Video Article

A High Content Imaging Assay for Identification of Botulinum Neurotoxin Inhibitors

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Abstract

Synaptosomal-associated protein-25 (SNAP-25) is a component of the soluble NSF attachment protein receptor (SNARE) complex that is essential for synaptic neurotransmitter release. Botulinum neurotoxin serotype A (BoNT/A) is a zinc metalloprotease that blocks exocytosis of neurotransmitter by cleaving the SNAP-25 component of the SNARE complex. Currently there are no licensed medicines to treat BoNT/A poisoning after internalization of the toxin by motor neurons. The development of effective therapeutic measures to counter BoNT/A intoxication has been limited, due in part to the lack of robust high-throughput assays for screening small molecule libraries. Here we describe a high content imaging (HCI) assay with utility for identification of BoNT/A inhibitors. Initial optimization efforts focused on improving the reproducibility of inter-plate results across multiple, independent experiments. Automation of immunostaining, image acquisition, and image analysis were found to increase assay consistency and minimize variability while enabling the multiparameter evaluation of experimental compounds in a murine motor neuron system.

Video Link

The video component of this article can be found at http://www.jove.com/video/51915/

Introduction

The bacterium *Clostridium botulinum* produces Botulinum neurotoxin, one of the most potent biological toxins known to man[1]. There are 7 distinct BoNT serotypes (BoNT/A-G). BoNT/A-Gall induce paralysis at the neuromuscular junction due to SNARE complex proteolysis [2,3]. SNARE proteolysis prevents neurotransmitter vesicle-membrane fusion and therefore blocks neurotransmitter exocytosis[4]. The specific SNARE target depends upon the particular BoNT serotype involved in the intoxication process. BoNT/A and BoNT/E cleave SNAP-25 whereas BoNT/C cleaves both SNAP-25 and syntaxin[5]. The remaining serotypes cleave synaptobrevin (also called Vesicle-associated membrane protein (VAMP). BoNT/A was chosen for assay development as it is responsible for a high proportion of naturally occurring botulism and has the longest duration of action[6]. Development of small molecule therapeutics against BoNT/A is a major goal for our drug discovery program and has utilized traditional target-based methods to identify active site proteolytic inhibitors[7,8-10]. However, the creation of active site inhibitors with broad spectrum activity against multiple serotypes and post-exposure efficacy will likely be challenging.

We have therefore implemented an innovative, phenotypic drug discovery approach that uses BoNT SNAP-25 cleavage as a functional endpoint to identify small molecules that can block BoNT-mediated motor neuron intoxication. SNAP-25 is required for neurotransmitter release, as degradation of SNAP-25 is predictive of paralysis and lethality *in vivo*. For example, cell-based screening could lead to discovery of new modulators of cellular factors responsible for toxin inactivation or inhibition of toxin pathways inside targeted cells. An important factor in phenotypic assay development is the selection of physiologically relevant biological models. We and others have described mouse embryonic stem (ES) cell-derived motor neurons that recapitulate the immunologic character of primary motor neurons, including the expression of SNAP-25[11-13]. Importantly, these cellular systems are highly sensitive to BoNT/A intoxication and demonstrate dose-dependent cleavage

of SNAP-25 in response to increasing concentrations of toxin[11,12]. The differentiated motor neurons are also produced in quantities that are sufficient for high throughput plate based analysis and allowed the design of an array of cellular assays.

The phenotypic assay is an immunofluorescence method utilizing two distinct antibodies to quantify cleavage of endogenously expressed full length SNAP-25 during BoNT/A intoxication of mouse motor neuron culture. A carboxyl terminal BoNT/A cleavage-sensitive (BACS) antibody that recognizes only full length SNAP-25 allows the assessment of BoNT/A mediated proteolysis of SNAP-25 expression in mouse motor neurons[10]. A schematic diagram of the HCI assay is depicted in **Figure 1**.

Protocol

Plate 20,000 differentiated mouse ES cells (MES) / well in a 96 well Poly-D lysine coated plates and maintain in motor neuron terminal differentiation media for 5-7 days.

1. Compound Administration and Intoxication with BoNT/A

Perform all of the following work in a BSL2 enclosure to maintain compliance with CDC/NIH guidelines.

