal models have yielded questionable translational value, particularly for psychiatric and neurodegenerative disorders, serving only to highlight the striking differences between lower and higher order species.

Recent advances in stem cell technologies offer an exciting alternative path where researchers can use human induced pluripotent stem cells (iPSCs) to create differentiated neurons and support cells (e.g., astrocytes, microglia). While this approach is in its infancy, the potential exists to build fully humanized, patient-specific, advanced cell models for neuroscience. To fulfill this promise, considerable work is required to optimize the reprogramming and differentiation methods, and to build and validate cellular bioassays that are representative of native human (patho) physiology. Phenotypic measurements that inform of functional outcomes and long-term plastic changes will be hugely useful in this regard.

To this end, we discuss live-cell analysis techniques and describe a suite of neurobiology applications that are amenable for studies in human iPSCs as well as primary cell models.

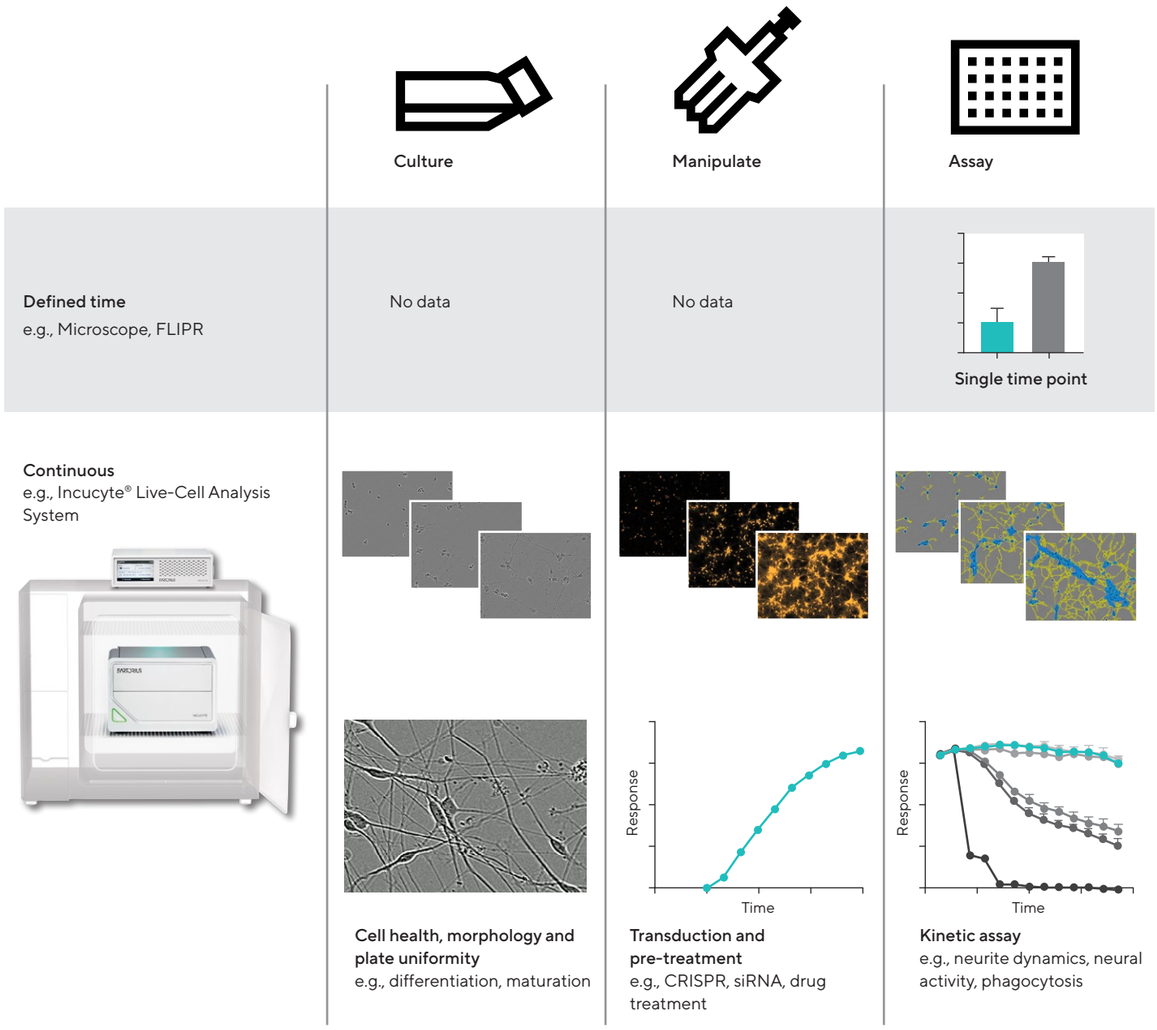

Figure 1: Workflow, QC and cell health. Comparison of continuous live-cell analysis and endpoint workflows. For live-cell analysis, images and data are collected in real time from within the cell incubator, throughout the culture, manipulation and kinetic assay phases.

