

High Throughput Cell Based Assays for Toxicity Assessment using SpectraMax® Paradigm Plate Reader

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Abstract

Development of predictive in vitro assays suitable for safety and efficacy testing is of high interest for improving the drug development process and reducing drug attrition. Recently, there has been a great interest in using stem cells as tools for screening compounds during early drug development. Cardiomyocytes, neuronal progenitors, hepatocytes, and hematopoietic stem cells have all been shown to be attractive cell models for performing a variety of assays. Typically, such assays are performed using high content imaging and analysis (HCA) that allows individual assessment of cell response and multi-parametric outputs. However, there is a need for higher throughput methods with similar sensitivity. Here we present results obtained from the SpectraMax® Paradigm® Plate Reader for performing toxicity assessment of various compounds using stem cell derived cell models in a high throughput manner. The system is configured for cell-based assays with a highly sensitive direct bottom fluorescence read. Plates can be scanned using "Well Scan" or "On-The-Fly" read types. Several viability markers were evaluated including Calcein AM, actin, and mitochondrial stain. We have characterized a number of known hepatotoxic compounds and IC50 values were determined. Excellent agreement was found between plate reader and HCA results.

Introduction

There has been a trend in the past decade from biochemical assays toward cell based assays. The former have been the traditional domain of plate readers and great strides have been made in the sensitivity, throughput, and ease-of-use of such assays. However, cell-based assays are in general more biologically relevant and can provide multiple readout parameters per well. Most researchers rely on automated imaging systems for their cell based assays. Such systems can acquire multiple images of different fluorescently tagged proteins or cellular components (see Figure 1). The combination of multiple images with multi-parametric analysis has been coined as high content analysis (HCA). The down side to HCA is it requires complex equipment and is very time consuming. The read time per plate can be 15 minutes to several hours, plus analysis can also take similar amounts of time, if not longer.

An attractive alternative is the SpectraMax® Paradigm® Plate Reader. This system is configured with direct optical coupling for reading fluorescence from the bottom of multiwell plates. The system can be setup with a variety of cartridges optimized for the most popular fluorophores. This includes the TUNE® Cartridge for tunable filter-based fluorescence reading at any visible wavelength. The sensitivity of the system for live cell dyes such as Calcein AM has been found to be comparable to imaging systems, while the plate read times (<1 min) are significantly faster. Here we present a comparison of assay performance between SpectraMax Paradigm reader read types, and to the ImageXpress Micro imaging system. The performance is based on a multiplexed hepatotoxicity assay and comparisons are based on assay windows, Z-prime factors, IC50 values, and read times.

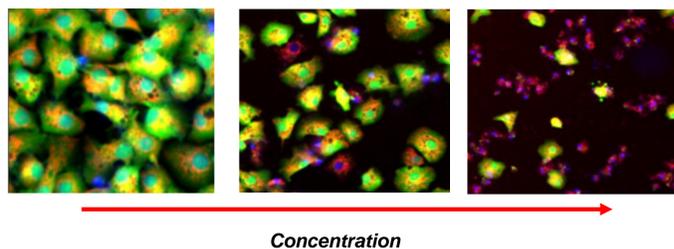


Figure 1. Images of hepatocytes incubated with increasing concentrations of staurosporine. Cells were stained with Calcein AM (Green), MitoTracker Orange (Orange), and Hoechst (Blue). Images were acquired on ImageXpress® Micro XL System.

Materials & Methods

Cell Treatment

- Aliquots of human iPSC-derived iCell® Hepatocytes from Cellular Dynamics International were thawed and plated at 10K cells/well into black-walled poly-D-lysine/laminin coated 384 well microplates according to the provided protocol.
- Cells were cultured for 3-21 days and treated with compounds for the duration of the specific experiment.
- Live cells were imaged under environmental (CO₂, humidity, and temperature) control
- Cells were stained with combinations of either:
 - Calcein AM
 - Mitotracker Orange
 - Hoechst (for imaging only)

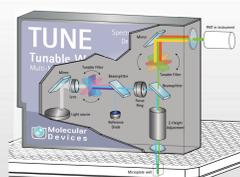
Plate Reader Acquisition

- SpectraMax Paradigm Plate Reader was operated using SoftMax® Pro Software ver. 6
- Three cartridges were used:
 - TUNE Cartridge tunable filter source
 - FLRH – Dual FITC and PE emission (485 excitation)
- Three read types were used
 - Standard single point fluorescence (Stop-and-Go) (2 min/384 well plate)
 - Multiple point On-the-Fly Performance (OTF) (1min/384 well plate)
 - Multiple point Well Scan (WellScan) (15 min/384 well plate)
- Data was analyzed in SMP or exported to Excel (Microsoft)

High Content Image Acquisition & Analysis

- Images were acquired with the ImageXpress® Micro XL System.
- Data was analyzed with MetaXpress® Software

High Throughput SpectraMax® Paradigm® Fluorescence Plate Reader



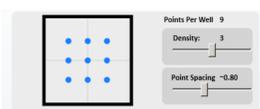
Industry's Only Tunable Filter-based Plate Reader