1. Prepare 10 mM stock solutions of each library compound in 100% DMSO in polypropylene 96 well plates. Use the 10 mM stock to prepare an intermediate dilution plate that is 10-fold greater than the desired screening concentration. Prepare 100 uM intermediate plate by dispensing 10 mM stock into 96-well plate and dilute it to 100 uM using culture media. Dispense 10 µl of the compounds on to 90 µl of media on the cells[11]. Test the compounds at 10 uM final concentration and ensure the final percentage of DMSO does not exceed 0.5%.
2. Perform all studies that utilize live cells and active toxin under biosafety level 2 conditions. Replace the old media in the 96-well plates with 80 µl of fresh terminal differentiation media using a 12 channel manual pipette. Carry out aspiration and dispense steps by rows to avoid exposure of the cells without media to the ambient air.
3. Pretreat cells with compounds prior to application of toxin by addition of 10 µl of 10x compounds from the source plate using a 12 channel pipette, followed by mixing by aspiration (twice). For each 96-well plate, treat two columns of 6 wells with 10x DMSO diluted in the media to serve as high signal and low signal controls. The column without BoNT treatment exhibits the highest level of the full length SNAP-25 (high signal control). Treat the other column with BoNT alone (without any compounds) as the low signal control. Use low control to observe the efficiency of BoNT cleavage of SNAP-25 and its detection with the BACS antibody (**Figure 2**).
4. Incubate cells with compounds for 30 min at 37 °C and 6% CO_2 in the cell culture incubator.
5. Prepare Botulinum neurotoxin A from a 1 mg/ml commercial source in Phosphate buffered saline (PBS) to a final 10x working stock by diluting with terminal differentiation media.
6. Initiate intoxication by adding 10 µl of 10x BoNT/A stock into wells designated for treatment and low signal controls using a multichannel pipette (**Figure 2**). Treat the wells designated as high control with media alone.
7. Decontaminate all plastic ware and other disposables that comes in contact with BoNT/A during the study by immersion in 0.825% hypochlorite solution in water for at least 20 min. Perform all procedures in biosafety cabinets and decontaminate working areas both before and after experiments with 5% detergent disinfectant cleaner such as MicroChem solution.
8. Label the plates clearly to denote toxin use and incubate plates treated with 1 nM/L BoNT/A for 4 hr at 37 °C and 6% CO_2 in a cell culture incubator. Display appropriate signage to inform colleagues that toxin is in use in the laboratory.
9. Stop BONT/A proteolytic cleavage by methanol fixation. Remove media containing toxin and compounds from each well with a multichannel pipette and discard in 10% bleach solution. Add ice cold methanol (100 µl) directly to each well.
10. Incubate plates for 15 min at room temperature (RT) to allow fixation to occur.
11. After fixation, safely handle the discarded reagents (considered neutralized) with biosafety level 1 protocols and discard accordingly.
12. Discard methanol and use 100 µl of PBS to wash the cells and rehydrate them prior to immunostaining. Perform two additional PBS washes.
13. After the final wash, leave PBS in the wells, seal plates with microplate adhesive film and store at 4 °C until analysis. Store plates at 4 °C for several days.

2. Immunostaining

The immunostaining procedure is a labor-intensive, multistep operation that includes repetitive reagent dispense/aspirate cycles and extensive plate washing which can lead to the potential introduction of significant intraplate and interplate variability. A semi-automated approach is applied to save the time of laboratory personnel, increase assay throughput, and minimize immunostaining variability.

1. Use an automated workstation equipped with a 96-well head capable of transferring volumes up to 250 µl and integrated with a robotic gripper arm to move labware into different locations on the modular deck (see Material and Equipment) for this protocol.
    1. The modular deck is designed to host different types of labware and can support up to 11 items at any one time. The automated microplate handler is equipped with a dual magazine microplate stacker in order to hold and manipulate up to 50 plates per cycle.
    2. The automated workstation is located inside of a Class II biosafety cabinet designed for laboratory automation equipment to provide sterility and safety. For automated immunostaining, the deck is arranged as shown in **Figure 3** to enable reagent access as needed and without human intervention.

2. Pre-load plates containing fixed cells into the plate magazine and transfer to the deck as needed for liquid handling operations. Use the 96 pipette head fitted with 250 µl tips to aspirate PBS from the wells down to 10 µl. Permeabilize cellular membranes to facilitate antibody binding to intracellular targets with permeabilization buffer (0.1% Triton-X 100). Dispense permeabilization buffer (100 µl) into each well before returning the plates to the second storage magazine of the microplate stacker for a 15 min incubation at room temperature (RT).
3. Remove permeabilization buffer and perform plate washing with PBS (twice) as previously described in step 1.13.
4. After the last wash step, remove the PBS buffer and dispense 100 µl of blocking buffer containing 1% horse serum and 0.1% Tween 20 in PBS into all microplates before returning them to the plate magazine for 1hr incubation at RT.

5. Use two primary antibodies for immunostaining: a rabbit polyclonal anti-mouse BACS antibody, for detection of full length SNAP-25 and a mouse monoclonal anti-III tubulin antibody for detection of neurons (see Material and Equipment). After 60 min of incubation, remove blocking buffer and dispense 50 µl of 4ug/ml of BACS antibody and 0.5 µg/ml of III-tubulin in blocking buffer into all plates.
6. Incubate for 1hr at RT remove the primary antibodies and wash 3 times with 100 µl of PBS containing 0.05% Tween (PBST).
7. Use an anti-mouse IgG labeled with Alexa Fluor 647 to visualize the -III tubulin and anti-rabbit IgG tagged with Alexa Fluor 568 to visualize the BACS antibody. Prepare both antibodies as a 2 µg/ml dilution in blocking buffer and dispense 50 µl of the mixture into each well.
   NOTE: The secondary antibodies selected for immunostaining are labeled with different fluorophores to allow detection of their emitted fluorescent light at distinct wavelengths using different channels within the detector of the High Content Imager.
8. Incubate for 1 hr at RT. Aspirate the secondary antibodies and wash 3 times with 100 µl of PBST.
9. After the final wash, add 100 µl of PBS containing Hoechst dye (32 µM) to stain the DNA for nuclei detection.
10. Seal the plates with a light impermeable film to protect the fluorophores from photobleaching. Plates can be stored at 4 °C for weeks without significant loss of signal.

3. Imaging

NOTE: Perform image acquisition using High Content Imaging System (See Materials and Equipment).

