Monitoring neurite morphology and synapse formation in primary neurons for neurotoxicity assessments and drug screening

Suk J. Hong and Richik N. Ghosh
Thermo Fisher Scientific, Pittsburgh, PA USA

Abstract

Synapse formation during nervous system development and degeneration in the pathogenesis of human neurological diseases are highly regulated processes. Subtle changes in the environment of the complex neuronal network may cause either breakdown or creation of synaptic connections. Drug discovery screening for neurological diseases and compound neurotoxicity evaluation would benefit from robust, automated, quantitative *in vitro* assays that monitor neuronal function. We hypothesized that (1) toxic insults to the nervous system will cause neuronal synapses to deteriorate in the early phase of neurotoxicity, eventually leading to neurite degeneration and neuronal cell death if the damage is severe; and (2) an *in vitro* functional assay for synapse formation and neuronal morphology could be used to monitor and identify such neurotoxic events. We thus developed an automated, functional, high-content screening (HCS) imaging assay to track and quantify the dynamic changes in neurites and synapses. This assay facilitates automation and streamlining of a laborious process in drug discovery screening and compound neurotoxicity assessment. The assay also enables quantitative comparisons between compounds that affect neuronal morphology and function, particularly in neurite- and synapse-associated events.

Introduction

Neurons in central and peripheral nervous systems function to transmit electrical signals from one location to the other to keep the brain and the body functioning properly. One of the critical structures in the neuron to maintain their proper functional network is the synapse, which is the junction between a nerve cell and the cell that receives an impulse from the neuron. The molecular network between these synapses controls not just synaptic signal transmission and synaptic plasticity but also regulates neuronal growth, differentiation, and death. The microstructure of synaptic junctions has been extensively studied to understand the relationship between synaptic activity and neuropathophysiology, as well as the molecular mechanism involved in synaptogenesis and regulation of the synapse.

Once synaptic function is disrupted by natural or man-made neurotoxic substances, it could lead to long-lasting and often irreversible neuronal damage. Synaptic damage has often been recognized as the first sign of neurodegeneration in many different pathological conditions, including traumatic nerve injury, ischemic stroke, and many neurodegenerative disorders such as motor neuron diseases, Alzheimer's, Parkinson's, and Huntington's diseases. Many synaptic proteins play an important role in the progression of neurodegenerative diseases. For example, amyloid beta precursor protein, presenilin, alpha-synuclein, huntingtin, ataxin-1, frataxin, and prion proteins are all involved in presynaptic or postsynaptic structure of the neuron and play a role in synaptic damage and neurodegeneration.

To measure the synaptic changes that occur in synaptogenesis or synaptic damage, we needed to develop a reliable, accurate, and efficient method to measure accurate synaptic loss, neurite changes, and neuronal death. Here we introduce a new way of measuring synaptic function utilizing the power of automated, quantitative, high-content, cell-based imaging and analysis.

Results

Thermo Scientific™ HCS synaptogenesis reagents combined with the Thermo Scientific™ ArrayScan VTI HCS Reader and Neuronal Profiling BioApplication enables the quantitation of neuronal morphology and synapses *in vitro* (Figure 1). On-the-fly automated image analysis and quantitation accompanying the automated image acquisition is done by the Neuronal Profiling BioApplication, which is an automated image analysis software module on the ArrayScan VTI HCS Reader. Using this technology and assay method, we could identify synaptic changes over time and measure synaptic and neurite parameters in an automated manner.

The assay identified primary neuronal cells by a neuron-specific marker and detected synapses on the spines of neurites with pre- and postsynaptic markers (Figure 2 and Table 1). The multiplexed targets, including a nuclear marker, were simultaneously detected with four fluorescent colors, and the fluorescent images of the labeled neurons and synapses were acquired by automated imaging (Figure 3).

The phenotypic features of neuronal morphology and the synapse were automatically identified and quantified in real time. Such features are potential indicators for neuronal development, differentiation, and neurotoxicity, and we could quantify changes in these features under different conditions and for different drug treatments (Figures 4 and 5). By monitoring changes in these features, we could also quantitatively evaluate compounds involved in developmental neurotoxicity (Figure 6).