The Basics of Live-Cell Analysis and Applications in Cell Health

Live-cell analysis is defined as the continuous, or semi-continuous, image-based measurement from cells without perturbing the sample. Unlike traditional endpoint readouts (e.g., high content imaging, flow cytometry), data is generated throughout the experimental workflow, enabling insights through cell preparation, differentiation, manipulation (e.g., gene editing) as well as the kinetic assay, all from the same cells (Figure 1). Typically, live-cell analysis is conducted with parallelized, automated time-lapse imaging within a cell incubator (e.g., Incucyte® Live-Cell Analysis System) for complete environmental control. Phase-contrast, brightfield and fluorescence images are analyzed and quantified in real time to report changes in morphology, movement, and other phenotypic parameters. Time-lapse videos can be created to verify the experimental outcomes. The critical attributes are (1) relevant, informative analyses based on the minimization of artifacts arising from cell perturbation, (2) long-term monitoring of biological events that unfold over days, weeks or even months, and (3) sufficient miniaturization, throughput and ease of use to enable replication, controls, and overall experimental productivity. The attributes of live-cell analysis are strongly aligned to the requirements of neuroscientists working with basic and advanced cell models.

As a simple illustration, Figure 2 shows Incucyte® live-cell analysis data from human iPSC-derived neurons (iGluta, CDI Wisconsin). In this case, following thawing of the cell vials into 96-well plates (20K cell per well, poly-D-lysine), phase-contrast images were captured every 12 h for 5 days (panel A), during which the characteristic neuronal morphology (high contrast cell bodies, elongated bipolar neurite projections) and consistency of cell plating was verified. Using a non-perturbing, fluorescent Annexin V apoptosis detection reagent (Incucyte® Annexin V Orange Reagent, added at day 10), the health of the culture (control wells, no | low fluorescence) was confirmed throughout the experiment. In wells treated with pathological concentrations of glutamate, an excitotoxic amino acid, a robust concentration- and time-dependent increase in the Annexin V fluorescence was observed. The annexin signal was concurrent with clear evidence of membrane, cytoskeleton and DNA damage. MK-801, a non-competitive receptor NMDA antagonist, attenuated the glutamate response. This experiment presents the basis for miniaturized, quantitative, and information-rich cell health and neurotoxicity assays that report the full time-course of events without washing, staining or removing cells from the incubator (e.g., Yu *et al.*, 2017).

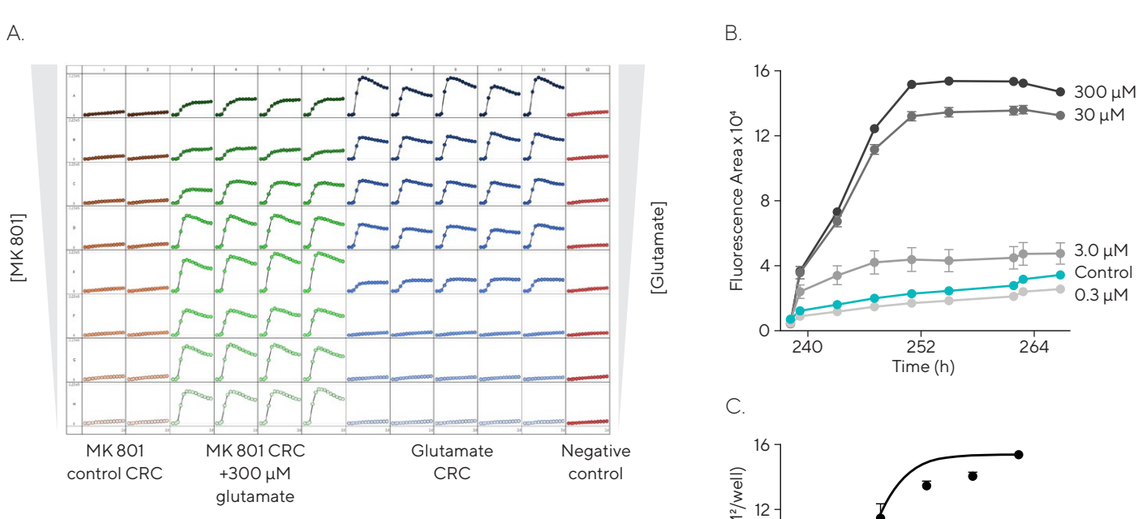

Figure 2: Live-cell analysis of neuronal health and apoptosis. Human iPSC-derived glutamatergic neurons (iGluta, CDI Wisconsin) were treated with increasing concentrations of glutamate alone or in combination with the NMDA antagonist MK-801. Annexin V Orange was included as a marker of apoptosis. (A) 96-well Microplate Graph showing the time-course of apoptosis (orange fluorescent area) over a 3 day period post-drug treatment. (B) Time-course plot of glutamate-induced excitotoxicity (mean ± SEM, 5–8 replicates). (C) Concentration response curve for glutamate-induced apoptosis (EC_{50} value 6.5 µM).

