Discovery of Toxic Metabolites by High-Content Imaging in the SciFlow 1000 96-well Microfluidic Culture System

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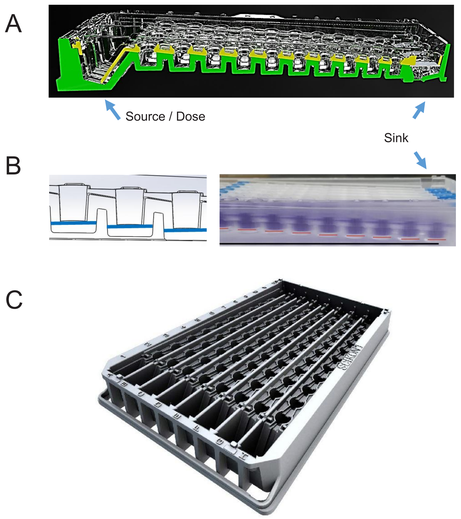
Abstract

We evaluated the utility of a newly released microfluidic culture system (SciFlow™ 1000) to support drug toxicity testing via parent compound, metabolite ID, and toxic cell metabolism.

Drug induced liver injury (DILI) is the leading cause of acute liver failure in the US, and is the most common reason cited for the withdrawal of a previously approved drug. DILI can result from toxicity caused by a parent compound or a metabolite of that compound (i.e. bioactivation). The ability to identify DILI inducing compounds or metabolites early in the drug discovery process results in significant potential savings to pharmaceutical companies. This requires a system that can both produce and retain drug metabolites, parent compounds, and measure the toxic effects on cells. The SciFlow 1000 system is a unidirectional gravity driven microfluidic tissue culture system based on a 96-well plate footprint, with all wells in a row linked by microchannels.

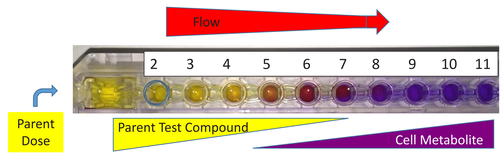
To demonstrate metabolic capacity and observe the pattern of metabolite production in SciFlow, substrates specific for 5 different CYP enzymes (1A2, 2B6, 2C9, 2D6 and 3A4) were introduced to SciFlow wells containing hepatocytes, and metabolite production was monitored over a 72-hour period. The fluorescent products of CYP metabolism begin to appear at the 24-hour time point and by 72-hours significant concentrations of these metabolites are measured in the downstream fluidic wells. Using these metabolite profiles, we set out to quantify the effects of metabolism on cellular toxicity. In a second experiment, a panel of drugs with varying mechanisms of toxicity were selected, and their effects on liver cell viability was monitored. We identified drugs that were hepatotoxic, and based on the pattern of cell death, those drugs whose toxicity was mediated through toxic metabolites. Additionally, we calculated the total drug exposure and determined an IC50 based on cell viability.

Technology



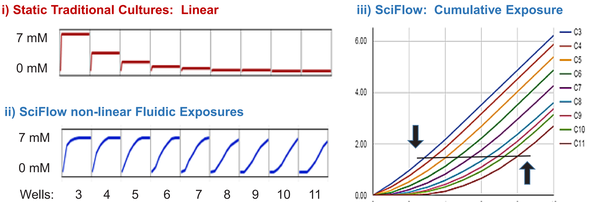
Panel A shows a profile view of one complete row of the SciFlow system with the source/dose and sink wells identified. **Panel B** shows a profile schematic of 3 wells (0.5mm offsets amid neighboring wells) and a picture of SciFlow with purple liquid filling one row to highlight the decreasing z-axis across the system. **Panel C** shows the whole SciFlow system as an SBS complaint 96-well format design, injection molded, using transparent tissue culture treated polystyrene.

