**SCIKON** INNOVATION INC

Basic Protocol for the SciFlow™1000 System
Acetaminophen (APAP) Setup & Example Protocol
Cell Viability in Real-Time Study (non-toxic)
## *using* CellTox™ Green (cell staining)

System Description:
The SciFlow™ 1000 Fluidic Culture System is a benchtop tool for *in vitro* use to mimic cell, organ, and living systems. SciFlow operates like a shallow river bed with a series of compartments for cell culture. The design allows for isolated and stagnant culture during cell seeding then delivers real-time fluid flow and compartment-to-compartment signaling over time. The entire system is contained within a 96-well formatted culture plate that includes 8 repeatable channels. Each channel has the capacity to connect 1-to-10 cell culture wells in a linear array. As a benchtop tool, SciFlow is configured for cell and tissue assessments allowing easy access to all culture wells and media streams. Additionally, SciFlow is compatible with microplate readers, high content imaging platforms, and microscopes. Beneficially, no external pumps, or tubes, or controllers are required.

Protocol Focus: There are many applications of the SciFlow 1000, this protocol focuses on measuring the changes in membrane integrity that occurs as a result of cell death. It is intended for assessing cytotoxicity in cell culture, in each of the 9 interconnected cellular compartments of the SciFlow system. Details relate to kinetic studies of cytotoxicity involving multiple plate measurements over a time course.

Overview: Culture areas within SciFlow 1000 are analogous to a ½ area 96-well plate (0.167 cm^2 per well). As purchased, all culture wells are tissue culture treated.
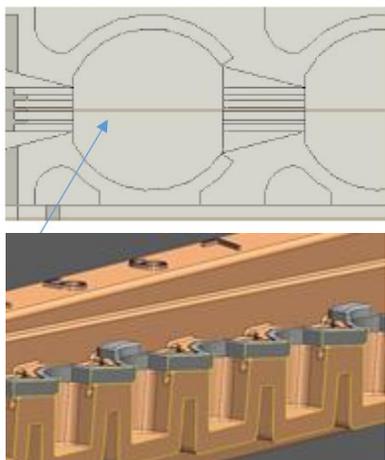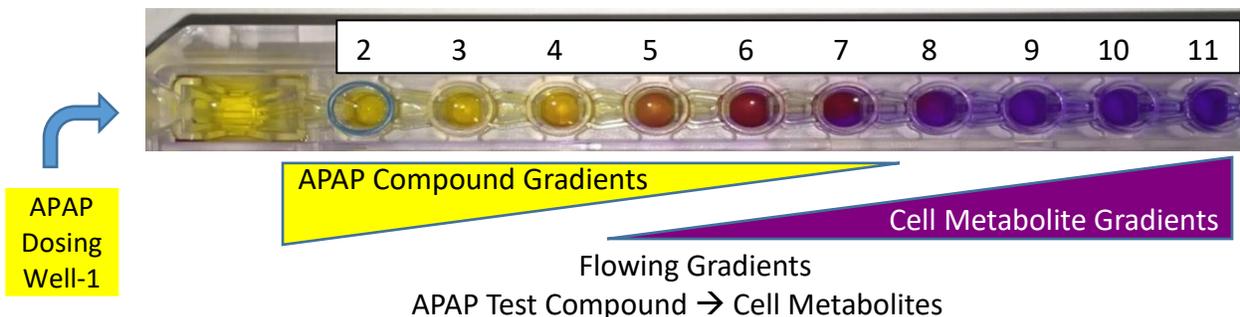