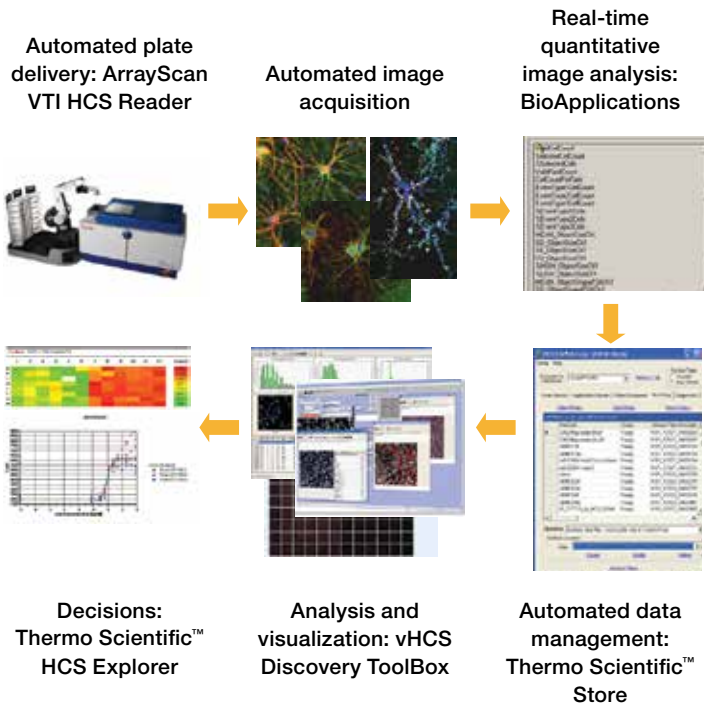


Figure 1. Seamless integration of steps in cellular analysis using the Thermo Scientific™ HCS platform.

Table 1. Potential synaptogenesis HCS assay targets can be detected in four different colors.

Fluorescence channel	Cellular entity targeted	Candidates for cellular target (best target screened in bold)	Fluorescent dye and color
Channel 1	Nucleus	**DNA**	DAPI
Channel 2	Cell body, neurite mask	**MAP-2**, ß3-tubulin, neurofilament	DyLight™ 488
Channel 3	Postsynaptic marker	**PSD95**, drebrin, spinophilin/ neurabin	DyLight™ 549
Channel 4	Presynaptic marker	**Synaptophysin**, synapsin1, synaptobrevin, synaptotagmin	DyLight™ 649

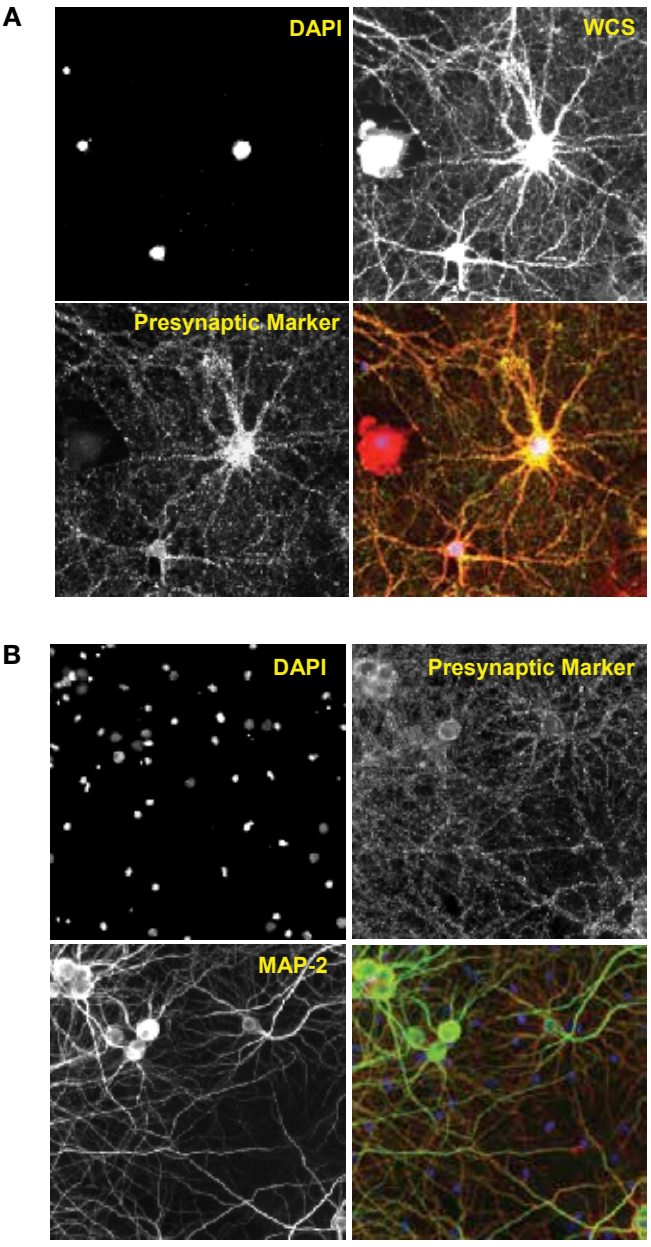
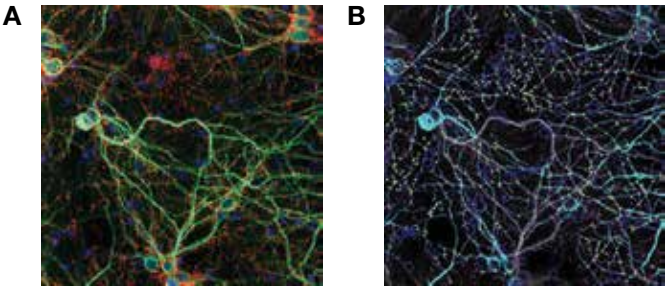


