Application Note

Serial Multiwell Gradient Exposures for Analyzing Effects of Changing Parent-Metabolite Ratios

SCIKON INNOVATION INC

TECAN.

The SciFlow System dynamic fluid gradients enable direct observation of the cellular effects of changing parent-metabolite ratios in cell culture

Introduction

The SciFlow™ Multiwell Cascading Fluidics Cell Culture System is a versatile system that enables the application of fluidic motion and gradient toxicant exposure to cell-based assays in a proprietary microplate reader-compatible 96-well format. Time-resolved dynamic exposure scenarios afforded by the SciFlow System is more in vivo-like and could enable more accurate assessment of adaptive vs toxic mechanisms. Moreover, the dynamic exposure scenario allows better prediction of concentration thresholds for response rather than extrapolating from endpoint dose-response curves.

The SciFlow System is an SBS formatted microtiter plate designed to be compatible with plate-readers, imagers, and automated liquid handling systems. In order to enable efficient flow of fluids across the plate, the SciFlow System was designed with variable well-heights. The wells are elevated at one end of the plate and subsequently the bottom surface of each well is 0.5mm lower than the previous well. This allows for a cascading flow of fluids across the plate (Figure 1). The Tecan Infinite® M1000 Pro plate reader features the ability to empirically determine the optimal height of each measurement.

To demonstrate the ability to measure fluorescence plate-based assays in the SciFlow System using the Tecan Infinite Pro, we measured the cytotoxic response of the hepatoma cell line HepG2 to acetaminophen using the DNA-binding reagent CellTox™Green.

Materials used:

HepG2 Cells (ATCC catalog # HB-8605)
SciFlow Multiwell Cascading Fluidics System (SciKon catalog # AA-1-50)
Culture media: DMEM (Thermo-LifeTech catalog # 31053-028) supplemented with 10% FBS (Sigma # F2442), 1X Glutamax (Thermo-LifeTech #35050-061), 1X Pen/Strep (Thermo-LifeTech # 30-002-C1)
CellTox™ Green (Promega catalog # G8731)
Acetaminophen (Sigma Cat # A7085)
Fluorescein salt (Sigma Cat # F6377)
Instruments: Tecan Infinite M1000 Pro Multimode plate reader

SciFlow System Features

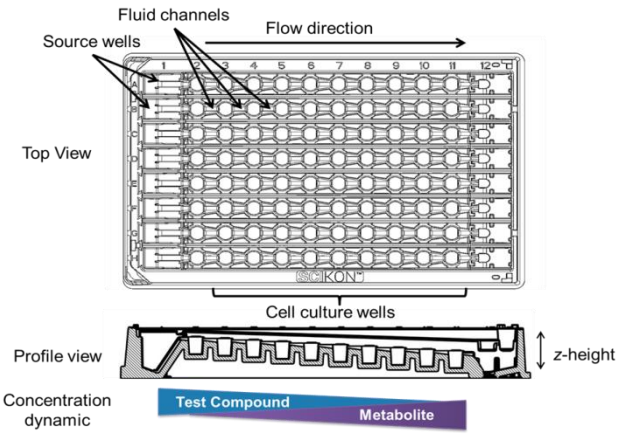

Figure 1. Top view and profile view of SciFlow™ Multiwell Cascading Fluidics system showing major features. Note the difference in Z-height of each well in profile view. Functionally, by applying test chemical to the source well, serial exposure to subsequent wells results in decreasing parent chemical and increases in metabolites or other cellular responses.

Methods

Optimizing Z-height focus in the Tecan Infinite M1000 Pro

To determine optimal height for fluorescence, HepG2 cells were plated to SciFlow and then lysed with detergent in the presence of CellTox™ Green to generate a maximal fluorescence signal at the well surface that served as a focal point for optimizing z-height. The z-heights determined for SciFlow are listed in Table 1. To improve speed of analysis, these optimal heights were then pre-set for each column rather than optimizing with each read.

Table 1	Optimized Z- Height for SciFlow using the Tecan Infinite M1000 Pro								
Well	3	4	5	6	7	8	9	10	11
microns	22735	22235	21735	21235	20735	20235	19735	19235	18735

Creation of a Fluorescein standard curve. A fluorescein standard curve is used to extrapolate concentrations of test compounds during the dynamic gradient formation in SciFlow System. To create the fluorescein standard curve, each row of the SciFlow System was filled with a single concentration from 0.001uM to 1uM of Fluorescein dissolved in complete media. Fluorescent signal was then read at ex485/em525 using the Tecan Infinite M1000 Pro using z-heights in Table 1. 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 2). The equation for this curve was determined using linear regression analysis. Gain settings were recorded and set in the software for use in later experiments.

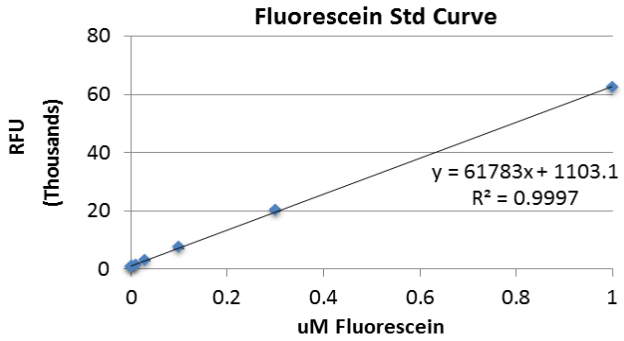

Figure 2 Fluorescein Standard Curve generation. RFU values were averaged from all wells in a single row at each concentration. Line was drawn using linear regression analysis. The line equation is shown. Experimental RFU are converted to Fluorescein concentration by solving the equation for "x".