Surface & profile images of linear aligned culture wells

APAP / Toxicant
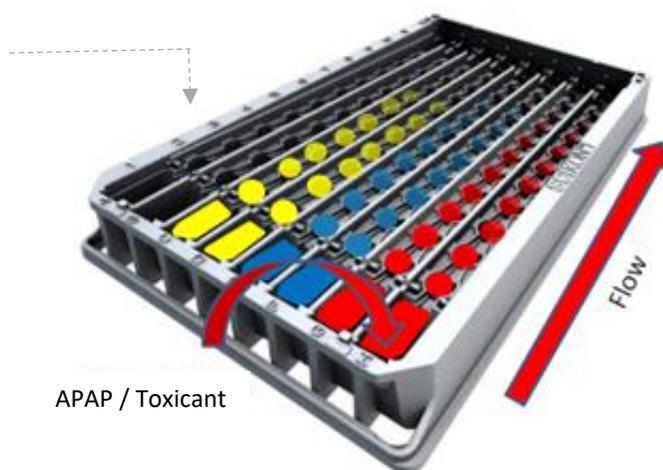
Flow

Background: Acetaminophen (APAP): Literature shows APAP is metabolized to a toxic intermediate, and under prolonged incubations result in toxicity and cell death. In the SciFlow System, cell metabolite ratios will increase in wells furthest from the dosing well (i.e. wells 6-11) after the parent compound (e.g. APAP) is infused into the dosing well (well-1).

SciFlow 1000 Fluidics Culture System: Displaying 1 of 8 rows (Row A)

APAP Dosing Well-1

APAP Compound Gradients

Cell Metabolite Gradients

Flowing Gradients
APAP Test Compound → Cell Metabolites

Assay to Evaluate Toxicity: The CellTox^TM Green assay uses an asymmetric cyanine dye that is excluded from viable cells but preferentially stains the DNA from dead cells. When the dye binds DNA in compromised cells, its fluorescence properties are substantially enhanced. Viable cells produce no appreciable increases in fluorescence. Therefore, the fluorescence signal produced by the binding interaction with dead cell DNA is proportional to cytotoxicity. NON-TOXIC: The CellTox^TM Green Dye is well tolerated by a wide variety of cell types and is essentially nontoxic. The signal remains constant after exposure of 72 hours, making it ideal for determining toxic effects of treatments throughout extended exposures. The dye can be diluted in culture media and delivered directly to the cells at seeding or dosing, allowing kinetic measures of cytotoxicity. The dye also can be diluted in assay buffer and delivered to cells as a conventional endpoint after an exposure period.

Materials: Promega CellTox^TM Green Cytotoxicity Assay Kit: There are three catalog numbers (G8741 at 10 mls, G8742 at 50 mls, G8743 at 100 mls) for the kit. The kit contains CellTox^TM Green Dye, a lysis solution and an assay buffer. The assay described here is a live cell assay and only uses the CellTox^TM Green Dye, which can be purchased separately (catalog number G8731). If you need to do a standard curve, the lysis reagent in the kit will be useful.

CellTox^TM Green Express Cytotoxicity Assay (Promega, cat. # G8731)

Reagent Stability and Storage: CellTox^TM Green Express Cytotoxicity Assay components are stored at -20C. When ready to be used, the reagent is thawed in a 37C water bath. For homogeneity, mix using a vortex mixer.

CellTox^TM Green Cytotoxicity Assay Manufactures Protocol:
https://www.promega.com/-/media/files/resources/protocols/technical-manuals/101/celltox-green-cytotoxicity-assay-protocol.pdf

Acetaminophen (Sigma Cat # A7085)

Equipment:

> SciFlow 1000 System
> Fluorescent Signals: Plate reader or imager or microscope (z-axis adjustable)
> > Fluorescence at 485nm excitation, 525nm emission
> Multichannel Pipette
> Pipette Tips
> Reagent Reservoirs
> Cells and Medium

Standard Curves/Correlations:

Fluorescein as a Tracer Dye for Drug Concentrations (Drug Surrogate): To estimate dilution of test compounds across a SciFlow channel, create a fluorescein standard curve that is calibrated to specific instrument settings. Then, fluorescein can be used as a tracer dye to extrapolate concentrations of compounds across the SciFlow plate.

Reagents and Supplies:
1. 100 uM Fluorescein: Make a stock solution by dissolving fluorescein sodium salt (Sigma F6377) in your base media without supplements.
2. Example: If your base media is DMEM, dissolve fluorescein in DMEM to 100 uM. Protect from light and maintain sterility. This stock solution can be stored at 4 degC wrapped tightly in foil.
   a. One SciFlow 1000 system.
3. Culture media. This is the media used to culture your cells. If you have different media for different cells, a standard curve calibration will need to be performed for each media.

