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Determination of a Brevetoxin Live-Cell Fluorescent Imaging Assay Using the *SOFI* Fluorescent Imaging System

Introduction

Brevetoxins are a family of toxins produced by the single-cell marine organism Karenia brevis. Brevetoxins produced during "red tide" blooms contaminate shellfish and finfish, resulting in neurotoxic shellfish poisoning when consumed by people. Brevetoxin binds specifically to voltage sensitive sodium channels on neuronal cells, leading to disruption of their activity. The physiological effects include gastrointestinal, neurological, and, in the most severe cases, cardiac symptoms.

Traditional methods to detect brevetoxin contamination requires injection of the sample into live mice (known as the mouse bioassay), which are then monitored over time. This method is insensitive, expensive and slow to run. Here, we reproduce an assay descripted in "A New Cytotoxicity Assay for Brevetoxins Using Fluorescence Microscopy" in which cultured cells are used in place of live mice[1].

The assay was adapted to be analyzed on the SOFI fluorescent imaging system, a new high-content imaging platform developed by Charted Scientific for high-throughput imaging-based screening assays.

Assay Method

A 96-well microtiter plate was seeded with 5,000 SCJRH30 cells per well. After plating, the cells were treated with brevetoxin in a concentration range from 0 nM to 10,000 nM and incubated at 37˚ C for 48 hours. The cells were then stained and then imaged on SOFI. In this assay, the total cell number and number of dead cells were used as the metrics for cellular toxicity.

In the study, three different combinations of fluorescent stains were used to identify cells and to differentiate live cells from dead cells. CellBright Orange, SYTO Orange, and CellBright Red were used to stain all cells in the samples. Dead cells were identified with either DRAQ7 or propidium iodide.

	Dye Combination 1	**Dye Combination 2**	**Dye Combination 3**
Total Cell Count	CellBright Orange	SYTO Orange 82	CellBright Red
Dead Cell Count	DRAQ7	DRAQ7	Propidium Iodide

The SYTO Orange dye labels nuclei of both live and dead cells in each sample, permitting a direct count. The CellBright reagents label both cell membranes and cytoplasm. As in the study, cells grew to near confluence, segmentation of individual cells was not possible based on the membrane labels. For these dyes, total area was used as a proxy for cell count rather than an actual count of individual cells.

1. Mar. Drugs 2014, 12, 4868-4882; "A New Cytotoxicity Assay for Brevetoxins Using Fluorescence Microscopy".

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As the SYTO dye labels RNA in the cell as well as the nuclear DNA, total area occupied was able to be quantified with that dye as well as counting nuclei (see Figure 1).

The cells were imaged prior to staining in order to evaluate any effects related to the toxicity of the dyes themselves on the cells. Without labels, no quantitative measures could be made, however, a marked change could be seen in the cell morphology as the level of brevetoxin increased from 5,000 nM to 10 µM (see Figure 2.).

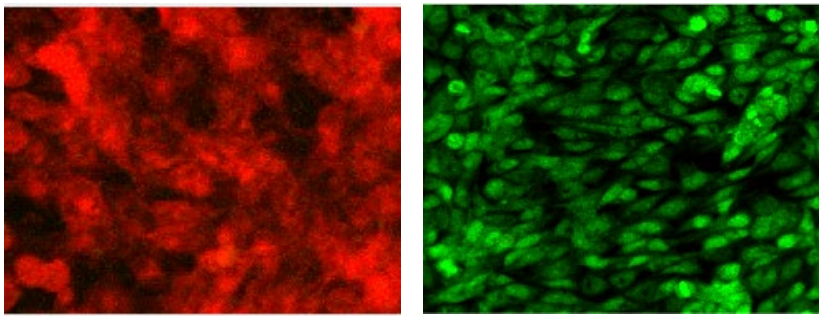


Figure 1. Cell membranes labeled with CellBright Red (left) and cell nuclei labeled with SYTO Orange (right).