Neurite Dynamics

As neurons develop, they produce new interconnecting, elongated projections termed neurites. Neurite length and branch point measurements are widely used to describe neuronal phenotype. Indeed, neurite parameters form the basis for many *in vitro* models of plasticity, development, and neurodegeneration. Neurites are typically quantified using endpoint high content imaging techniques coupled with 'fix and stain' immunocytochemistry methods. However, the sample preparation and wash steps that are required can perturb fragile neurites, and temporal changes cannot easily be determined.

With live-cell analysis, neurite dynamics can be measured non-invasively over extended time periods, either with or without fluorescent labels (e.g., Hong *et al.,* 2018). For simple monoculture systems, neurite parameters are derived 'label-free' using phase-contrast images. Figure 3A shows an example of image segmentation in a range of neuronal cell types with the corresponding neurite outgrowth time plots. By normalizing the neurite length to the number of cell bodies, it is possible to compare directly the rates of outgrowth. Neuronal fluorescent labeling methods are required in co-culture systems, where phase-contrast images alone are not able to discriminate the neuronal projections from the background support cells.

The main concerns here are that the labeling is neuronal-specific, long lasting, and not detrimental to cell health. Phototoxicity associated with repeat exposure to short wavelength lights must also be avoided, so longer wavelength fluorophores (e.g., orange, red) are preferred (Laissue *et al.,* 2017).

Figure 3C shows the use of Incucyte® Neurolight Orange Lentivirus, a lentivirus encoding a fluorescent protein driven off a synapsin promoter to strengthen neuronal expression and minimize non-neuronal crossover. Here, rat cortical neurons are co-cultured with rat astrocytes and transduced with Incucyte® Neurolight Orange in a single step protocol. Neurite development was initially followed for 7 days using live-cell analysis to establish a baseline measurement. Cultures were then treated with different concentrations of hydroxydopamine (6-OHDA), a selective neurotoxin that is used to destroy dopaminergic neurons and induce Parkinsonism in laboratory animals. 6-OHDA caused a clear time- and concentration-dependent neurotoxicity over a further 7–10 days. This experiment illustrates how live-cell analysis is applicable to long-term temporal monitoring of neurite dynamics, and can be used to assemble a human co-culture model of dopaminergic neurobiology.

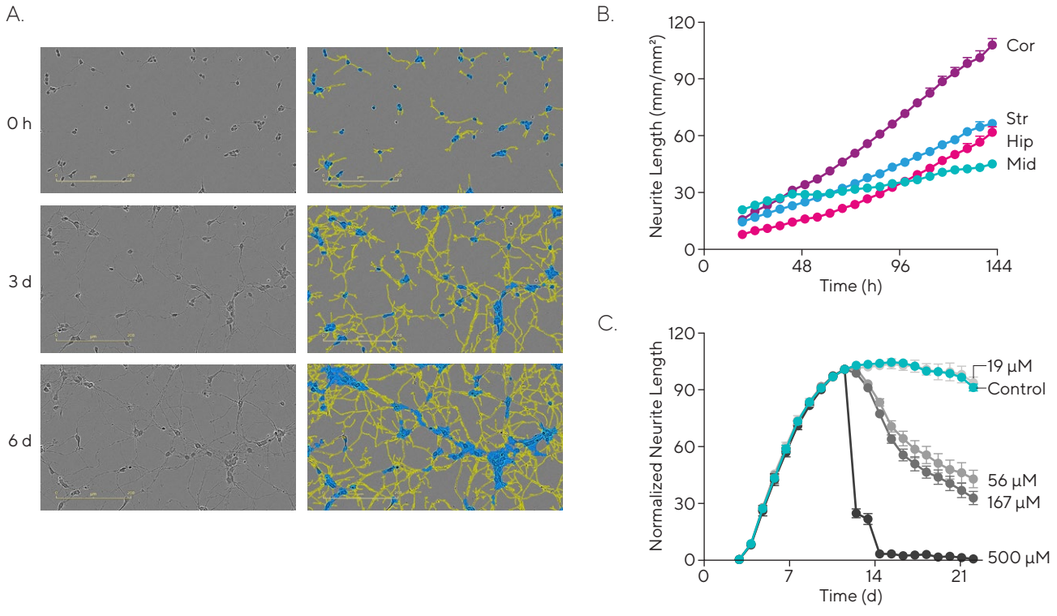

Figure 3: Live-cell analysis of neurite dynamics. (A) Incucyte® images of rat cortical neurons at 0, 3, and 6 days post-plating (left) with corresponding image masks (center: cell bodies (blue) and neurites (yellow)). (B) Differential time-courses of neurite outgrowth for neurons isolated from four rat brain regions: cortex (Cor), striatum (Str), hippocampus (Hip) or mid-brain (Mid). (C) 6-OHDA-induced neurite disruption in rat cortical neurons co-cultured with rat astrocytes. Neurons were transduced with Incucyte® Neurolight Orange to enable identification and segmentation of neurites using fluorescence imaging. 6-OHDA, added at day 10, produced a concentration- and time-dependent disruption of the neurite network. Data are expressed as % neurite length, normalized to pre-treatment values (mean ± SEM, 3 replicates).