Procedure:
1. Create a dilution curve from 1uM to .001uM Fluorescein in 2mLs dilutant. When creating dilutions, dilute into complete culture media (Base plus any serum or additives). Always protect from light as much as possible. Fluorescein rapidly quenches when left exposed to light.
2. Remove a sterile SciFlow™ System from packaging.
3. Load one concentration of dye along an entire row, 100ul per well with 500uL in the source well (well-1) and 350uL in the sink well (well-12). For example:
   a. Row A: 1uM
   b. Row B: 0.3uM
   c. Row C: 0.1uM
   d. Row D: 0.03uM
   e. Row E: 0.01uM
   f. Row F: 0.003uM
   g. Row G: 0.001uM
   h. Row H: Media only, no dye

4. Set fluorescent plate reader instrument to optimize gain based on 1uM Fluorescein. Read plate at excitation 485nm, emission 520nm

    a. Coefficient of variance across each row should be calculated from data. If the plate reader allows adjustment of z-height focal length, make z-height adjustments until CV is at lowest point. See Key Dimension specifications of the SciFlow plate to aid in creating appropriate z-height settings.

    b. **IMPORTANT**: Note the gain level that the instrument chooses for 1uM Fluorescein. This will be the gain level that must be used for all subsequent experiments

    c. **For some instruments, the gain is preset**. Call your manufacturer to clarify how to determine and set the gain appropriately

5. Plot data by averaging fluorescent units across rows against concentration of fluorescein.
6. Create a line equation that will be used to predict fluorescein concentrations based on fluorescent units collected at the gain level developed in step 5.
7. Record this line equation with the gain setting for future use
8. With new stock reagents, changes in culture media, or from time to time, recalibrate the gain level by using a new plate and re-optimizing gain based on 1uM Fluorescein. Always store equation and gain levels together for accuracy.

Fluorescein Standard Curve Example

Below is an example of creating a Fluorescein standard curve. In this example, a Tecan Infinite M1000 Pro was used to generate RFU. 100uL of each dilution was added to all the wells of a single row. The average and standard deviation was used to create a line equation for subsequent experiments.

Parameters

Mode	Fluorescence Top Reading	
Excitation Wavelength	485	nm
Emission Wavelength	525	nm
Excitation Bandwidth	5	nm
Emission Bandwidth	5	nm
Gain	88	Manual
Number of Flashes	10	
Flash Frequency	400	Hz
Integration Time	20	µs
Lag Time	0	µs
Settle Time	0	ms

Raw Data

uM Fluorescein	2	3	4	5	6	7	8	9	10	11
1	61722	62521	63000	63000	62569	62916	62620	62820	62364	63000
0.3	18967	19849	20243	21107	20361	21105	20220	20112	20182	21087
0.1	6917	7468	7669	7535	7633	7673	7720	7696	8677	6787
0.03	2683	2952	2886	3044	3121	3008	2953	3021	2948	3049
0.01	1437	1580	1507	1417	1505	1548	1621	1493	1574	1628
0.003	1055	1063	1040	1056	1042	1017	1026	1057	980	1051
0	806	799	803	782	811	818	793	744	762	726

Summary Stats

uM Fluorescein	Average	Stdv	%CV
1	62653.2	399.2	0.6
0.3	20323.3	663.0	3.3
0.1	7577.5	509.8	6.7
0.03	2966.5	119.7	4.0
0.01	1531	71.9	4.7
0.003	1038.7	25.2	2.4
0	784.4	30.7	3.9