Figure 2. Staining of presynaptic marker synaptophysin, whole cells, and MAP-2. (A) DAPI and Thermo Scientific™ Whole Cell Stain (WCS, red) is used to detect the nuclei and the fine structure of neurites, respectively (mouse cortical neurons, 18 divisions). Synaptophysin staining is in green. (**B**) Synaptophysin (red) and MAP-2 (green) staining for presynaptic vesicle and neurite detection (rat hippocampal neurons, 22 divisions). Images were acquired using the ArrayScan VTI HCS Reader.



Raw image
Presynaptic marker synaptophysin (red)
Neuronal marker MAP-2 (green)

Analyzed image
Branch point (white)
Localized synaptophysin (purple)
Neuronal trace (blue)

Figure 3. Automated, simultaneous measurement of presynaptic vesicles, postsynaptic structures, and neurites. Rat hippocampal neurons (21 divisions) were stained for synaptophysin, PSD-95, and MAP-2, then imaged and analyzed. **(A)** Neurite detection with MAP-2 staining. **(B)** Postsynaptic marker spot detection with PSD-95 staining (magenta spots) and colocalization with synaptophysin spots (green spot). Colocalized spots (green) represent the location of potential synapses.

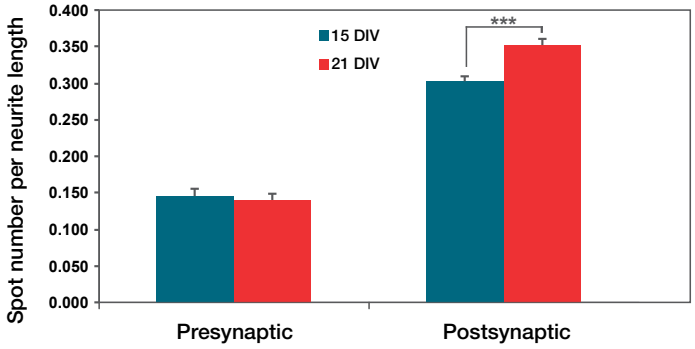


Figure 4. Punctuated PSD-95 stain increases by maturation of neurons. Mouse cortical neurons were cultured for 15 or 21 divisions and stained for synaptophysin, PSD-95, and MAP-2, then imaged and analyzed. Only postsynaptic spots stained with PSD-95 antibody increases in division 21 neurons compared to division 15 neurons (Student's *t*-test, $P < 0.001$). Presynaptic spots show no significant change.

