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Unraveling the Complexities of Neurological Disease and Injury with Real-Time, Live-Cell Analysis

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Introduction

The pathophysiology of neurological disease and injury is admittedly highly complex and temporally dynamic. This poses a daunting challenge for the development and appropriate use of physiologically relevant models for investigative research. Subtle fluctuations occur continually, and degenerative processes can impact cell health and function over long periods of time. These kinetic changes are challenging to capture, quantify, and interpret, but they hold valuable clues for understanding disease pathology, identifying therapeutic targets, and characterizing drug pharmacology. This is further complicated by issues surrounding the limitations of neurological models and their appropriate use, which was the focus of a recent editorial, "Neurological disease models made clear" (*Nature Medicine*, 2015). The editorial requested that authors of submissions provide more information about the models that were used and why they were chosen. This highlights the need for a greater characterization of neurological models, their performance, and also their limitations.

Real-time kinetic studies can generate quantifiable information and are valuable within the framework of neurological models, linking subtle changes in cell health and function, either from disease or therapeutic intervention, with observable phenotypic effects. This real-time, quantifiable information can enhance understanding of disease etiology, the identification of therapeutic targets, and the characterization of drug mechanisms. Additionally, there is a requirement to maintain cellular integrity throughout the course of an experiment. This is advantageous in light of calls for increased emphasis on rigor and reproducibility for *in vitro* models. Live-cell analysis can be used to monitor the health and function of cell lines, permitting the detection of subtle, cumulative changes that may lead to unwanted phenotypic effects over time with increased passage.

This communication is focused on the use of the Incucyte® Live-Cell Analysis System as a valuable tool for use in neurological disease model development, the study of disease pathology, neuroinflammation, nervous system injury, and drug discovery.

This system uses real-time, automated imaging and analysis, combined with microplate throughput, to supply kinetic measurements of cellular health, cell number, function, and neurite outgrowth, among others. As amply illustrated with examples from peer-reviewed literature that follows, many investigators have already begun incorporating live-cell analysis into their experimental workflows. They have uncovered key information and new functional insights spanning the breadth of neurological conditions. Such timely work fills a much-needed gap in our understanding of the dynamic changes underlying neurological disease development and system repair. The integration of real-time kinetic monitoring and cell health measures into experimental workflow can augment the development and interpretation of more biologically relevant neurological disease models. This technology will better-equip researchers to unravel the complexities of neurological disease, expedite workflow, and aid the discovery and analysis of new therapeutics.

Neurological Disease Model Development and Refinement

Challenges with Disease Models

A wide variety of *in vitro* and *in vivo* models have been employed in neurological research to explore cell growth activities, morphological changes, genetic regulation, debris clearance, regeneration, and drug targets. Each model has both positive and negative attributes, and the selection of the appropriate model system, or combination thereof, merits careful consideration (for guidance, see *Nature Medicine* editorial, referenced below). Animal models may incur substantial costs over time and suffer from a lack of translational relevance, thus the current efforts to humanize translational research with *in vitro* models to better recapitulate the complexity of human diseases. The development and use of human pluripotent stem cells (hPSC) has given translational research new hope for the capacity to create more biologically relevant models, opening the exploration for the development of cells for personalized therapeutics and lineage reprogramming (Ferreira and Mostajo-Radji, 2013). However, for *in vitro* cell lines, there are still additional considerations to address, such as the requirement for homogeneity of an expandable cell line, cell synchronization to a post mitotic state, and phenotypic evidence of a mature neuron (Schlachetzki *et al.*, 2013). Further, problems may arise in long-term cultures with increasing passage, including cross contamination with other cells, misidentification, selective pressures, and genetic drift that may alter cell health and function, confounding results (Hughes *et al.*, 2018).