Graph and Line Equation

Average by Row

$y = 61783x + 1103.1$

$R^2 = 0.9997$
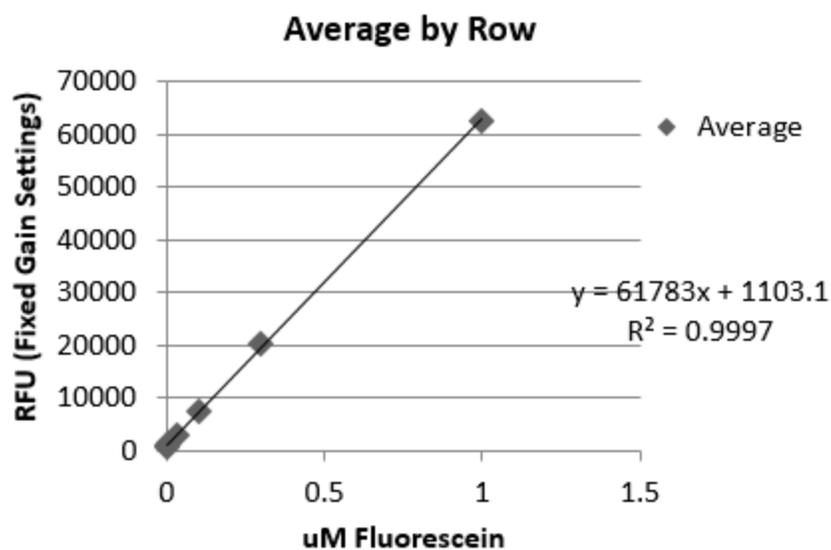
Once the standard curve is generated, a line equation is calculated. This equation will allow back calculation of an absolute fluorescein concentration which is then correlated to the starting concentration of your drug or toxicant.

In order for this approach to be effective, users must first optimize gain settings for the highest concentration of fluorescein (recommended starting concentration is 1 uM). The starting concentration may vary depending on instrument sensitivity and settings. Then, users must manually set the gain levels (i.e. fixed gain) to be the same for each subsequent experiment.

In one example, the starting concentration of fluorescein was 1uM (dose) and the starting concentration of APAP was 50mM. To extrapolate APAP concentrations, 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. To account for both time and concentration dependency, calculated concentrations at each time point were converted to average concentration per hour of the experiment (i.e. radiation type exposure modeling). Expectedly, the dose-response curves constantly change as APAP transfers serially through the SciFlow wells (C vs. T), also indicating a change in compound-parent to cell-metabolite ratios.

CellTox^{TM} Green Outcomes: Cell cytotoxicity data can be normalized against control cultures, such as vehicle only cultures within the same SciFlow system.
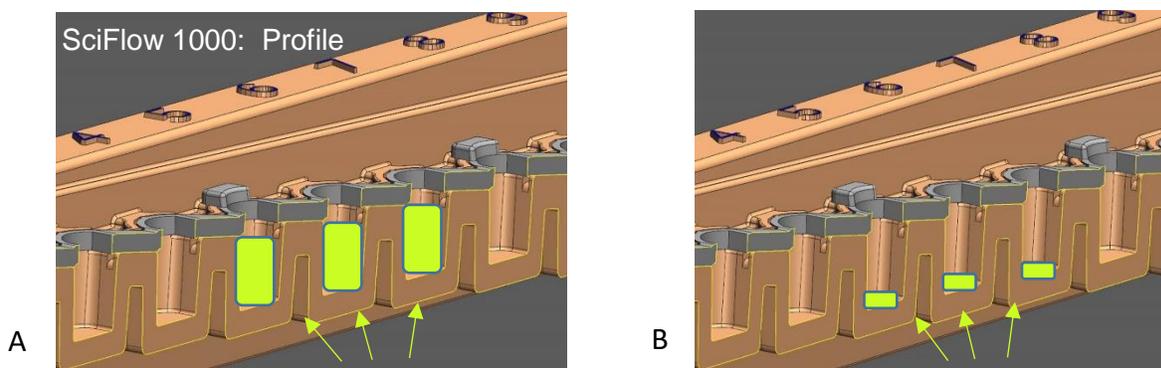
SciFlow 1000 System z-height adjustments									
Column	3	4	5	6	7	8	9	10	11
microns	+2000	+1500	+1000	+500	0.0	-500	-1000	-1500	-2000

A) Fluorescein: Media/Signal with larger volumes for reader data acquisition. **B)** Cell-Stain/Signal with smaller signals for reader data acquisition. Due to location of signal, use bottom reads. **C)** SciFlow is configured similar to a 96-well culture format. SciFlow also incorporates z-height steps to 500 microns between neighboring columns.

Signal/Image Accuracy:

To determine signal/image accuracy of all z-heights, when using CellTox^{TM} Green, cells were plated in SciFlow then cells were lysed with detergent in the presence of CellTox^{TM} Green to generate a maximal fluorescence signal at the well surfaces.