Neuronal Activity Measurements

Of course, the most fundamental function of the nervous system is the transmission and integration of information via electrical signals that pass along axons and dendrites, and between cells at synapses. Activity measurements are therefore critical for true insight into neuronal behavior. Traditionally, these are made using sophisticated microelectrode electrophysiology techniques. Patch-clamp measurements of action potentials and synaptic currents provide exquisite resolution of electrical changes down to the single cell, and even single ion channel, level. Unfortunately, they are not amenable to monitoring long-term changes (days | weeks) and require deep operator expertise. Extracellular multi-electrode array (MEA) methods address these problems to some extent but sacrifice spatial precision and can require high cell densities.

As a new approach, we have developed a neuronal genetically encoded fluorescent Ca^{2+} indicator (GECI) and combined this with novel live-cell acquisition and analysis tools (available for Incucyte® Live-Cell Analysis System configured with an Orange/NIR or a Green/Orange/NIR Optical Module) that are purpose-built for long-term activity measurements. Neuronal Ca^{2+} transients at the individual cell (or cell cluster) level report the integrated spontaneous and synaptically driven excitability events. Data can be analyzed for insight into network connectivity and synaptic plasticity. This live-cell analysis workflow is illustrated in Figure 4. First, neuronal | astrocyte co-cultures are treated with Incucyte® Neuroburst Orange Lentivirus, a lentivirus encoding neuronal targeted GECI protein that is optimized for long-term, non-perturbing Ca^{2+} measurement. Full expression of the fluorescent protein typically takes 2–3 days, and is stable for > 1 month. A series of fluorescent images is then captured for up to 3 minutes (3 fps) in each well (Incucyte® Stare Mode acquisition). Single cells or cell clusters are identified and processed for changes in Ca^{2+} (orange fluorescence) over the image sequence. Several hundred active 'nodes' are typically present in each field of view—many more than MEA—and can be overlaid to visualize the coordination within the network. Importantly, these 3' sampling epochs can be repeated over many days and weeks to build understanding of the development of the network and any long-term plastic changes.

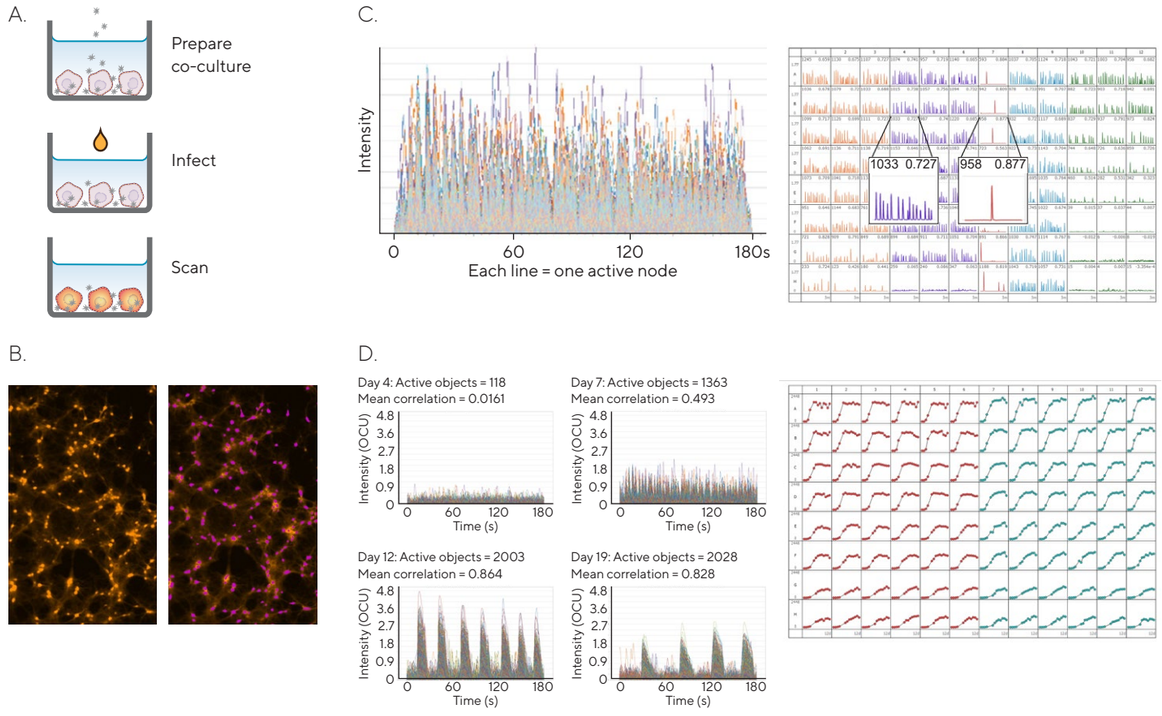

Figure 4: Neuronal Activity Assay—concept, workflow, and analysis. Neurons are transduced with Incucyte® Neuroburst Orange and then placed into an Incucyte® Live-Cell Analysis System for live-cell imaging and analysis. (B) A rapid series of fluorescent Ca^{2+} images are captured in each well (up to 3 minutes, 4X magnification), and active nodes (typically 500–1500) are identified and analyzed. (C) Ca^{2+} traces from each node are overlaid to visualize Ca^{2+} oscillations in the network, both at the single well and microplate level. (D) Network activity changes are monitored over days and weeks for changes in bursting and synchronicity patterns.