Refinement and Comparison of Models

Live-cell imaging and analysis of cell health, function, and morphology may be combined with *in vitro* neurological models to provide valuable insights for their comparison and improvement. Microglia have been implicated in neurological diseases such as MS and Alzheimer's (Ransohoff and El Khoury, 2015). Bohlen *et al.* (2017) undertook a study to characterize and optimize primary microglia culture. Using live-cell analysis, the phagocytosis of myelin debris by microglia was evaluated in the context of exposing microglia to serum, as might occur when the blood-brain barrier is compromised (i.e., stroke or contusion). Data presented showed that exposure to serum increased the phagocytic activity of microglia, and this effect was intrinsic and sustained; attributable to a larger factor(s) that would be unable to cross an intact blood-brain barrier, but could do so if the barrier was compromised. He *et al.* (2018) attempted to optimize microglial culture, examining three methods for their isolation from mouse brain, which included shaking, trypsinization, and MACS® sorting with CD11b microbeads. The MACS®-isolated primary cells and BV2 cells were sequenced and subjected to transcriptional profiling, which suggested a possible difference in chemotaxis between the two. This was validated with measurements of chemotactic migration towards C5a, generated by Incucyte® Live-Cell Imaging and Analysis of Incucyte® 96-Well Clearview Plates. The primary microglia displayed enhanced migration as compared to cells from the BV-2 cell line, revealing an important difference between these models. This illustrates how live-cell imaging and analysis can be used to examine specific characteristics of models to better understand model performance for model selection.

Development of New Models

New models are continually under development in order to better recapitulate disease mechanisms, especially when trying to elucidate patient specific genetic factors. This process can be augmented by incorporating live-cell analysis into the workflow to capture dynamic changes that may otherwise go unrecognized. Kobayashi *et al.* (2018) developed systems for purifying and culturing retinal ganglion cells (RGC) for *in vitro* use to study RGC and regenerative medicine applications. Incucyte® Neurotrack Analysis Software was used to measure and analyze neurite outgrowth of RGCs from 3D retinal organoids, revealing an increase in neurite length with time, out to 90 h after plating. Long-term culture of RGCs may be useful to study neuroprotective drugs for glaucoma with additional applications for transplantation therapy. Nguyen *et al.* (2018) investigated the activity of miR-146 (implicated in neurodevelopmental disorders) in the differentiation and neural lineage determination of neural stem cells. Expression analysis from autism spectrum disorder (ASD), post-mortem human brain samples was combined with *in vitro* functional data from human NSC (neural stem cells) modified to overexpress miR-146. The Incucyte® Live-Cell Analysis System and Incucyte® Annexin V Dye were used to collect proliferation (confluence) and apoptosis data from H9 human NSC cells that overexpressed miR-146a, as well as to conduct differentiation analysis. The proliferation rate decreased with an increase dendritic branching and extension. This suggested that miR-146a upregulation may disturb normal transcription, possibly impacting cortical disorganization, neuron numbers, and dendritic spine density, which are morphological changes noted in autism spectrum disorder (ASD). Cavaliere *et al.* (2017) also used live-cell analysis to analyze neurite outgrowth of neuronal and astrocytic abnormalities due to human-derived α-syn aggregates in a model of Parkinson's disease with Lewy Body particles. Even simple output measures, such as confluence and growth assessment, can yield valuable information when strategically incorporated into experimental model design. Bressan *et al.* (2017) measured confluence and growth to assess the effects of genetic manipulation from CRISPR/Cas9-assisted gene targeting on NSC. This opened new possibilities for research on CNS development and pathology by performing scalable genome editing in a neural stem cell model. Taken together, these studies illustrate how live-cell imaging and analysis can be utilized to characterize new models that examine disease genetics.

Human Pluripotent Stem Cell Models

Live-cell imaging and analysis can be useful for the measurement of a variety of parameters in the development of human induced pluripotent stem cell models, which are gaining traction due to their promise for recapitulation of patient-specific phenotypes. These models are growing in popularity due to their capability to generate multiple cell types and a large capacity for self-renewal, making them an attractive option for use in high-throughput drug screening (Zhu and Huangfu, 2013). In a study by Robinson *et al.* (2017), neurite length and branch points were measured on human induced pluripotent stem cells (hiPSCs) when the crosslinking agent, genipin, was added to fibrin scaffolds. The genipin-crosslinked fibrin scaffolds had enhanced neurite outgrowth and were more stable, with possible applications for 3D printing. In another example, the migratory behavior of the human cortical neurons derived from human induced pluripotent stem cells (hiPSCs) was captured using live-cell imaging from a lissencephaly patient sample as compared to control, providing new information on human CNS development and brain formation (Bamba *et al.*, 2017). Ogura *et al.* (2018) attempted to create a 3D spinal cord induction model to study spinal cord development using hPSCs. Using DF Brightfield (a proprietary optical technique for capturing images with an extended depth of field) time-lapse imaging, they creatively utilized the Incucyte® Spheroid Analysis Software Module to capture images of differentiating hPSCs to aid in model characterization. The model displayed induction of dorsal and ventral interneurons, and was able to generate centers resembling