Protocol:

Promega CellTox^{TM} Green is sold as a concentrate. This protocol uses the Express, No-Step addition, that is intended for kinetic studies of cytotoxicity involving multiple plate measurements over a time course.

Brief Synopsis:

This process is for examining the effects of applying APAP to cells using a time-resolved gradient.
1. Briefly, cells are plated in 50 ul media/well (no flow conditions). Seed your cells in traditional culture media, <u>without</u> CellTox^{TM} Green Dye.
2. Following attachment, all media is changed to media containing 1:2000 dilution of the cytotoxicity indicator CellTox^{TM} Green (2.5ul to 5ml media; optimize per cell type).
    a. 350 ul into the sink (well-12)
    b. 100 ul per well into culture wells (11, 10….2)
    c. 350 ul into the dosing well (well-1).
3. The SciFlow System is incubated for 30 minutes to allow for connection of fluidic channels between culture wells.
4. Once SciFlow channels are fully connected, 150 ul of test compound, vehicle control, or 1 uM fluorescein is added to the source well.
    a. APAP Test Compound: 150 ul volume created as 50mM concentration.
    b. Vehicle Control: 150 ul volume created with vehicle control concentration
    c. Fluorescein: 150 ul volume created as 1uM concentration
5. To maintain fluid flow over multiple days, 150 ul is removed manually from well-12 and new drug, vehicle, or fluorescein standard is added to the appropriate dosing well 3-times per day (8am, noon, 4pm).
    a. This results in complete turnover of media over four days while providing a constant infusion of increasing concentrations of chemical in each group.
6. Both fluorescein and CellTox^{TM} Green signals are monitored using fluorescent data acquisitions using reader/imagers that have pre-set and optimized z-height and gain settings.

Generic Notes:
1. Each row of the SciFlow System holds 1.85 mls of media. An entire SciFlow System (8 rows) holds 15 mls of media.
    Well-1: 500 ul   Wells 2 – 11: 100 ul/well   Well 12: 350 ul

Detailed example of APAP SciFlow Study (using one SciFlow System)

1. Determine the volume amount of CellTox™ Green Dye reagent required.
   a. One SciFlow system requires one-time priming with 14.8ml
   b. Twenty media changes correspond to 24mls of media
   c. Summation 14.8 + 24 = 38.8ml; rounding up to 40ml
      i. Create a 1:2000 dilution (2.5ul to 5ml media)
   d. Need 20ul CellTox™ Green Dye reagent placed into 40ml culture media
2. Day 0: Seed SciFlow using culture media **without** CellTox™ Green Dye reagent
   a. Seed in 50ul culture media per well. Wells 3 -11.
3. Day 1: After cell attachment, remove all media from SciFlow.
   a. SciFlow can be emptied by inversion and flicking into an appropriate waste container. Care should be taken to keep the plate sterile. Alternatively, vacuum suction the media by aspirating from Well 12. It may be necessary to tilt SciFlow, allowing all media to flow into Well 12, while performing media aspiration
   b. Add media containing CellTox™ Green Dye reagent.
      i. Add media in this order:
         1. The sink, well-12 (350ul)
         2. Internal wells following this order 11, 10, 9……3, 2 (100ul each)
         3. The dosing well, well-1 (350ul).
   c. Place SciFlow in 37degC humified incubator for 30 minutes to allow fluid connections between wells.
      i. Remove SciFlow from incubator.
      ii. Confirm that all channels are fluid-connected amid SciFlow wells.
   d. Add drug/toxicant/vehicle into the dose well (well-1). This 150ul volume also contains CellTox™ Green Dye reagent.
      i. 150ul x 8 rows = 1.2ml media plus 0.6ul CellTox™ Green Dye reagent.
         1. APAP Test Compound: 150 ul volume created as 50mM.
         2. Vehicle Control: 150 ul volume created with vehicle control
         3. Fluorescein: 150 ul volume created as 1uM concentration
   e. Monitor fluorescence at any time (real time data acquisition)
4. Day 2 through Day 8: Three times per day (e.g. 8am, noon, 4pm), remove 150ul from sink (well 12).
   a. Add drug/toxicant/vehicle to dose well (well-1). This 150ul that also contains CellTox™ Green Dye reagent.
      i. 150ul x 8 rows = 1.2ml media plus 0.6ul CellTox™ Green Dye reagent.
         1. APAP Test Compound: 150 ul volume created as 50mM.
         2. Vehicle Control: 150 ul volume created with vehicle control
         3. Fluorescein: 150 ul volume created as 1uM concentration
   b. Monitor fluorescence at any time (real time data acquisition)
5. End protocol