Flow Dynamics



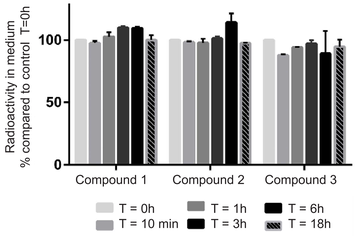
Repeated dosing generates a gradient of decreasing compound concentration across the connected wells of a row. Cellular metabolism further decreases the compound concentration within a given well. Fluid flow causes the metabolites and cellular responses to also flow downhill. Downstream wells are exposed to metabolites yet may never see significant concentrations of parent compound.

Compound Exposure Kinetics



i) Static plates (non-flow) are at fixed concentrations based on serial dilutions. Ii) SciFlow dosing creates gradients of compound concentrations over time. The concentration of compound increases in each well until the equilibrium concentration is reached, then that concentration is maintained for the duration of the experiment. Wells at different distances from the source well have very different concentration exposure profiles (Concentration vs. Time). iii) SciFlow exposure is a cumulative radiation-exposure where total exposure over time is determined (e.g. mM-hours).

Non-Specific Binding to Compounds (Novartis Study)



To evaluate binding of selected compounds to SciFlow 1000 System; culture surfaces are pre-coated with collagen I.

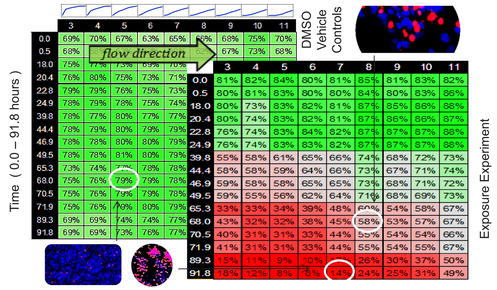
Each of the three compound were dosed at a 2 µM concentration. Compounds characteristics relate to low binding (compound 1), mid-range binding (compound 2), and high binding (compound 3) traits.

The control, no binding values are established at time = 0 (T=0h). Thereafter, radioactivity in the medium was evaluated at 10min, 1h, 3h, 6h, and 18h.

The radioactivity data confirms very limited, if any, non-specific bindings of compounds to the SciFlow system. A highly successful outcome.

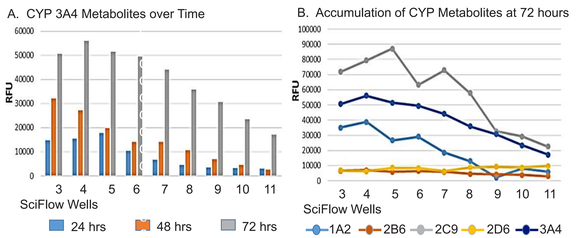
	Clog(P)	Polarity	MW (g/mol)	pKA
Compound 1	4.73	49.33	296	4.1
Compound 2	2.44	74.35	405	5.2
Compound 3	2.04	112.22	443	2.3/7.2/10.0

Data Centric



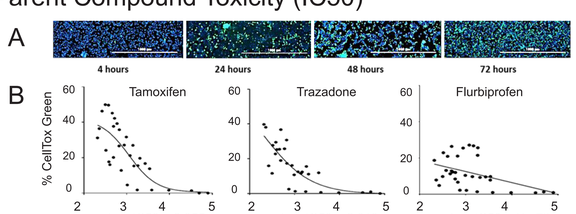
Heat Maps of % Viability (Real Time Metrics): Data metrics are quantified to create response or alerting heat maps of cell activity during the escalation of gradients to quickly and efficiently explore large data sets. Dual color images (Hoechst and CellTox Green).

CYP Metabolism Across a SciFlow Channel



Primary Human Hepatocyte CYP Metabolism in SciFlow. A) Accumulation of an isozyme-specific fluorogenic CYP 3A4 metabolite in SciFlow over time (24, 48, 72hr). Fresh substrate was added every 24 hours. B) Primary hepatocyte production of CYP1A2, CYP2B6, CYP2C9, CYP2D6, and CYP3A4 metabolites over 72 hours.