roof plates. Akhtar *et al.* (2018) combined iPSC neural progenitors and inducible gene expression with *ex vivo* cell engineering to produce a method to modulate GDNF secretion. GDNF could be induced or reversed with doxycycline in human induced pluripotent stem cells that had been derived from neural progenitors. Live-cell analysis was used to assess reporter kinetics by capturing live-cell images of human iNPCs (human induced pluripotent stem cell-derived neural progenitor cells) that had been nucleofected and grown with luciferin. Nucleofected iNPCs were also transplanted *in vivo* into the brains of NOD-SCID mice to study gene induction. The development of this novel methodology could have applications for the delivery of proteins for neuroprotection.

In a *Nature Medicine* publication investigating the genetic engineering of hPSCs, Ihry *et al.* (2018) discovered that Cas9 can cause double-strand breaks that can kill human hPSCs. Incucyte® Live-Cell Analysis was used to quantify confluency for toxicity studies. Toxicity was found to be dependent on P53/TPS3. Cas9 toxicity has the potential to cause problems during engineering involving clonal expansion, as P53 mutations could develop over time. This could be problematic when CRISPR/Cas9 is used for the production of hPSCs used in cell replacement therapies.

Taken together, the incorporation of live-cell analysis with human pluripotent stem cell models provides important information for use in model assessment for factors such as neurite outgrowth, migration, and assessment of genetic manipulations.

Neurological Disease Mechanisms and Pathology

Tracking experimental manipulations in real-time can provide information that might be otherwise missed with conventional methods. Muñoz *et al.* (2018) developed a cell culture method to study proteolytic fragmentation of apoE, which plays a role in the clearance of β-amyloid (Aβ) and neuronal signaling. Live-cell imaging and analysis was used to assess confluence and neurite length in SH-SY5Y neuroblastoma cell lines. Neuritogenesis was stimulated by full-length apoE as well as a 25-kDa apoE fragment, which is regulated by HtrA1 under physiological conditions and was found to have a neurotrophic function. Constantinescu *et al.* (2017) examined PAS (plasminogen activation system) clearance of large, pathological protein aggregates from the body. Cell viability of mouse brain and lymph node cultures were measured during the interaction of PGPFs (cytotoxic plasmin-generated protein fragments) incubated with clusterin (CLU) and 2-macroglobulin. Cytotoxicity was measured in EOC 13.31 (microglial-like) and SVEC4-10 (endothelial-like) cells with live-cell imaging and analysis. Clusterin and 2-macroglobulin bound to the PGPS to reduce toxicity. This integrated analysis suggested that the plasminogen activation system, along with extracellular chaperone proteins, may enhance the clearance of protein aggregates.

Neuroinflammation and Neuroprotection

Neuroinflammation combines the complexity of both the nervous and immune systems, and it is advantageous to incorporate a number of kinetic measurements to assess this process. Zorina *et al.* (2018) examined whether binding of human recombinant IgM antibody (HIgM22) could mark damaged myelin for phagocytic clearance by microglia in multiple sclerosis (MS). Live-cell imaging and analysis revealed that IgM stimulated phagocytic activity. Combined with other analysis, it was discovered that this process required actin polymerization, activity of the IgM Fc domain, and CR3 (Complement Receptor 3). Manresa-Arraut *et al.* (2017) utilized live-cell analysis in an ischemic stroke model to investigate the role of RhoA in T cell transmigration. The migration rate of RhoA−/− T-cells was slower than RhoA+/+ T-cell across the endothelial cell layer, such as encountered in the BBB (blood-brain barrier), which can correlate with disease severity. T cell transmigration was reduced with atorvastatin and fasudil treatment, independent of Rho expression, which was attributed to a more global inhibition of the RhoA-ROCK pathway. Live-cell analysis was also utilized to study the involvement of RhoA in T cell activation was also explored in an animal model of MS (Manresa-Arraut *et al.*, 2018). Live-cell analysis can also be used to characterize neuroprotection and neuroinflammation. Peng *et al.* (2017) studied HSV (Herpes Simplex Virus) to examine why this viral infection is not commonly associated with peripheral nerve destruction. Long-term kinetic imaging of cultivated human fetal DRG neurons was performed with an IL-17c gradient, NGF, or BDNF. Neurite length and number of branch points were assessed with Incucyte® Neurotrack Analysis Software Module. Human IL-17c acted as a neurotrophic cytokine through stimulation of neurite outgrowth and branching, reduction of apoptosis, and provided a neuroprotective role during HSV reactivation. Cheng *et al.* (2018) studied a variant of TREM 2 receptor (R47H TREM2, linked to Alzheimer's disease) using a TRM2 R47H transgenic mouse model and TREM-2 activating antibody. Live-cell analysis was used to assess confluence of microglia, macrophages, BMDM (Bone Marrow Derived Macrophages), as well as microglia invasion in response to the TREM2 antibody. The antibody caused increased signaling in wild type (WT) myeloid cell function.