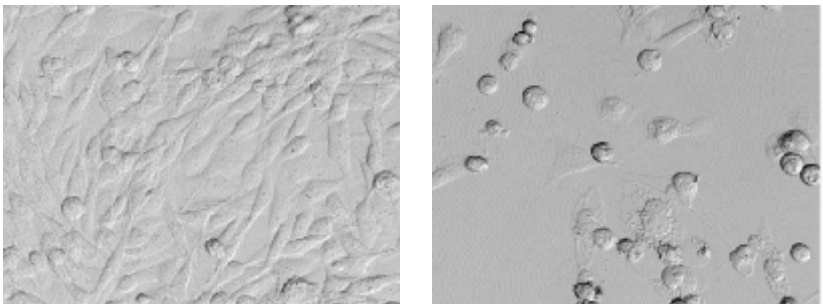


Figure 2. Transmitted light images of SCJRH30 cell: control, with no brevetoxin (left) and cells treated for 48 hours with 10,000 nM brevetoxin concentration (right).

The cells were imaged using the SOFI imaging platform from Charted Scientific. SOFI provides excitation at 532nm and 638nm and detects fluorescence at three different wavelengths as shown in the table below:

Fluorescent Label	Excitation	Emission
SYTO Orange	532nm	565nm – 588nm
Propidium Iodide	532nm	565nm – 588nm
DRAQ7	638nm	700nm – 730nm
CellBright Orange	532nm	565nm – 588nm
CellBright Red	638nm	660nm – 680nm

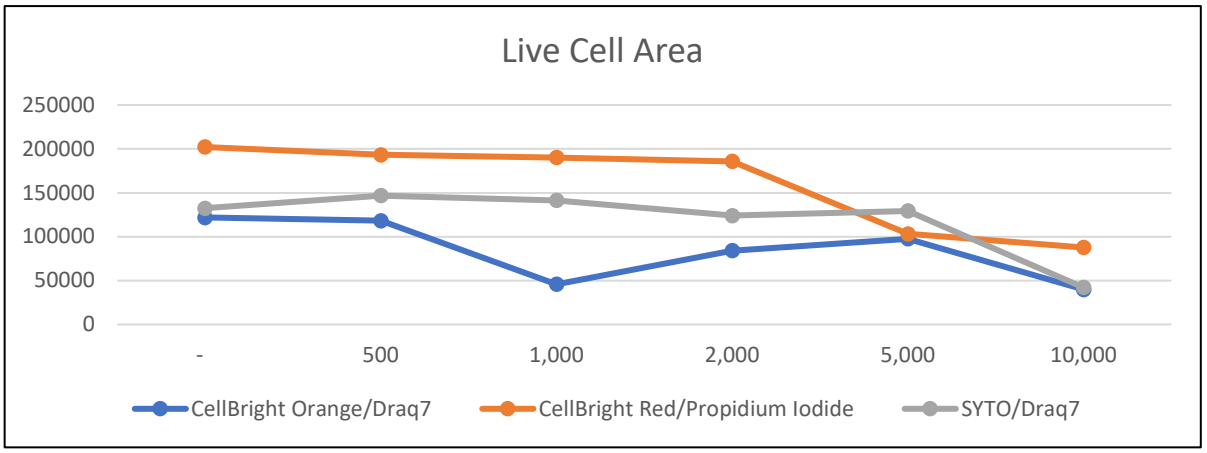
Brightfield images are produced by measuring transmitted light at either of the excitation wavelengths. With a 20X objective, SOFI delivers spatial resolution greater than 1 micron.

The images produced from SOFI were analyzed using CellProfiler 3.1.9. Cell counts were obtained using the nuclear stains identified as primary objects. Total cell area was obtained by applying an adaptive threshold to the fluorescence image.