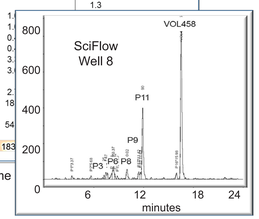
Parent Compound Toxicity (IC50)



To compare the effect of compounds on HepaRG cells in SciFlow, we used **A)** nuclear DNA staining and a live/dead cell stain to leverage the time resolved nature of compound exposure, allowing the collection of time lapse and long-term compound exposure data. **B)** Three compounds (Tamoxifen, Trazadone, and Flurbiprofen) of different hepatoxicity were dosed. Plotting the percentage of live/dead cells vs. exposure to compound allows a calculation of 50% effect number (IC50).

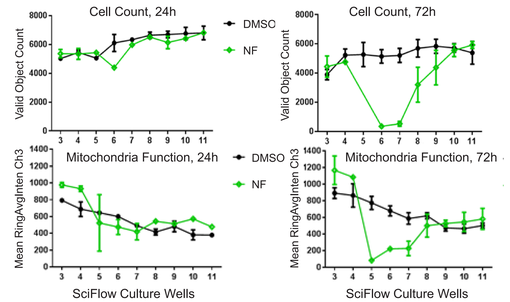
Metabolite ID (Novartis Study): 2D vs. SciFlow

Peak #	2D 20 hr.	SciFlow day 1 2x changed.			SciFlow day 2 4x changed.			SciFlow day 3 9x changed.		
		C3	C6	C8	D3	D6	D8	D11	H5	H8	H11
P1	1.8										
P2	1.5										
P3	7.1					0.9	1.				
P4	2.7					1.8	1.				
P5	1.4					1.1	0.				
P6	17.8	1.4				1.4	0.				
P7	1.8	1.4			4.2	2.4	3.				
P8	5.3	0.9	2.3		3.6	3.3	3.				
P9	6.1	0.8				1.0					
P10	4.1	0.8			3.2	2.0	2.				
P11	21.6	4.3	5.2	4.7	13.2	14.2	18				
P12						1.4					
parent	15.1	82.2	79.9	88.1	67.5	55.7	54				
total dpm	62175	23516	8688	3730	15176	20385	183				



2D (static) vs. SciFlow (fluidic): SciFlow developed some unique metabolites. SciFlow may be a tool of choice when evaluating the effect of <stable> metabolites on pharmacology or toxicology.

Toxic Cell-Metabolite Effect: Nefazodone (NF)



NF Exposure in HepaRG cells: High content imaging was used to monitor cell number in wells treated with nefazodone. **A)** Cells were fixed and stained with Hoechst nuclear stain, and counted. In the NF treatment, after 72-hours, the cell number drastically decreases in wells 6 and 7. The decrease in cell number is not seen at the highest drug concentration (wells 3 & 4). The downstream decrease in cell numbers is characteristic of a toxic metabolite effect, with upstream cells remaining viable. **B)** Cytotoxic effects of NF are attributed to mitochondrial damage (stress). To demonstrate this, cells were stained with Mitotracker Orange. After 24 hours of culture, all wells stained similarly. By 72-hours, there is a marked decrease in mitochondrial staining in NF treated cells in columns 5-7. Again, this demonstrates that damage is observed in downstream wells, signifying a toxic metabolite effect.

Conclusions

The SciFlow™ 1000 fluidic culture system exhibits more biologically relevant (in vivo-like) compound exposure data in a time-resolved fashion. SciFlow integrates the generation of parent compound and metabolite gradients across a plate with standard biochemical and high content imaging techniques.

This powerful methodology, using the combination of metabolically active liver cells, the SciFlow 1000 Fluidic Culture System, and high content imaging demonstrates the ability to identify cellular toxicity and distinguish between parent compound vs. metabolite effects without any prior knowledge of the compound's metabolite profile, allowing earlier identification of a compound's mechanism of toxicity.

The SciFlow 1000 system is a versatile fluidic culture system which is compatible with plate readers, high content imagers, and commercially available biochemical assay kits.

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