1. Specification of High Content Imager Definitions:
   1. Select Plate type, layout, number of fields, objective: Greiner u clear, 96 well format, 16, 20X water respectively.
   2. Select channels: nucleus excitation EX: 405 nm as channel 4; cytoplasm EX: 644 nm as channel 2; antigen specific antibody channel EX: 561 nm as channel 3 and Set up excitation power of each laser, Setup experiment as 2 exposures, Exposure 1 with one excitation at 644 nm, Exposure 2 with two excitations at 405 nm and 561 nm. Select binning as Bin2.
   3. Use high control wells for exposure optimization with maximum intensity as SNAP-25 channel and low control wells for minimal intensity.
   4. Adjust the exposure, so that the intensity of each channel is about half of the saturation level (2,000-2,500 relative units).
   5. Identify the optimal Z plane on cell mask channel using delta correction script. See **Figure 5** for results after immunostaining and imaging. Export data to the server where the image analysis software is residing.

4. Image Analysis (Figure 6)

NOTE: The following steps describe application of the Columbus software algorithms.

1. Select an appropriate algorithm to segment the primary objects (nuclei). If required, adjust background threshold and contrast parameters. The Columbus software provides 4 nuclei detection methods. Select the method that segment nuclei accurately by visually inspecting segmented nuclei (in F**igure 6a** method M is selected).
2. Select the neurite algorithm (CSIRO Neurite Analysis 2) to segment the neurites If required, adjust background threshold and contrast parameters to remove background. See **Figure 6b** for parameters used to detect neurites.
3. Identify the neurite regions (neurite segments) based on secondary objects (neurites) using a -3 pixel expansion of the β-III tubulin channel (Alexa Flour 640) as mask (**Figure 6c**).
4. Calculate intensity of the signal channels (SNAP-25, (Alexa Flour 568) for the neurites region and β-III tubulin channel (**Figure 6d** & **6e**).
5. Select desired parameters for export, such as neurite length, mean intensities of SNAP-25, β-III tubulin, nuclei number, neurite segment number, *etc.* (**Figure 6f**).
6. Transfer plates from plate stackers using the robot and load into the High Content Imager for image acquisition.

5. Data Analysis:

To assess the robustness of the designed assay, calculate the following parameters from the plate-based experiment.

1. Signal (S) to background (B):S/B = mean intensity of high signal control) / mean intensity of low signal control
2. Coefficient of Variation (CV) of signal and background (to measure the uniformity of the specific signal): CV = (Standard Deviation (SD) / mean of sample)*100(%), to determine the variability in liquid handling, reagents, etc.
3. Signal to Noise: S = (mean intensity of signal – mean intensity of low signal control) / SD of low control
   1. Calculate the Z' factor with intoxication of one half of a 96-well plate and a mock intoxication of the other half. Z' = 1 - 3 * (SD of high signal control + SD of low signal control) / (mean of high signal control – mean of low signal control). For the further analysis of the screening data, normalize some of the primary raw parameters on a plate basis to allow comparison between plates and between different days of experiments.
4. Percent of cleavage, reflecting the reduction of the signal associated with full length SNAP-25 detected by specific antibody (BACS) : % Cleavage = 100% * (mean intensity of high signal control - intensity of sample) / mean intensity of high signal control.
5. Percent of Inhibition of cleavage: % Inhibition = 100% * (intensity of sample – mean intensity of low signal control) / (mean intensity of high signal control - mean intensity of low signal control).

Representative Results

Data from high and low controls created two distinct populations with the difference of two medians exceeding 3 standard deviations **(Figure 7A**). The goal of the screening process is to find the compounds within the sample population with values closer to positive control population,

assuming a normal distribution within the sample population (**Figure 7B, (i)**). Data points that are 3 standard deviations beyond the mean are considered statistically different from the noise and classified as an active "hit" (**Figure 7B(ii)**, red boxes). The compounds that are classified as "hits" will be subjected to further confirmation testing in future studies. **Figure 7B (ii)** shows the detection of positive hits in the population of the samples from a representative subset of the screening plates. All 4 points that had values lower than the (median samples - 3*SD of the samples) belonged to the same inhibitor that was spotted in different locations within 4 different screening plates. This inhibitor demonstrated robust SNAP-25 protection against BoNT/A, as shown in **Figure 5**.