To demonstrate the value of this, we explored the long-term effects of the microtubule stabilizer paclitaxel (Taxol) on neuronal activity. The use of Taxol as a cancer chemotherapeutic is limited by chronic sensory and motor toxicity side effects. In rat cortical neuron and astrocyte co-cultures, spontaneous neuronal activity developed and stabilized over the first 7 days *in vitro* (Figure 5). Treatment with paclitaxel caused a slow, progressive, concentration-dependent reduction in neuronal activity. Inhibitory effects were observed at concentrations as low as 10 pM, but only after prolonged treatment (10–15 days).

In side-by-side live-cell analysis, effects on morphology (neurite length) were only observed at higher concentrations. Overall, the neuronal activity metric was > 20-fold more sensitive to the effects of paclitaxel compared to the structural change. This finding highlights the importance of extending beyond morphological measurements and into functional readouts to gain greater insight into the ongoing biology. Overall, this neuronal activity application provides a powerful, relevant phenotypic readout for long-term network changes in advanced cell models.

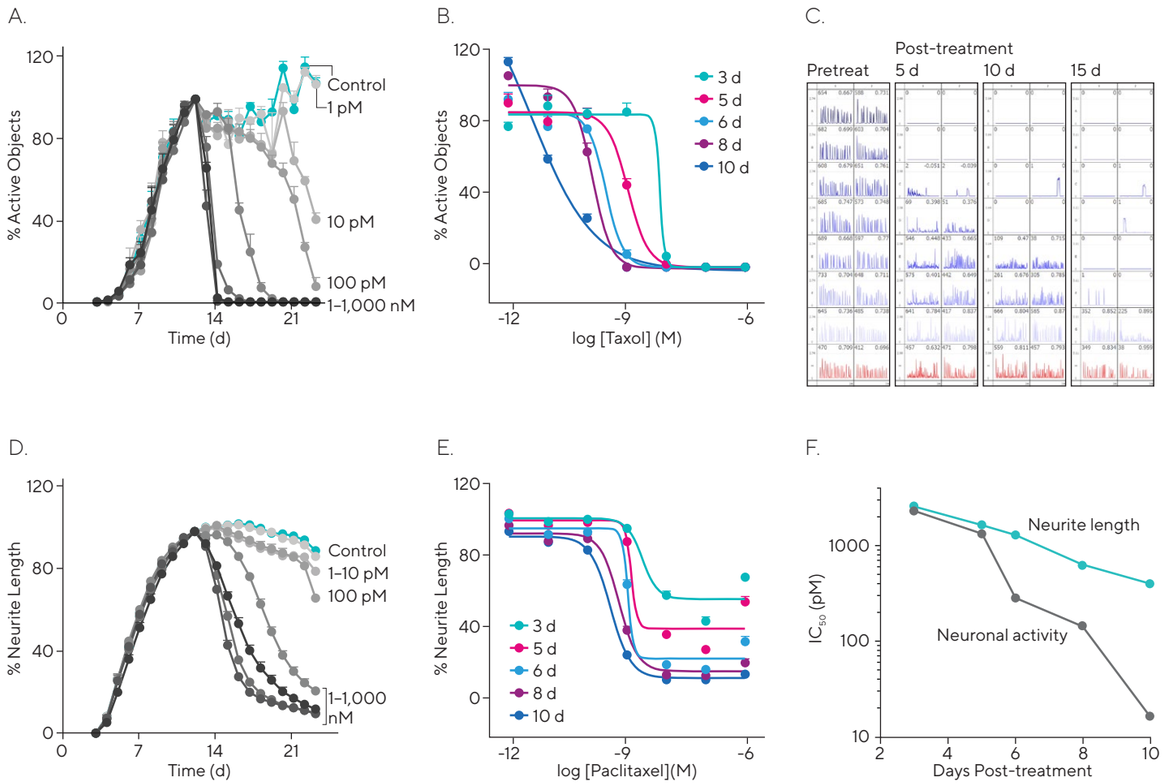

Figure 5: Incucyte® functional (A–C) and structural (D–E) readouts of Taxol-induced neurotoxicity. Rat cortical neurons were co-cultured with rat astrocytes and transduced for neuronal activity Ca^{2+} assays (Incucyte® Neuroburst Orange) or neurite dynamics (Incucyte® Neurolight Orange) at day 3 in culture. Live-cell analysis measurements were made each day using Incucyte® Live-Cell Analysis System. After 11 days, neural networks had fully formed and stabilized. Taxol (1 pM to 1 µM) or vehicle control was then added, and cultures were monitored for a further 15 days. Time-courses of neuronal activity and neurite development (D) prior to and after the addition of control or increasing concentrations of Taxol. Data is expressed as % active objects or neurite length, normalized to the pre-treatment value (mean ± SEM, 3 replicates). Concentration response curves determined for neuronal activity (B) or neurite length (E) at 3, 5, 6, 8 and 10 days post-treatment. (C) Neuronal activity traces at pre-treatment and at 5, 10, and 15 days post-treatment. (F) Potency (IC_{50} values) plotted against time post-treatment for neuronal activity (grey) and neurite dynamics (yellow). Note, initially similar inhibitory potency are observed for both readouts, however the marked time-dependent increase in potency (1000-fold) for neuronal activity yields IC_{50} values in the low pM range.

