Differences in predicted EC-50s using time-resolved, non-linear toxicant exposure compared to static exposures in vitro

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ABSTRACT

Standard 2-dimensional static cell culture systems are limited tools for toxicology evaluations in vitro. Cells grown in monolayers do not form appropriate cell-cell contacts and cells grown in static culture wells are subjected to increasing amounts of waste products and decreasing amounts of dissolved oxygen leading to potential stress responses and toxicity not related to the toxicant added. To overcome these limitations, researchers are turning to 3D scaffolds, spheroids, hydorgels, and fluidics systems to improve the physiological relevance of the environment to which cells are introduced. We show here the evaluation of a fluidics system engineered to connect 10-wells of each row in a 96-well plate together with a microfluidic channel. This system sequentially links wells together to form a cascade of cell chambers through which drugs or toxicants can be applied. Toxicants interact with cells in the upstream compartments creating metabolites that will mix and interact in downstream wells forming a parent-metabolite gradient in a time-resolved fashion. Such a system, termed SciFlow, enables concentration by time kinetics of toxicity measurements in a more life-like environment. To demonstrate the value of this system, we have evaluated the effects of Tamoxifen on HepG2 cells in comparison to static conditions using the reagent CellTox™Green. Using a fluorescein tracer molecule we could demonstrate that exposures in the fluidic system were non-linear and shaped similar to an expected plasma curve in vivo. Comparisons of AUC between static and flow systems revealed dramatically different dose-response curve shapes and EC-50s with HepG2 cells in the flow system approximately 4 fold less sensitive to Tamoxifen than cells in static systems.

BACKGROUND

A new paradigm in predictive toxicology includes assessment of early initiating cellular events due to toxicant or drug exposure. One of the major challenges of this paradigm is determining whether such initiating events are adaptive or will lead to toxicity. Cell culture models that relate cellular events, such as changes in mRNA levels, with cytotoxic outcomes are often used to distinguish an adverse response from an adaptive response. The exposure levels required to produce each of the responses is then extrapolated to probable in vivo exposure scenarios through dose-response curves.

The SciFlow™ Multiwell Cascading Fluidics Cell Culture System is a versatile system that enables application of fluidic motion and gradient toxicant exposure to cell-based assays. Time-resolved dynamic exposure scenarios afforded by SciFlow is more in vivo-like and could enable more accurate assessment of adaptive vs toxic mechanisms. Moreover, the dynamic exposure scenario allows ascertainment of actual thresholds rather than extrapolating from endpoint dose-response curves.

To demonstrate the use of SciFlow and the effects of gradient exposures on cytotoxicity, we conducted a series of experiments examining the effects of Tamoxifen and Acetaminophen (APAP) on HepG2 cells comparing cytotoxicity between several experimental approaches.

OBJECTIVES

- Evaluate total time-resolved exposure cell death EC-50 for Tamoxifen or Acetaminophen (APAP) treated HepG2 cells using SciFlow vs. standard static fluid culture.

OVERVIEW OF SCIFLOW DESIGN

Figure 1. Overview of the Features and Use of the SciFlow system

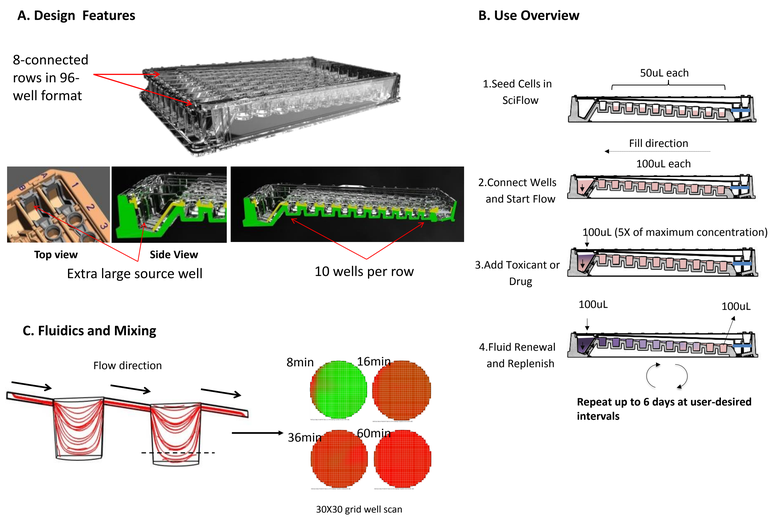

Figure 1. A. The Microtiter plate format was re-designed to feature an extra large source well capable of holding up to 600uL of media. Each subsequent well of each row is connected together by a microchannel running across the top surface of the plate. A row cover serves to create a closed channel system with open wells to allow full access to both the media and cells in the system. A porous wick at well 12 serves as a sink for excess fluid. **B.** Overview of how SciFlow is used to seed cells and tissues, initiate flow, and renew and replenish for long term culturing. **C**. Fluid vector lines showing the direction of fluid moving throughout an individual well. Images acquired using the Wellscan feature of the Clariostar Multimode Reader from BMG LabTech. A 30X30 2D grid was measured for fluorescence intensity at indicated times to measure mixing kinetics. By 60 minutes, the %CV across the grid was less than 2% indicating nearly complete mixing.