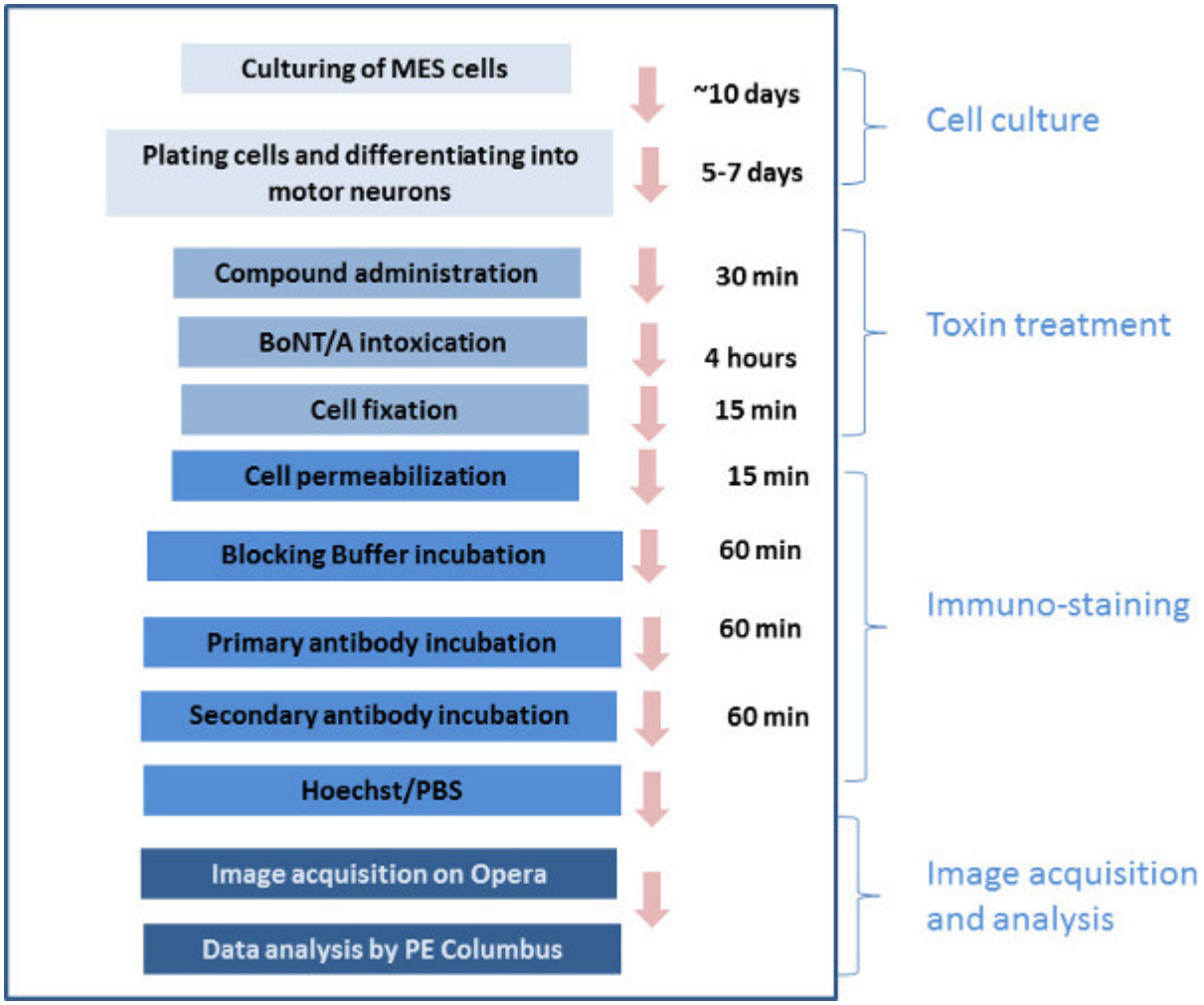
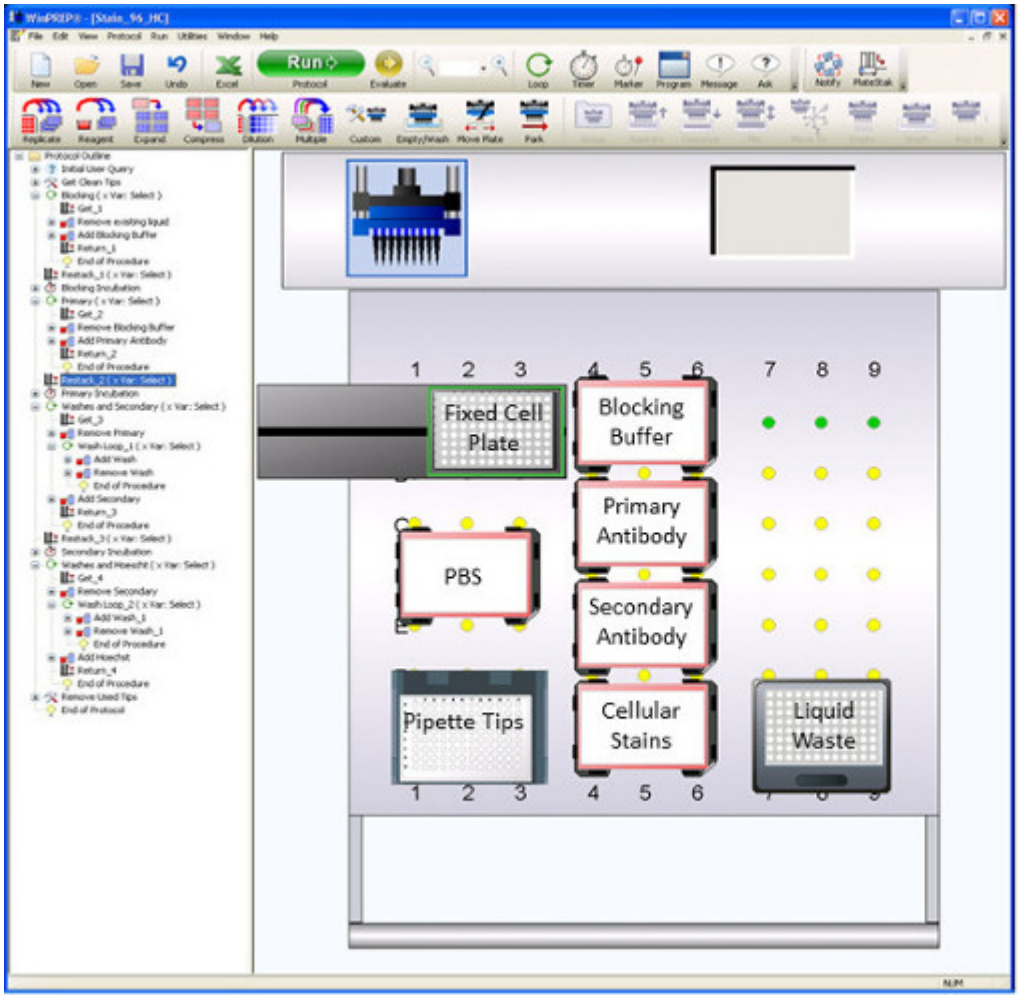