Results

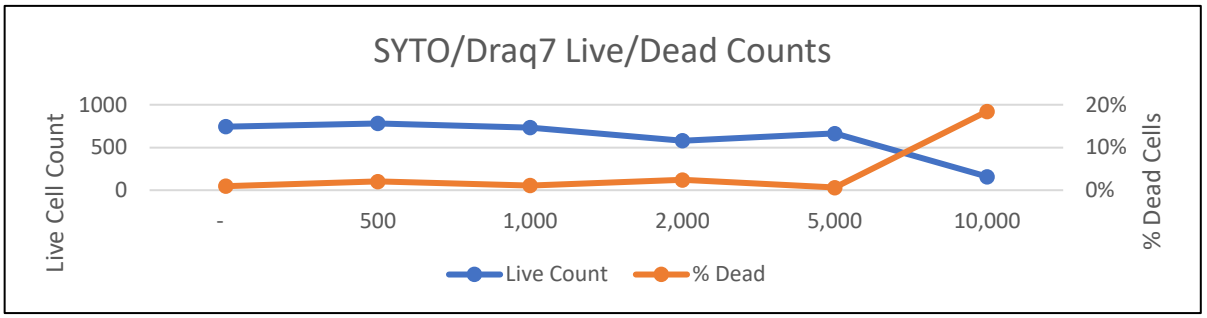
Using the total area metric, each of the assay methods determined that there is a sharp decrease in viable cells associated with brevetoxin concentrations between 5,000 nM and 10,000 nM.

The dead cell count does not provide useful information by itself. The brevetoxin inhibits cell growth rather than having a cytoxic effect on cells. A total cell count, or a total count of viable cells, provides a better indication of toxin concentration effect than the dead cell count.



The large drop in live cell area at the 1uM concentration seen in the CellBright Orange/DRAQ7 samples was caused by an effect independent of the brevetoxin treatment. The cells in that well had detached from the well plate and become rounded before the treatment, indicating possible contamination of the sample.

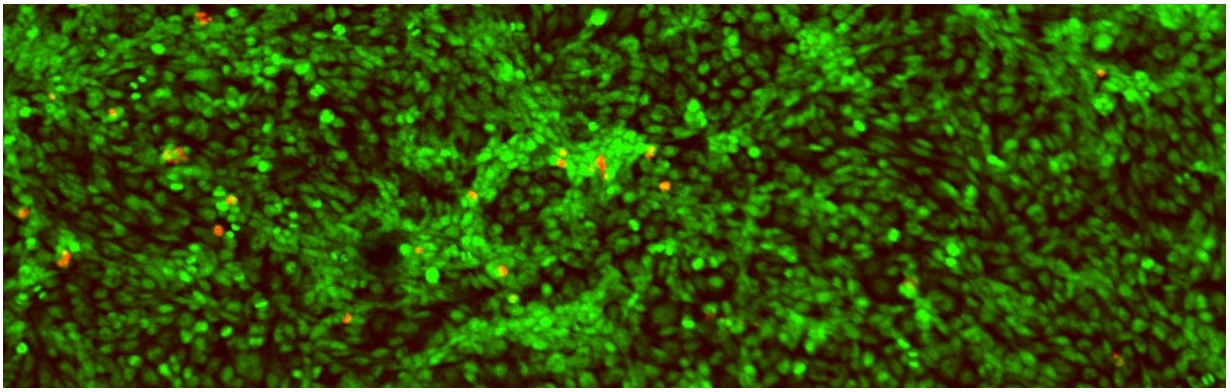
The count of live vs dead cells using the SYTO Orange and DRAQ7 dye combination produced the same result as the total area measure. As with the other two dye combinations, the dead cell count by itself does not provide useful information.



Discussion

All three of the labeling strategies serve a useful function in measuring the number of of viable cells. For highly confluent cells such as the SCJRH30 cells used in this experiment, the SYTO Orange/DRAQ7 combination is particularly effective because it provides an accurate means of segmenting and counting both live and dead cells. The ability to measure live cell count is a novel consideration in the mechanism of brevetoxin activity given the low level or consistent levels of apoptosis/cell death between all concentrations.

SOFI's ability to produce transmitted light/brightfield images is particularly useful as a built-in control. An initial measure of cell count or confluence can be used to confirm findings and to flag samples that had problems before the treatment and stains are applied.



Acknowledgments

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BioStatus provided samples of DRAQ7.

Attogene Corporation prepared, treated and stained all the samples used in this experiment at Attogene's laboratory in Austin, Texas. Attogene supplied cultured cells as well as the brevetoxin used for treatment.