Outcome: The application of the SciFlow System dynamic fluid gradients enables direct observation of the cellular effects of changing parent-metabolite ratios in cell culture.

Protocol (Alternative using smaller amounts of CellTox^{TM} Green reagent):

In some cases, it is desirable to retain the original media in SciFlow (i.e. don't remove media without CellTox^{TM} Green). This protocol uses a 10x stock of CellTox^{TM} Green that is added directly to individual culture wells and does not require media exchange.

Thaw CellTox Green
1. Make a 10x stock by dilution: 1:200 into cell culture media (5 ul / ml media).
2. Assuming each well already contains 100 ul of media, add 10 ul of the 10x CellTox^{TM} Green stock to each well.
    a. Note/Hint: The volumes in different wells of the SciFlow1000 may be different, with wells in higher columns (i.e., 10 and 11) having larger volumes and the lower number columns (i.e., 3 and 4) having less volumes. The CellTox^{TM} Green will have slightly different concentrations in the different wells due to different dilutions. CellTox^{TM} Green is only fluorescent when bound to the DNA of dead cells, so the exact concentration is not of utmost importance. Normalization to control wells treated in the same fashion will help reduce any experimental artifacts from DNA staining in this manner.
3. Incubate for 30 minutes in 37degC incubator.
    a. Monitor fluorescence (real time data acquisition)
4. For additional data-points, over time, repeat steps 1-3.
5. End Protocol (Alternative)

Data Analysis Option (CellTox^{TM} Green replicates in the SciFlow System):

One can automate the data outcomes (real time cell responses) by normalizing data against a vehicle-control within the same SciFlow System (e.g. Row B)

Table 1: Experimental Setup of a SciFlow System											
	Source	2	3	4	5	6	7	8	9	10	11	Sink
A	Fluorescein Tracer	R	Acellular Fluidic Tracer used to determine compound dose concentrations (C vs. T)									
B	Vehicle Control	R	cells	cells	cells	cells	cells	cells	cells	cells	cells	
C	Compound 1	R	cells	cells	cells	cells	cells	cells	cells	cells	cells	
D	Compound 1	R	cells	cells	cells	cells	cells	cells	cells	cells	cells	
E	Compound 2	R	cells	cells	cells	cells	cells	cells	cells	cells	cells	
G	Compound 2	R	cells	cells	cells	cells	cells	cells	cells	cells	cells	
G	Compound 3	R	cells	cells	cells	cells	cells	cells	cells	cells	cells	
H	Compound 3	R	cells	cells	cells	cells	cells	cells	cells	cells	cells	

Table 2: Normalized Outputs of a SciFlow System											
	Source	2	3	4	5	6	7	8	9	10	11	Sink
A	Fluorescein Tracer	R	Acellular Fluidic Tracer used to determine compound dose concentrations (C vs. T)									
B	Vehicle Control	R	1	1	1	1	1	1	1	1	1	
C	Compound 1	R	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	
D	Compound 1	R	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	
E	Compound 2	R	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	
G	Compound 2	R	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	
G	Compound 3	R	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	
H	Compound 3	R	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	≤ 1	

Tips and FAQ: This section outlines some very useful techniques for handling SciFlow 1000.

Removing Media (if required): SciFlow can be emptied by inversion and flicking into an appropriate waste container. Additionally, the entire row can be emptied via vacuum aspiration through the sink well.

Adding Media (if required): When adding fluid to SciFlow, it is best to begin additions at the lower end of the plate (Column 12), followed by columns 11, 10 then 9…. This will result in downstream wells being filled before the upstream wells are filled, and result in a much more controlled fluid flow.