Figure 1: Workflow for compound screening in the BoNT/A HCI assay.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		High control	SAMPLE	SAMPLE	SAMPLE	SAMPLE	SAMPLE	SAMPLE	SAMPLE	SAMPLE	Low Control	
C		High control	SAMPLE	SAMPLE	SAMPLE	SAMPLE	SAMPLE	SAMPLE	SAMPLE	SAMPLE	Low Control	
D		High control	SAMPLE	SAMPLE	SAMPLE	SAMPLE	SAMPLE	SAMPLE	SAMPLE	SAMPLE	Low Control	
E		High control	SAMPLE	SAMPLE	SAMPLE	SAMPLE	SAMPLE	SAMPLE	SAMPLE	SAMPLE	Low Control	
F		High control	SAMPLE	SAMPLE	SAMPLE	SAMPLE	SAMPLE	SAMPLE	SAMPLE	SAMPLE	Low Control	
G		High control	SAMPLE	SAMPLE	SAMPLE	SAMPLE	SAMPLE	SAMPLE	SAMPLE	SAMPLE	Low Control	
H												

Figure 2: The 96-well plate map for the HCI assay. High control wells (pink) were treated with 0.5% DMSO as control only and low control wells (blue) were treated with 1 nM BoNT/A and 0.5% DMSO. Sample wells (green) were treated with 1nM toxin and 10 µM of compounds in 0.5% DMSO. The outer columns and rows (grey) were not used due to incompatibility with the optimized plate format and the inability of the 20X water-Immersion objective to image edge wells.



Liquid Waste: Vacuum manifold connected to a bulk liquid waste recovery tank

Figure 3: The design of the automated work station deck for the immunostaining assay. Blocking buffer, primary antibody, secondary antibody and cellular stains were dispensed in 96-well polypropylene plates to reduce dead volume reagent loss. PBS was dispensed in a tray capable of holding 300 ml. The manifold used for liquid waste aspiration is shown on the inset image.

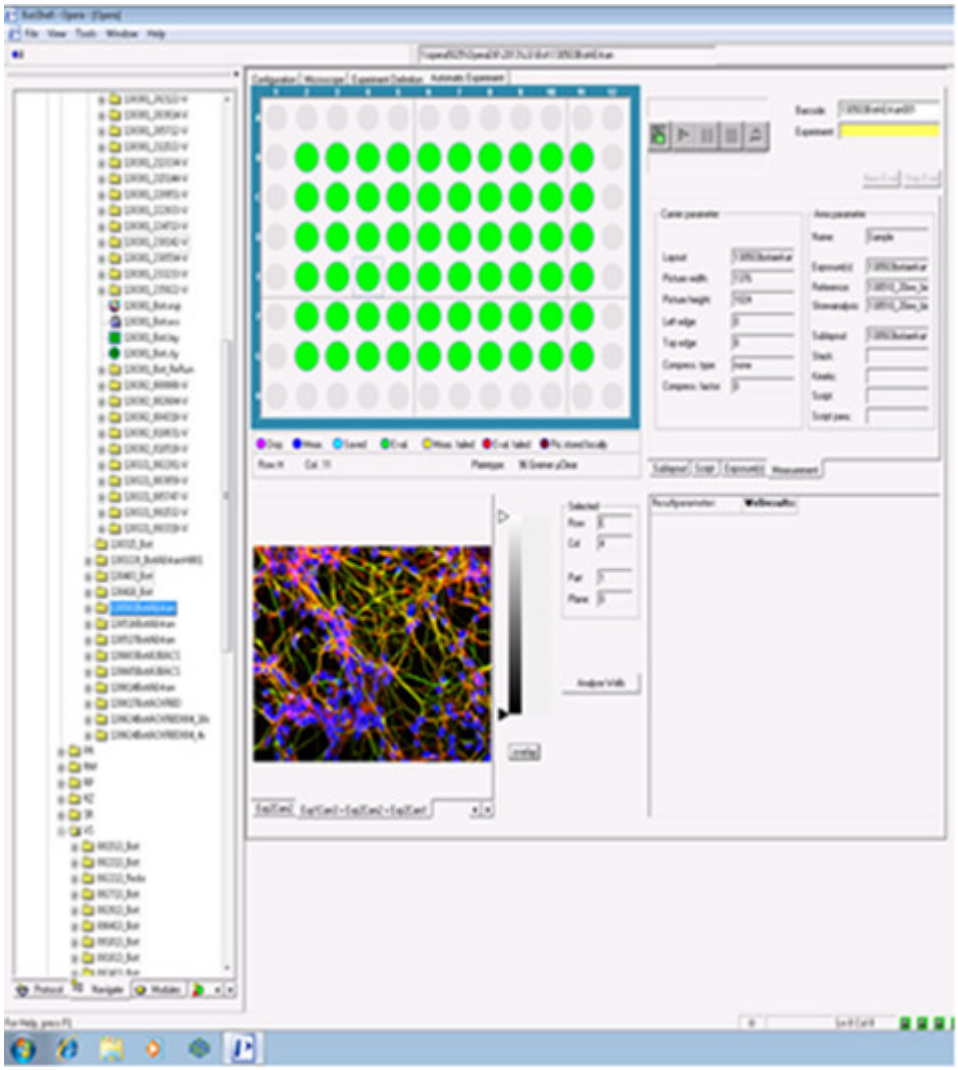


Figure 4: Screen shot of the High Content Imager experimental set up.