Applications in Neuroimmunology

The importance of the interface between the nervous and immune systems during development, homeostasis, and disease is increasingly recognized. The resident macrophages of the brain, microglia, are now known to profoundly impact synaptogenesis and synaptic wiring, as well as control pathogen defense and the clearance of dying cells. Human iPSC-derived microglia have been developed and exciting microglial drug targets for neurodegenerative and neuroinflammatory diseases are beginning to emerge (e.g., TREM2, SIRP1A).

Phagocytosis is the process of ingestion and engulfment of cells or particles by phagocytes and is a critical function of microglia. Assays for microglial phagocytosis are essential for dissecting basic biological mechanisms and for screening new microglia-based treatments. One approach that lends itself to live-cell analysis is the use of pH-sensitive fluorescent dyes (e.g., pHrodo® Orange Cell Labeling Dye for Incucyte®). These can be attached to targets for engulfment (e.g., dying cells, protein aggregates, pathogens, synaptosomes) and increase in fluorescence as the particle is internalized and processed through the acidic environment of the cellular lysosome (e.g., Sellgren *et al.*, 2017; Brosius Lutz *et al.*, 2017; Bohlen *et al.*, 2018; Zorina *et al.*, 2018).

Figure 6 illustrates live-cell assays for human iPSC-derived microglia (Axol BioScience) as they engulf pHrodo® Orange labeled apoptotic neuroblastoma cells (N2A—panels 1-4) and aggregates of beta amyloid (Aβ). In the image sequence, clear internalization of the apoptotic cell can be observed, accompanied by an increase in orange fluorescence. Using fluorescent area as a metric, the phagocytic signal is rapid and proportional to the number of apoptotic cells added. Similarly, microglia engulfment of Aβ aggregates gives rise to robust and concentration-dependent increases in fluorescence. In control experiments on non-phagocytic cells (e.g., HT-1080 fibrosarcoma) we observe no signal change over the same time period.