Cell Number: The SciFlow 1000 is a ½ area 96-well plate, the number of cells seeded in each well should be adjusted accordingly. See cell seeding table at the end of this protocol.

Compound Addition: When initiating a gradient, the most reproducible method is to add a small volume of a more concentrated stock solution into the source well. When adding the more concentrated stock, do not pipette up and down (to mix) as this will change the flow dynamics. Add gently.

Feeding/Dosing: For experiments requiring incubations longer than 24 hours or for repeated exposures to the compounds of interest, additional media/compound must be added to the source well. Volumes between 50μl – 250μl can be used for dosing/feeding, in most cases 150μl is an appropriate volume. Whatever volume is chosen, the same volume should be initially removed from column 12 (waste) and a replicate volume of fresh media or 1x compound in fresh media added into the source well of the SciFlow 1000.

Tracking Flow: Fluorescein can be used as a tracking dye to monitor the flow during an experiment and to approximate compound concentrations in each well, not accounting for cellular metabolism. See Application Note for detailed instructions on quantifying fluorescein and compound concentrations.

Moving SciFlow 1000: SciFlow is a fluidic system and if the plate is tipped along the lengthy axis (x-axis), this can disrupt or modify both flow and any established gradients. Reasonable care should be taken when moving the plate to minimize unintended flow caused by tipping the plate.

Evaporation: Though the SciFlow 1000 does have a lid, evaporation can be observed, and for experiments over 7 days, a 10 – 20% larger volume can be added to the source well than is removed from the sink, to combat decreasing volumes in the source.

Sampling: It is possible to sample from the wells during the experiment. 5 – 10μl aliquots can be removed from each well in a row using a multichannel pipette, to simultaneously remove sample from all of the wells.

Running Assays in SciFlow: Cell based assays can be run in the SciFlow 1000. It is important to remember that once the fluidics are engaged they will stay engaged. Here are a few assay suggestions:

- For endpoint assays remove all media from the plate by inversion and flicking the plate over an appropriate waste container.
- Keep volumes of buffer and assay reagents less than 50μl in order to reduce in possible flow from well to well.
- Shorter incubation times are preferable, minimizing inter-well flow.

Sample Plate Map:

	Source	2	3	4	5	6	7	8	9	10	11	Sink
A	Fluorescein Tracer		Acellular Fluidic Tracer									
B	Compound 1		Cellular Model (e.g. HepaRG cells @ 40,000 cells/well)									
C	(triplicate											
D	rows)											
E	Vehicle		Cellular Model (e.g. HepaRG cells @ 40,000 cells/well)									
F	control											
G	(triplicate)											
H	Fluorescein Tracer		Acellular Fluidic Tracer									

Example Cell Seeding Densities

Table of Example Cell Seeding Parameters:	SciFlow culture well areas are ½ the size of traditional 96-well culture surface areas (0.167cm^2 or 16.7mm^2).					
Cell Seeding Examples, 2D monolayers	Number of cells per SciFlow plate	Number of cells per well	How many culture wells	Seed Time	Initial confluence	Adjustment
Primary human hepatocytes with collagen coating	2.0 E6	27,500	72 (3 – 11)	Overnight	Confluent	By viewing
Primary rat hepatocytes with collagen coating	1.0 E6	14000	72 (3 – 11)	Overnight	Confluent	By viewing
Primary mouse hepatocytes with collagen coating	6.5 E5	9000	72 (3 – 11)	Overnight	Confluent	By viewing
Primary duck hepatocytes with collagen coating	6.5 E5	9000	72 (3 – 11)	Overnight	Confluent	By viewing
Primary canine hepatocytes with collagen coating	1.0 E6	14000	72 (3 – 11)	Overnight	Confluent	By viewing
HepG2	2.2 E6	30000	72 (3 – 11)	Overnight	80%	By viewing
HepaRG	2.9 E6	40000	72 (3 – 11)	Overnight	80%	By viewing
HepaRG (no spin)	2.9 E6	40000	72 (3 – 11)	Overnight	80%	By viewing
Cell Line MCF7	7.2 E5	10000	72 (3 – 11)	Overnight	20%	By viewing