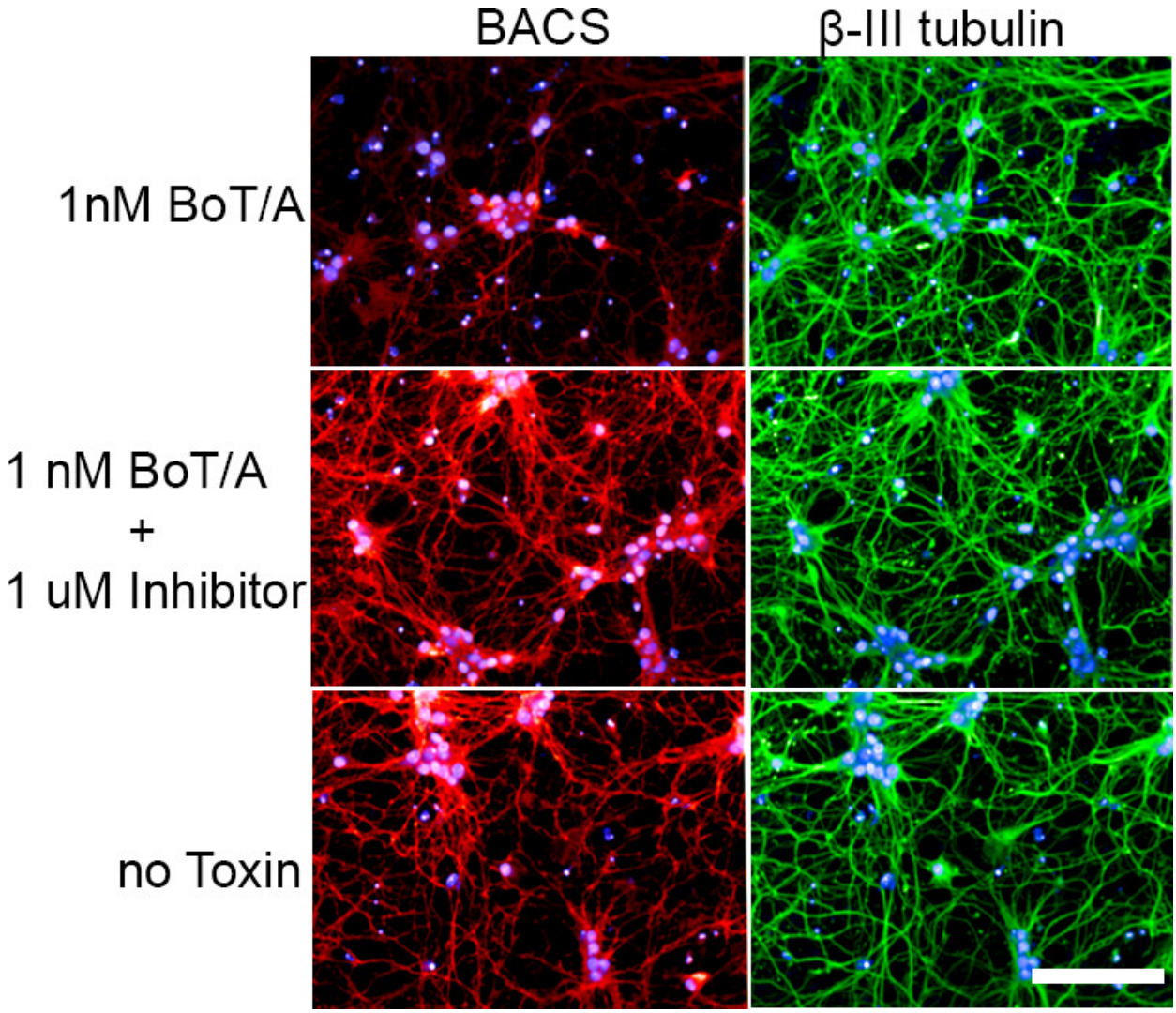


Figure 5: High content imaging of SNAP-25 cleavage in mouse ES-derived motor neurons. Cells were treated with 1 nM BoNT/A with and without addition of 1 μM of inhibitor (Toosendanin)[14] or left untreated without toxin. SNAP-25 was detected with the BACS antibody. In another channel, neurons were detected using a β-III-tubulin antibody to create mask of neurites for SNAP-25 image analysis. This is a single representative image from one of the 16 images taken from a given well. Blue indicates nuclei stained with Hoechst 33342, red is the BACS signal and green is the β-III-tubulin signal. Images were obtained with the Opera as described. Size bar: 10 μm