- User configurable cartridges
- Sub-picometer sensitivity
- Designed for Automated Screening

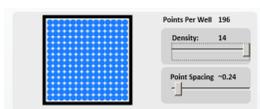
Read Type Optimization

There are multiple read types available for plate readers. The most common read type is a single point per well measurement which can be done either in a "stop-and-go" mode or "on-the-fly". SnG is typically used when the read time is long compared to plate movement (i.e., time to transverse a well) or when multiple reads are to be averaged in each well. However, it has a drawback of requiring longer plate read times. OTF is used when fast plate read times are desired and the read time per well is short compared to plate movement. The majority of assays developed for plate readers have a uniformly distributed signal. However, in the case of cell based assays the signal is not always uniform. In this case, WellScan has an advantage in that multiple read points are taken in each well and averaged together. While this overcomes the issue of non-uniformity, it can lead to much longer plate read times since it operates using SnG. The Paradigm has a unique read type which combines the multiple sampling of WellScan and the speed of OTF called Performance On-The-Fly. Users get improved precision for cell-based assays with fast read times desired for high throughput screening. A comparison of performance between WellScan and Performance OTF for a hepatotoxicity assay is shown in Figure 3

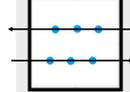
SpectraMax Paradigm Read Types



WellScan with a 3x3 grid for full well coverage in 15 minute read time for 384 well plate.

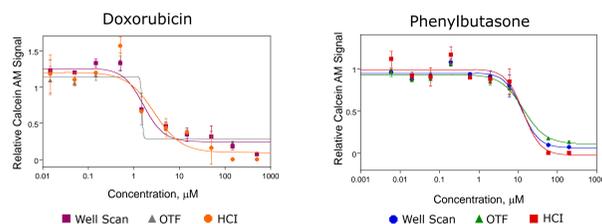


WellScan with 0.25 um step size to maximize the coverage for each well (196 points).



Representation of On-the-fly Performance mode where plate movement is continuous along arrows and read points are synchronized with well position.

Figure 2. Schematic representations of read types available with the SpectraMax Paradigm Plate Reader.

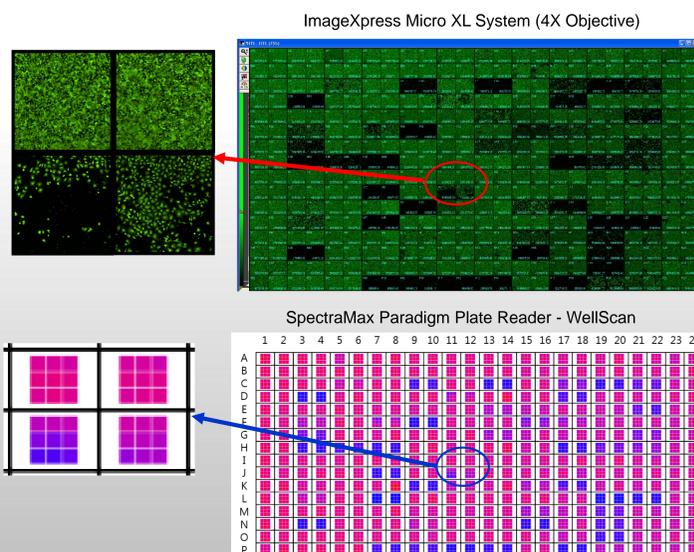


	3x3 Well Scan		Performance OTF		High Content Imaging	
	Window	Z-Prime	Window	Z-Prime	Window	Z-Prime
Phenylbutasone	11.3	0.76	6.0	0.70	1330	0.67
Doxorubicin	9.4	0.65	6.5	0.61	5841	0.89

Figure 3. Comparison of hepatotoxicity concentration response curves and assay performance for two read types and high content imaging (HCI). Assays were performed in 384 well plates. Imaging results were acquired using ImageXpress Micro XL system with a single site per well. Window and Z-Prime values were calculated using data from the three lowest and two highest concentrations.

Plate Reader versus Imaging

We have demonstrated a multiplexed assay model for assessing general and specific hepatotoxicity that is well-suited for automated screening environments. This assay monitors changes in cell viability (Calcein AM), nuclear shape (Hoechst), and mitochondrial potential (MitoTracker Orange) associated with different types of toxicity. It is also possible to use a fluorescent actin stain to look at cytoskeletal integrity as an indicator of cell health. With HCA, well images are analyzed on a cell-by-cell basis using multi-wavelength cell scoring. This provides outputs on total live cells, mitochondrial integrity of cells, number of apoptotic cells, and other parameters. The SpectraMax® Paradigm plate reader was also used to determine toxicity response and a comparison between these methods is presented. A composite image from the ImageXpress Micro System and a heat map from the SpectraMax Paradigm Plate Reader for Calcein AM from a plate used in a hepatotoxicity assay is shown in Figure 4. Good correlation is found between the heat map intensity and the cell distribution. Assay performance was also compared between HCA and Plate Reader for a hepatotoxicity concentration response and those results are shown in Figure 3. Imaging can provide a much larger assay window primarily due to lower background, but Z-prime and IC₅₀ values were found to be very comparable.