METHODS

- Optimize Z-height focus for measuring fluorescence along rows of SciFlow
- Create Fluorescein standard curve for extrapolating real-time concentration gradients
- Determine EC-50 for Tamoxifen and APAP induced cell death using HepG2 cells and CellTox™Green

RESULTS

Figure 2A: Optimal Z-height Focus

Well	Z-Height optimized (um)
3	20858
4	20545
5	20545
6	20544
7	20231
8	19918
9	19918
10	19605
11	19605

Figure 2B: Fluorescein Std Curve

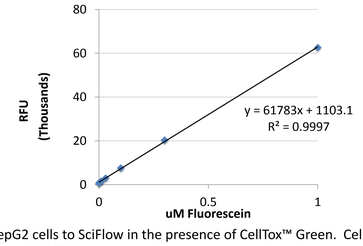

Figure 2. A. Optimal Z-Heights were determined by plating HepG2 cells to SciFlow in the presence of CellTox™ Green. Cells were then lysed to generate maximum signal. The Tecan Infinite M1000 Pro z-scan settings were used to identify optimal focal height. **B**. To create the Fluorescein standard curve, each row of the SciFlow was filled with a single concentration from 0.001uM to 1uM of Fluorescein dissolved in complete media. Plate was then read at ex485/em525 using the Tecan Infinite M1000 Pro using z-heights in table. The instrument was set to determine optimal gain settings for the 1uM dilution. The values from each row were averaged and plotted against concentrations resulting in a linear standard curve (Figure 1). The equation for this curve was determined using linear regression analysis. Gain settings were recorded for use in later experiments.

RESULTS

Figure 3. Concentration x Time Analysis

A. Experiment Plate configuration

	Source	Regulator	3	4	5	6	7	8	9	10	11	Syphon
A	1uM Fluorescein		Acellular Wells (no metabolism)									
B												
C	Vehicle Control		HepG2 cells, 30000 cells/well									
D												
E												
F	Treated (150uM Tamoxifen or 50mM APAP)		HepG2 cells, 30000 cells/well									
G												
H												

B. Data Analysis

- RFU values in rows A&B are converted to [uM] Fluorescein using the standard curve.
- Calculated uM Fluorescein (X_{FL}) is converted to "Expected" toxicant concentration (X_E) by:

  $X_E = X_{FL} * X_I$: where X_I is the initial toxicant concentration applied to source well in SciFlow
- Area-under-the-curve (AUC) concentrations are calculated by using the linear trapezoidal method for each timepoint. (PKSolver: Computer Methods and Programs in Biomedicine Volume 99, Issue 3, September 2010, Pages 306–314)

Figure 3. A. Plate configuration. Acellular wells were treated exactly the same as cells seeded with 30000 HepG2 cells. After seeding and attachment, media was changed to media containing 1:2000 dilution of CellTox™Green. Toxicant, Vehicle, or 1uM Fluorescent tracers was applied to source wells as indicated**. B.** Overview of data analysis procedure to obtain an EC50 based on AUC exposure.

Figure 4. Effects of Concentration X Time Exposures on EC-50

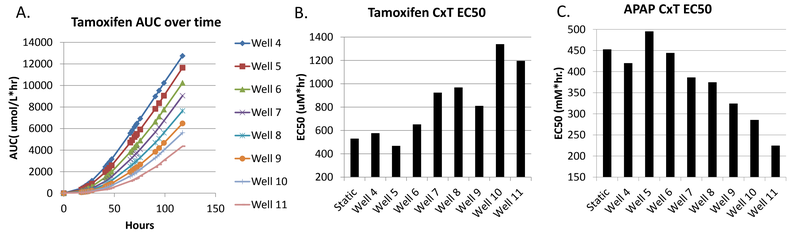

Figure 4. Concentration X Time effects on cytotoxicity. **A**. HepG2 cells in SciFlow were treated with increasing concentrations of Tamoxifen over time. **B**. Calculated EC50 for each well following repeated infusion of Tamoxifen. 150uM of Tamoxifen was infused into the SciFlow source well 3 times per day. Two acellular rows were infused with 1uM Fluorscein to enable calculation of absolute concentration at any given time (see Figure 3) . The reagent CellTox ™Green was used to monitor cellular toxicity. Area under the curve values for each timepoint were obtained and plotted against effect expressed as a % maximum of (treated/control). EC50 values were calculated by Sigmoid EMax model in PKSolver. **C.** Same as B using 50mM APAP.

CONCLUSIONS

- The SciFlow system enables generation of robust time-resolved toxicant gradients
- *Observed* EC-50 of Tamoxifen and APAP in downstream wells were different from *expected* when using standard static culture methods
- Tamoxifen *observed* EC50 suggests deactivation whereas APAP *observed* EC50s suggest bio-activation of a toxic intermediate consistent with what is known about these toxicants behavior in HepG2 cells.

ACKNOWLEDGMENTS

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