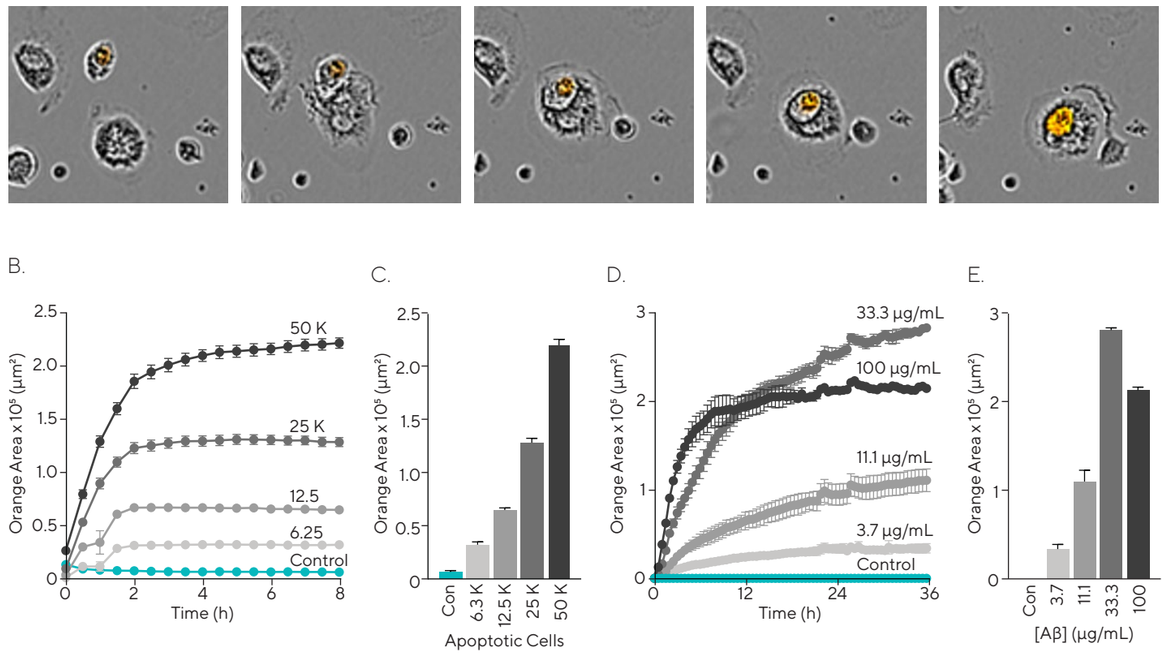

Figure 6: Microglial phagocytosis of apoptotic cells and disease-related protein aggregates. iPSC-derived microglia (Axol Bioscience) were seeded in 96-well plates and pHrodo® labeled apoptotic cells or protein aggregates were added prior to image capture and analysis (Incucyte® Live-Cell Analysis System). (A) Image series (8 minute intervals) depicting the engulfment of a pHrodo® labeled apoptotic Neuro2A cell. Note the development of orange fluorescence upon engulfment and transition to the acidic phagosome. Time-course of increasing efferocytosis of pHrodo® labeled apoptotic Neuro2A cells (B) or pHrodo® Orange labeled β amyloid (Aβ) aggregates (D). Bar graphs of orange fluorescent area showing the target cell number-dependence of the efferocytic signal (C) or the Aβ concentration-dependence of the phagocytic signal (E).

In other experiments on the microglia cell line, BV-2, we compared the pharmacology of engulfment of apoptotic cells and Bioparticles® (pHrodo-labeled bacterial wall proteins; Figure 7). Interestingly, while both responses were abrogated by cytochalasin D, only the cellular engulfment signal was inhibited by the integrin αVβ3 and αVβ5 inhibitor, cilenglitide. While the full explanation for this is unclear, the observation demonstrates that different signaling pathways may be involved in these two processes and that live-cell analysis is able to readily distinguish them. In its broadest context, the pH-sensitive dye and live-cell analysis approach provides a flexible and insightful method that can be applied to questions across a range of neurobiological areas.

Recent studies have shown that microglia are highly motile cells and migrate to areas of inflammation and tissue injury in the brain. The directed migration, or chemotaxis, of microglia can also be measured with live-cell analysis (e.g., He *et al.,* 2018). In Figure 8, C8-B4 cell migration toward complement C5a is measured using specialized Incucyte® Clearview 96-well Plates that enable the visualization of cells as they move toward the chemoattractant. As with the earlier applications, this approach affords the full time-course of biology with morphological insight. As another upside, considerably fewer (5 to 10-fold less) cells are required compared to traditional Boyden chamber transwell assays.