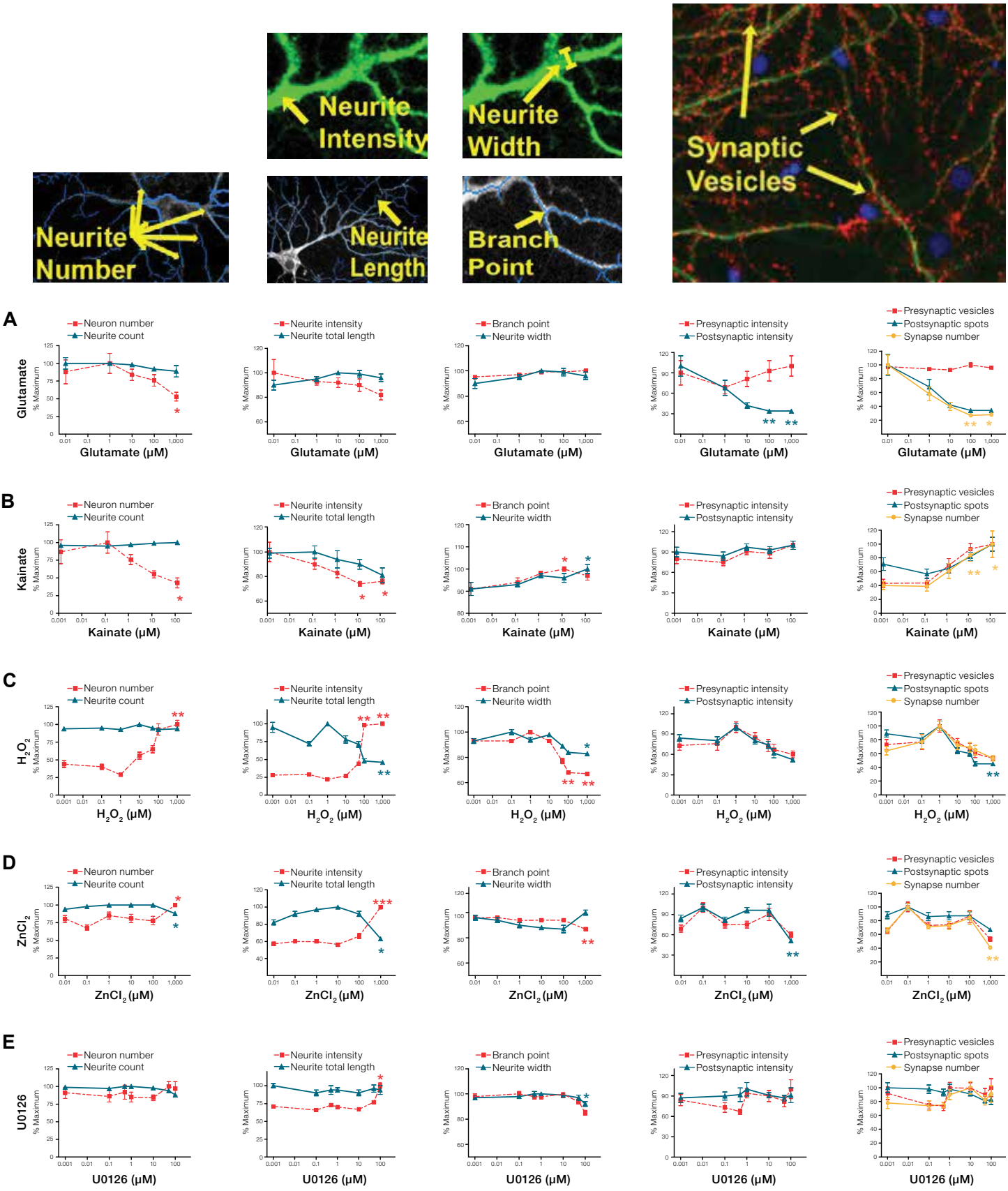


Figure 5. Neurite and synaptic changes in response to drug treatments. Mouse, rat cortical, or hippocampal primary neurons were cultured for 21 divisions, and the dose-dependent responses of compounds towards various properties of these neurons were investigated. **(A)** Glutamate with 10 mM glycine in HBSS was added to cells for 30 min, then the cells were washed and solution replaced with culture media. After a 24 hr incubation, neurons were fixed, stained, and analyzed. **(B)** Kainate, **(C)** H_2O_2, **(D)** $ZnCl_2$, and **(E)** U0126 were added to cells for 24 hr in culture media and then fixed, stained, and analyzed (Student's *t*-test, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

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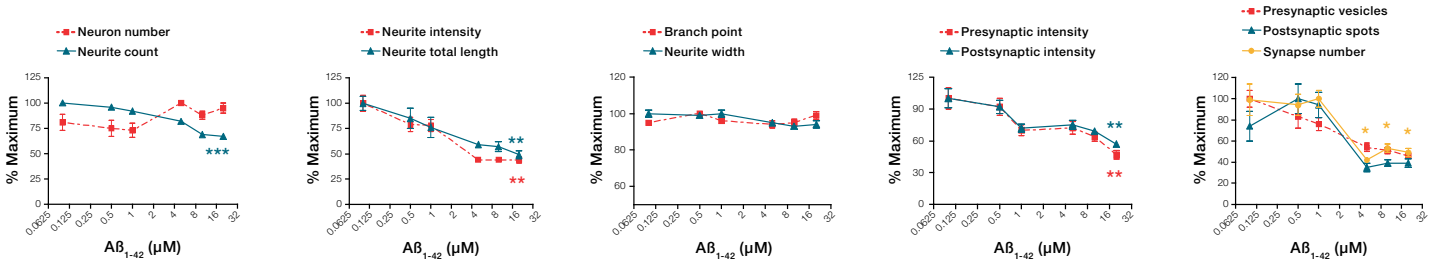

Figure 6. Neurite and synaptic changes in response to $A\beta_{1-42}$ aggregates. Rat hippocampal primary neurons were cultured for 50 divisions. 500 mM $A\beta_{1-42}$ was incubated at 37°C in media for 3 days to induce oligomerization. Neurons were incubated with the $A\beta_{1-42}$ oligomers for 48 hr, and then fixed, stained, and analyzed. $A\beta_{1-42}$ toxicity leads to synapse loss. (Student's *t*-test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

Conclusions

The multiparameter synaptogenesis assay simultaneously identifies and quantifies neurites, pre- and postsynaptic structures, and synapses in an automated manner.

- Neurotoxicity from neurotoxic substances is accurately detected
- Substances only affecting synapses can be detected
- Assay works for acute or chronic cell models of neurodegenerative disease

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