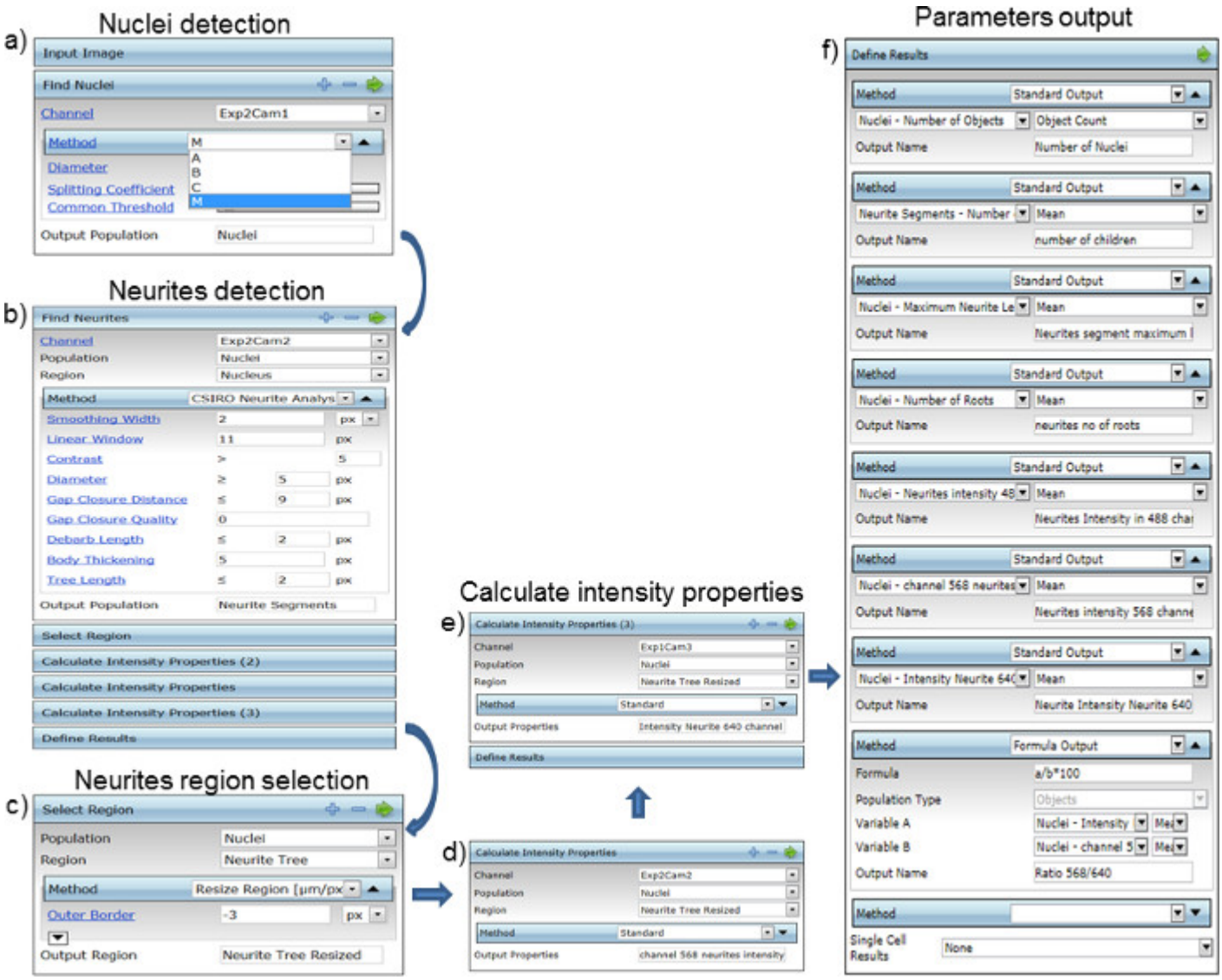


Figure 6: Screen shots showing various steps in image analysis pipeline.