Nervous System Injury

Much work is needed to advance our understanding of nervous system repair and the development of more effective therapies. Kinetic information, phagocytosis for debris clearance, and cell health measures are especially important for regenerative studies. Brosius Lutz *et al.* (2017) explored strategies to enhance myelin clearance following CNS injury. Schwann cells from a mouse sciatic nerve crush model were cultured in medium with pHrodo® -labeled crude PNS myelin. Schwann cell phagocytosis of myelin was measured using the Incucyte® Live-Cell Analysis System. Two phagocytic receptors, Axl and Mertk (TAM family of receptor tyrosine kinases) were required for clearance of the myelin debris, involving both autophagy and Schwann cell-mediated myelin clearance. This shed new light on the pathways involved in myelin clearance following CNS injury, introducing new avenues for therapeutic development. Li *et al.* (2017) explored transcription factor manipulation for neuron regeneration, testing if KLF7 could regenerate DPSN axons and form synapses following spinal cord injury. This *in vitro* study tested the effect of AAV-KLF7 on neurite outgrowth on rat neurons. Incucyte® measurements of neurite outgrowth revealed significant, dramatic increases in neurite length and branch points. This was explored further an *in vivo* mouse model, where mice received a T10 contusion injury followed by treatment with AAV-KLF7. KLF7 was expressed in the injured spinal cord, and treatment improved several output measures including the regeneration of axons and synapses, muscle weight, and even recovery of motor function, as assessed by the animal's ability to walk a grid. This creative study demonstrates how an *in vitro* model assessment using live-cell analysis can guide translational experimental design for assessment of therapeutic potential *in vivo*.

Identification of Drug Targets and Therapeutic Development

Drug Screening

Neurological models can be refined by using live-cell imaging and analysis, leading to the improvement of target selection for drug screening. As related in a recent Nature Communications paper, Jin *et al.* (2018) developed an *in vitro* screening method for the screening anti-Aβ antibody drug candidates for treatment of Alzheimer's disease, which could aid in the development of more effective immunotherapies. The goal was to provide more information to the field about the capacity of antibody candidates to recognize toxic Aβ. Using an aggregate-preferring mAB, IC22, they compared the binding of this agent against two murine precursors of bapineuzumab (mAb 3D6) and solanezumab (mAb 266). They developed a sensitive bioassay that combined the use of brain extracts from AD brain extracts with Incucyte® Live-Cell Imaging and Analysis of induced neurons (iN) from human iPSCs generated through modification of the Ngn2 protocol (Zhang *et al.*, 2013). Baseline measurements were established for the iNs, with an increase in neurite length and branch points measured using Incucyte® HD phase contrast imaging. This timeframe was also associated with increases in tau, synapsin 1, synaptophysin, 1, GluA1, and PSD-95. The neurons were then treated with Aβ-rich soluble AD brain extracts and live-cell imaging was performed again, revealing a time- and dose-dependent decrease in neurite length and branch points on the same cells used for the baseline. The IC22 antibody, 3D6, and 266 were then also tested by this new quantitative method, incorporating live-cell analysis to assess neurotoxicity. IC22 protected the iPSC-derived human neurons from human Aβ toxicity more effectively than 3D6 (bapineuzaumab) or 266 (solanezumab). This study introduced new and exciting methodology for therapeutic antibody screening to the field of drug discovery, which could enable more rapid screening of therapeutic antibodies for disease such as AD. This method was more quantitative through use of live-cell imaging and analysis to measure neurotoxicity and

attenuation, which could be incorporated into mathematical estimation of antibody levels required to neutralize the toxic Aβ and possible dosing.