Experimental Plate Layout

	Source	R	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 (50mM APAP)		HepG2 cells, 30000 cells/well									
G												
H												

Figure 3 Experimental overview. After seeding and attachment in 50uL in wells 3-11, media is changed to 100uL media containing 1:2000 dilution of the cytotoxicity indicator, CellTox™Green. After allowing fluidic channels to fill, test compound is added to the source well only. To enable continuous media replenishment 100uL of media was removed from well 11 and additional media containing test compound, vehicle control, or fluorescein standard was added to the source well at least three times per day.

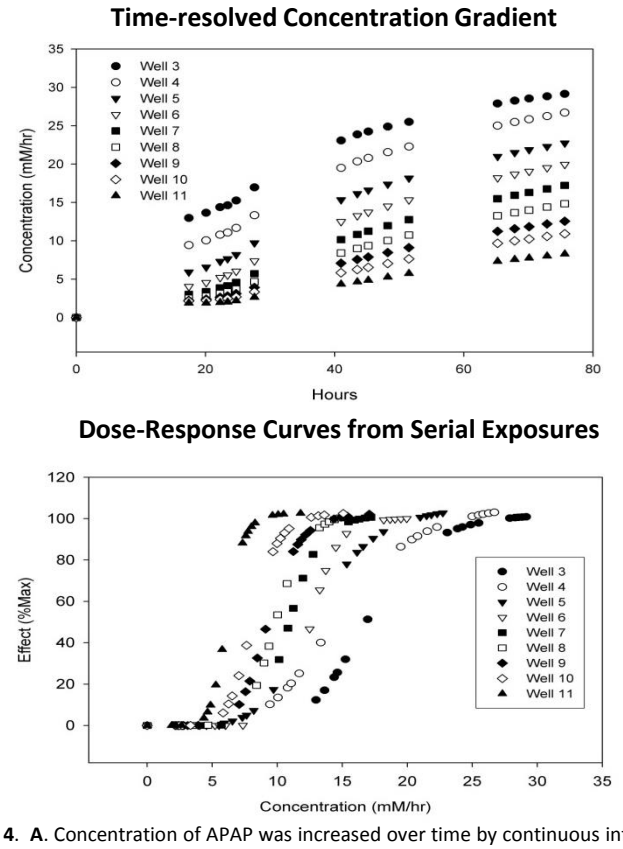

Figure 4. **A**. Concentration of APAP was increased over time by continuous infusion of fresh APAP in the source well. The slope of the increase was greatest in the wells adjacent to the source well. **B**. Cytotoxicity was queried in each well over time until a maximum signal from the CellTox™Green reagent was reached. To account for total time differences between maximum responses in each well, concentrations were converted to average concentrations per hour. The left-shift in the dose-response curves more distal from the source well shows increasing toxic potency of the media.

Effect of time-resolved gradient exposure on response of HepG2 cells to Acetaminophen.

Acetaminophen (APAP) is metabolized to a toxic intermediate that under prolonged incubations results in toxicity and cell death. In the SciFlow System, metabolite-to-parent ratio will increase in wells furthest from the source well where parent is infused (see Figure 1). Therefore toxicity profiles may also show differences based on well location.

The experimental process for examining the effects of applying APAP to HepG2 cell using a time-resolved gradient is outlined in Figure 3. Briefly, HepG2 cells are plated as noted in 50uL media. Following attachment, media is changed to media containing 1:2000 dilution of the cytotoxicity indicator CellTox™Green in 100uL per well. 400uL of media containing CellTox™Green was applied to the source well. The plate was incubated for 30 minutes to allow for connection of the fluidic channels. Once fully connected, 200uL of test compound, vehicle control, or 1uM fluorescein was added to the source well. To maintain fluid flow over multiple days, 100uL was removed manually from well 11 and new drug, vehicle, or fluorescein standard was added to the appropriate source well three times per day. This resulted in complete turnover of media over four days while providing a constant infusion of increasing concentrations of chemical in each group. Both fluorescein and CellTox Green signals were monitored on the Infinite Pro using the pre-set optimized z-heights and gain settings (Figure 4A).

For determining the concentration at any given time the signal obtained from the a-cellular fluorescein control rows were averaged for each column 3 through 11 and an actual Fluorescein concentration at each location was determined using the equation derived from the fluorescein standard curve (see Figure 2). The starting concentration of fluorescein was 1uM and the starting concentration of APAP was 50mM. To extrapolate APAP concentrations therefore, the amount of calculated fluorescein in each well was multiplied by the starting concentration of APAP to obtain the corresponding expected concentration of the test compound.

Using this extrapolated APAP concentration, dose-response curves were generated for each well position (Figure 4B). To account for both time and concentration dependency, calculated concentrations at each time point were converted to average concentration per hour of the experiment. As seen in the graphics, the dose-response curve shifted to the left as APAP was transferred serially through the wells in SciFlow indicating the change in parent to metabolite ratios is creating a solution that is more toxic to cells than the parent alone.

Conclusions:

- The application of the SciFlow System dynamic fluid gradients enables direct observation of the cellular effects of changing parent-metabolite ratios in cell culture.
- The Tecan Infinite M1000 Pro with z-height optimization enables robust measurement of plate-reader cytotoxicity assays such as CellTox™Green

For ordering:

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Email: Info@scikoninnovation.com
Web Product Page: http://scikoninnovation.com/shop/category/96-well-waterfall-culture-plate/

Product Number	Description
AA-1-50	SciFlow™ 1000 (Pk of 5)

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