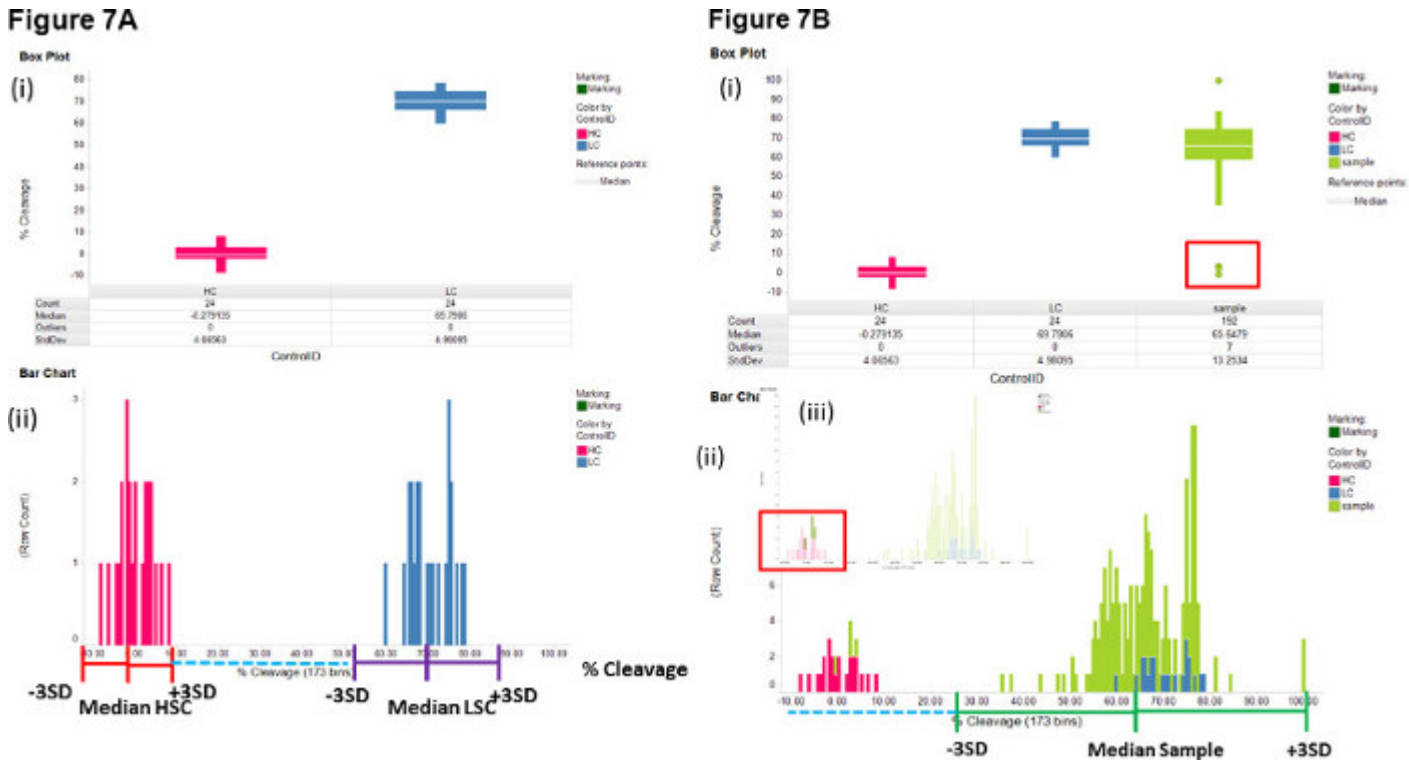


Figure 7: Statistical visualization of the data arising from the HCI screen. (A). (i) Box plot analysis of the % Cleavage calculated from control wells; shown as median values with their respective SD (n = 16). Pink represents the high signal control (HSC); blue represents the low signal control (LSC). Z' = 0.97 (ii) Histogram distribution of the same values as in (i) to demonstrate the distance between two control populations. Median and 3 SD were calculated from the statistical values associated with the corresponding box-plot. **(B)**. Distribution of the 192 compound treated samples from the same experiment. (i) Box-plot demonstrating the statistical values for the % cleavage for samples in comparison to controls as well as statistically significant outliers. (ii) and (iii), Histogram distribution of the same values in population of all data from the screen (green color). Hits (highlighted in red square), were selected from the outliers that have % cleavage values less than 3SD from the median of the sample population (<25.89 % cleavage). The four hits highlighted during selection (iii) on the histogram of all data points have similar values to the high signal control and represented the same compound, spiked into the plate for the tests, a known BoNT/A inhibitor (Toosendanin) that blocked SNAP-25 cleavage.

Discussion

The high potency of Botulinum neurotoxins and the relative ease of their weaponization has resulted in their classification as Category A (highest priority) biothreat agents by the U.S. Centers for Disease Control and Prevention. Unfortunately, there are no FDA approved therapeutics to counter BoNT intoxication after the toxin has been internalized by the motor neurons. Any druggable mechanism that promotes neuronal recovery from BoNT intoxication could lead toward the development of a potential therapy to protect both the armed services and public against this biological threat. In this article, we present a detailed HCI assay protocol for screening inhibitors of lethal toxins such as BoNT/A. An unusual aspect of this assay is scrupulous handing of toxins and implementation of strict biosafety protocols to ensure laboratory safety. Extreme care must be exercised while active toxin is in use. BoNT/A inactivation via methanol fixation and microplate decontamination with 10%bleach and 5% surface wipes are key steps that allow microplates to be removed from biosafety cabinets for downstream evaluation in biosafety level 2 laboratory environments.

Due to the ever expanding size of compound libraries coupled with slow motor neuron expansion (cell number), HCI assays for neurotoxin antagonists tend to run over several weeks. This requires that the variability between the plates and across time must be eliminated or minimized. In this protocol we provide automation methods for immunostaining, image acquisition and analysis to reduce the assay variability. Further reliability and consistency can be achieved by the automation of routine tasks associated with this protocol including cell plating, washing, and fixation. Our group is currently evaluating the impact of these improvements with the intent of incorporating them into our protocol in the near future.

In this report, we describe a SNAP-25 cleavage assay using high content imaging to measure relative fluorescence of intact SNAP-25 in motor neurons. This assay uses the BACS and β-III tubulin antibodies to detect full-length SNAP-25 and β-III tubulin respectively[11]. β-III tubulin is used as a marker to specifically identify neurons (**Figure 4**). While BoNT/A cleaves SNAP-25 and reduces the SNAP-25 signal, small molecules that inhibit any part of this process improve SNAP-25 signal in the imaging assay. As this assay depends on the fluorescence signal generated from the SNAP-25 distributed across many tiny neurites, high quality images are absolutely necessary. Hence, automated microscopes that produce high resolution confocal images are recommended (**Figure 4**). We routinely capture 6-16 fields/well in a 96-well format while using the 20X water-immersion objective. The number of field's imaged are dependent upon the overall number of neurites required to generate statistically significant results.

Modular image analysis algorithms were utilized to extract multiparameter data (**Figure 6**). The Columbus modular image analysis algorithms are relatively easy to generate for simpler analysis scenarios. For more complicated analysis or design of specific parameters for complex readouts, Acapella based scripts can be used within the Opera environment as well as inside the context of Columbus. In addition to the fluorescence intensities obtained from the SNAP-25 and β-III tubulin channels, we routinely collect other parameters such as total cell number (indirect measure for cytotoxicity), neurite length, neurite branching, and other endpoints to quantify neuronal health during the assay. The raw endpoint data are exported to statistical analysis software for further data analysis and hit selection (**Figure 7**). The data presented here demonstrates the ability of the HCI assay to be efficiently utilized for phenotypic screening in search for BoNT/A inhibitors using a physiologically relevant motor neuron model.

Disclosures

Krishna P Kota is an employee of Perkin Elmer Inc. Waltham, MA, that produces instruments and software used in this manuscript. The content of this publication does not necessarily reflect the views or policies of the U.S. Department of Health and Human Services, the U.S. Department of Defense, the U.S. Department of the Army, or the institutions and companies affiliated with the authors.

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