Multiplexed Toxicity Response

We demonstrate several assays assessing general and specific hepatotoxicity that are well-suited for high throughput screening environments. First, Calcein AM is used to monitor changes in cell viability using both WellScan and Performance OTF modes. Second, cytoskeletal toxicity is monitored by measurement of actin signal using F-Actin staining. Third, mitochondrial integrity is measured using MitoTracker Orange. Data was acquired for the latter two assays in WellScan mode only. A number of known hepatotoxic compounds were evaluated and IC50 values determined. Excellent agreement was found between the various assays and methods for IC50 values. Calcein AM provided the highest assay windows and statistical separation between positive and negative controls.

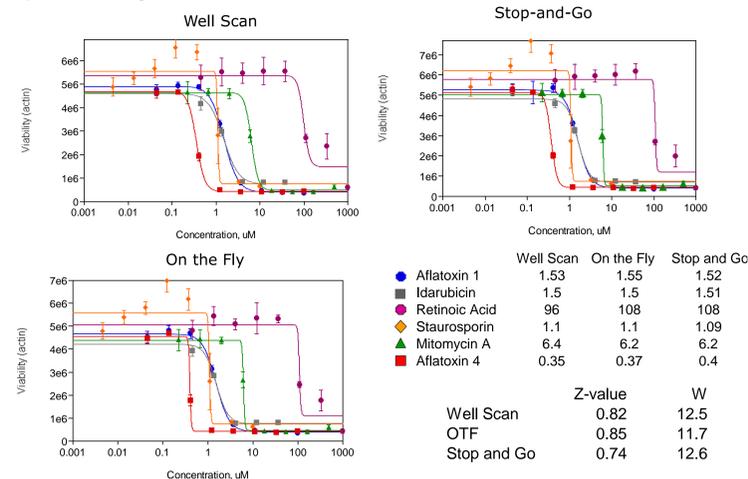


Figure 5. Comparison of hepatotoxicity results between different read types and assay outputs. Top: concentration response curves for F-actin intensity using three read types on the SpectraMax Paradigm Plate Reader. Bottom: Table of IC50 results for three assay outputs. All assays were performed in 384 well plates.

High Throughput Hepatotoxicity Screening

Hepatotoxicity is one of the main safety concerns in drug development. Therefore, highly predictive in vitro assays suitable for safety and efficacy testing are extremely important for improving the drug development process and reducing drug attrition. We have tested predictivity of the assays using Screen-Well™ Hepatotoxicity library (ENZO Life Sciences) that consists of more than 200 compounds representing different mechanisms of toxicity and demonstrated that method has significant value for compound screening and early safety evaluation in drug development process. Parallel plates were run using ATP luminescence (CellTiter-Glo, Promega) and live cell fluorescence (Calcein AM, Invitrogen). Correlations between data sets were found to be very high with R2 values typically >0.9. The fluorescence based assay provides the fastest read time and is also compatible with further analysis by imaging. This could provide a significant advantage where "hits" identified by a fast plate reader could then be subjected to HCA to gain more detailed understanding of phenotypic changes and mechanisms of action.

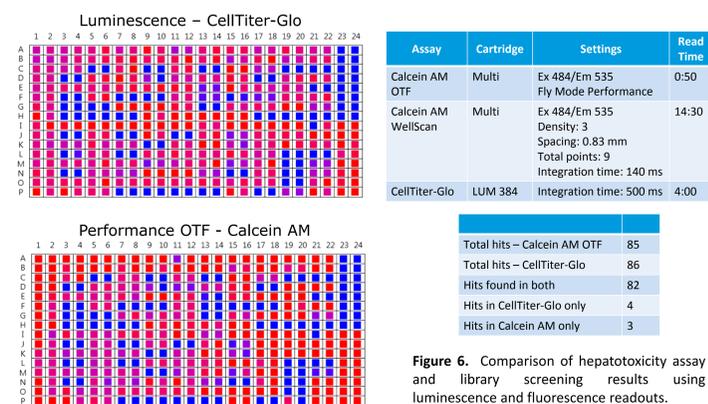


Figure 6. Comparison of hepatotoxicity assay and library screening results using luminescence and fluorescence readouts.

Summary

- The SpectraMax Paradigm plate reader has multiple read types that are well suited for bottom read cell based fluorescence assays. WellScan provides detailed information on cell distribution while Performance OTF allows plates to be read in <1 min with similar output
- We demonstrated performance of this system with cell-based assay models using iPSC derived hepatocytes for assessing hepatotoxicity with multiple fluorescence readouts
- Cell toxicity assays can improve drug safety by defining toxic effects early in drug development. The SpectraMax Paradigm plate reader can accelerate those assays and make them suitable for high throughput environments