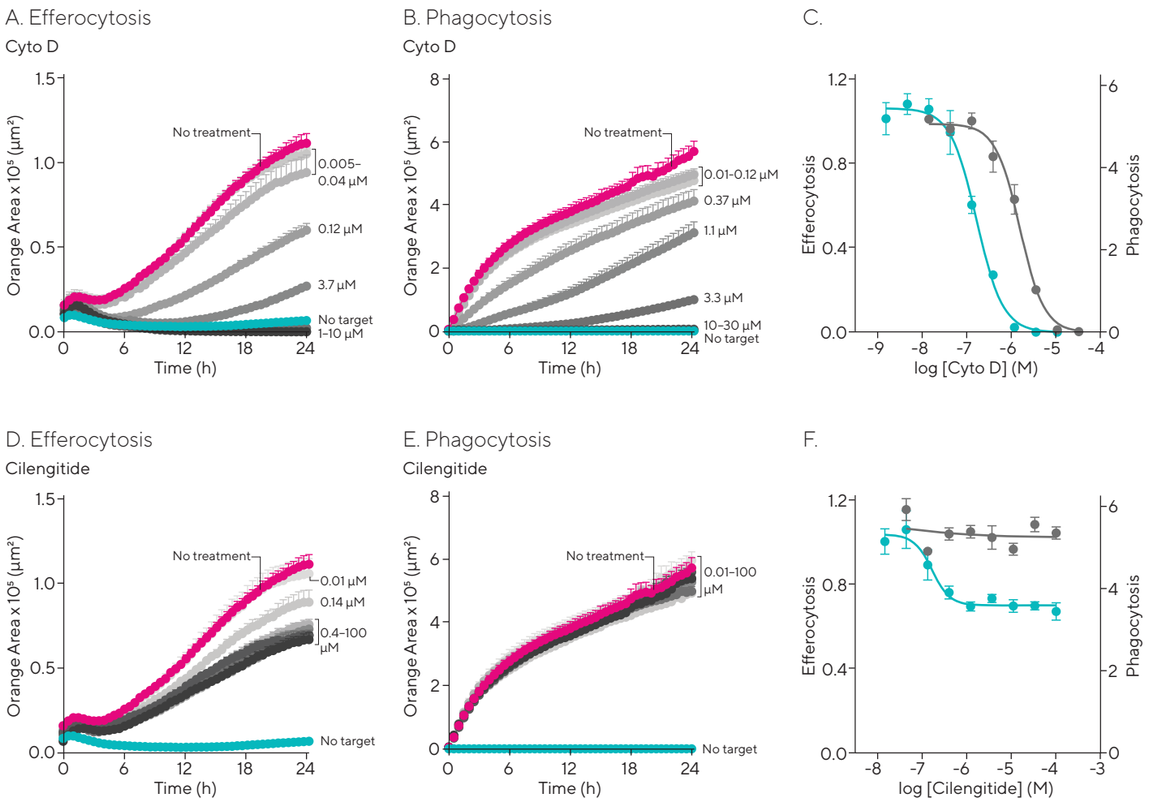

Figure 7: Differential mechanisms of phagocytosis and efferocytosis. BV-2 effector cells phagocytose apoptotic Neuro2A cells or E. coli bioparticles. Cytochalasin D, a potent actin polymerization inhibitor, elicits a concentration-dependent inhibition of both efferocytosis (A) and phagocytosis (B). Concentration-response analysis (C) yields IC_{50} values of 0.16 µM and 1.5 µM, respectively. In contrast, cilengitide, an inhibitor of αVβ3 and αVβ5 integrins, selectively attenuates efferocytosis (D), while inducing little or no effect on phagocytosis (E) at the highest concentration tested (100 µM). The differential pharmacology is highlighted in the concentration-response analysis (F) where cilengitide yields an IC_{50} value of 0.17 µM against efferocytosis.

Summary and Conclusions

In this white paper we have described a range of applications of live-cell analysis for the neuroscientist—these include basic cell health measurements, assays for neurite dynamics and neuronal activity, and readouts for neuroimmune functions such as phagocytosis and chemotaxis. These applications are illustrated with experiments in human iPSC-derived, primary and immortalized cells, which in many cases have been assembled in advanced co-culture models. Where fluorescent reagents are required, the examples shown deploy longer wavelength fluorophores (orange | red) that have been validated as non-perturbing to neurons and other cells.

Overall, the attributes of live-cell analysis are very well aligned to the requirements of neuroscience researchers, particularly those working with stem cell models.

Specifically, the non-invasive approach allows quantitative monitoring of cells throughout the culture, expansion and differentiation workflow and alleviates the need to select arbitrary assay endpoints. The method is cell sparing such that researchers can learn quickly even with a small number of cells. The images and time-lapse movies provide deep insight into changes in cell morphology and cell | cell interactions over time.

We conclude that live-cell analysis is a powerful and versatile method for neurobiology and provides a valuable compliment to established techniques such as high content imaging and electrophysiology. Going forward, live-cell analysis will play a key role in building and validating translational cell models for the discovery of novel neurotherapeutics.

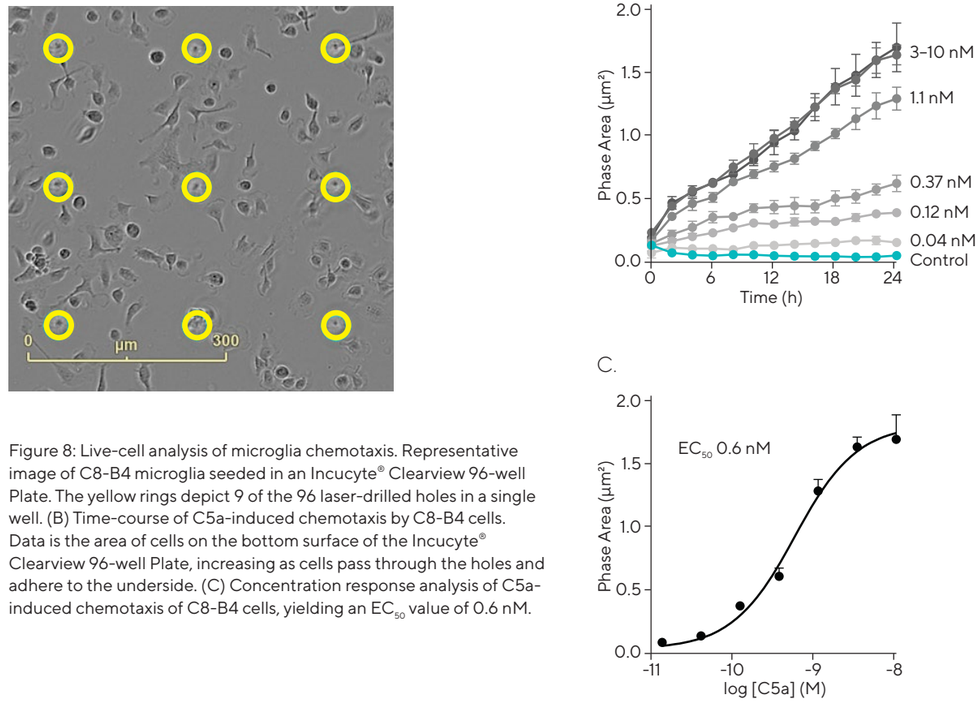

Figure 8: Live-cell analysis of microglia chemotaxis. Representative image of C8-B4 microglia seeded in an Incucyte® Clearview 96-well Plate. The yellow rings depict 9 of the 96 laser-drilled holes in a single well. (B) Time-course of C5a-induced chemotaxis by C8-B4 cells. Data is the area of cells on the bottom surface of the Incucyte® Clearview 96-well Plate, increasing as cells pass through the holes and adhere to the underside. (C) Concentration response analysis of C5a-induced chemotaxis of C8-B4 cells, yielding an EC_{50} value of 0.6 nM.

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Publication No.: 8000-0652-B00 Version: 12 | 2020