With regard to toxic amyloid fractions, Hong *et al.* (2018) further developed gentle homogenization techniques to isolate toxic Aβ oligomers prepared from post-mortem AD human brain extracts. Live-cell analysis was again used to collect phase contrast images of iPSC derived human neurons exposed to toxic Aβ oligomers in the brain extract followed by measurement of processes and cell bodies of the neurites using the Incucyte® Neurotrack Analysis Software Module. The diffusible, toxic Aβ fraction, isolated by gentle extraction, retained the neurotoxic activity as measured by a decrease in neurite length, with a temporal concordance to a loss of plasticity as measured by LTP recording (Long-Term Potentiation). The isolation of this toxic Aβ could enable more effective target selection, further emphasizing the value of incorporating live-cell analysis and functional assays into research workflows.

Identifying Drug Targets

Live-cell imaging and analysis has been used to identify drug targets for a variety of other diseases, emphasizing the value of quantitative kinetic outputs. Yu *et al.* (2017) investigated lowering the accumulation of cytotoxic defective protein from the HTT gene in Huntington's disease (HD). Two positive modulators of mHTT, HIPK3 and MAPK11, were found to modulate HTT levels *in vitro* and *in vivo*. The Incucyte® Live-Cell Analysis System was utilized to assess confluence (cell shrinkage and death), as well as measurement of Capsase-3 activity of Q73 neurons. Possible new drug targets for HD were identified as well as new ways to approach target discovery. Oiknine-Djian *et al.* (2018) combined live-cell analysis with gene expression studies in a model of HCMV (human cytomegalovirus) to show that a new drug candidate, artemisone, acted earlier in the viral replication cycle than a conventional ganciclovir treatment. Additional studies have been conducted using live-cell analysis for the investigation of HDAC inhibition for neuroinflammation and neurodegenerative disease (Durham *et al.*, 2017), the action of lithium on Schwann cells (Piñero *et al.*, 2017), and the functional analysis of mutations of GRIN2a (NMDR gene) in epilepsy-aphasia syndrome (EAS) patients (Addis *et al.*, 2017).

Examining Drug Toxicity

Quantitative measurements can be combined to determine treatment effects on normal cell health and function. Zhou *et al.* (2017) investigated the use of an isoform-selective p38α MAPK inhibitor, MW150, in an AD mouse model. Several Incucyte® live-cell assays applications were used in combination to study the response to MW150 in a BV2, a murine microglial cell line, including proliferation (confluence), migration, phagocytosis of BV2 cells and their response to MW150. The study showed that MW150 was able to modulate the neuroinflammatory responses in a selective manner without negatively impairing microglia physiological functions (proliferation, migration, and phagocytosis).

Identifying New Therapeutic Applications

Finally, live-cell analysis can also provide insights for how existing, approved medications may be useful for new therapeutic areas. Paik *et al.* (2018) demonstrated that somatostatin (SST) is a potential therapeutic for AD by preserving the organization of tight junction proteins (TJP), maintaining the blood-brain barrier against damage from Aβ, and regulation of LRP1 and RAGE expression. The Incucyte® Live-Cell Analysis System and Incucyte® Caspase 3/7 Green Dye were used to measure apoptosis in hCMEC/D3 cells treated with Aβ1-42 with or without SST. Cells treated with Aβ1-42 + SST displayed a dose-dependent reduction of Caspase-3/7 as compared to cells treated only with Aβ1-42, indicating drug inhibition on caspase activity as a mechanism. Sellgren *et al.* (2017) created patient-specific, *in vitro* models of microglia-mediated synapse and neural progenitor cell (NPC) engulfment to investigate the role of C4 in microglia-mediated pruning. Reprogrammed, microglia-like cells (hiMGS) and monocyte-derived macrophages were co-cultured with pHrodo-labeled synaptosomes and NPCs (neural progenitor cells) and imaged real-time with the Incucyte® Live-Cell Analysis System, revealing an increase in phagocytosis of synaptic structures and NPCs by the microglia-like cells, and lower uptake by macrophages. Cells were then treated with C4, implicated in schizophrenia, and the engulfment of synaptic structures was observed. This model suggests the possibility for the creation of patient specific models amenable to drug screening. Taken together, these examples illustrate the power of applying live-cell kinetic analysis to re-examine mechanisms of action for existing drugs and creating new screening assays, the results of which can be used to guide additional research investigations.

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