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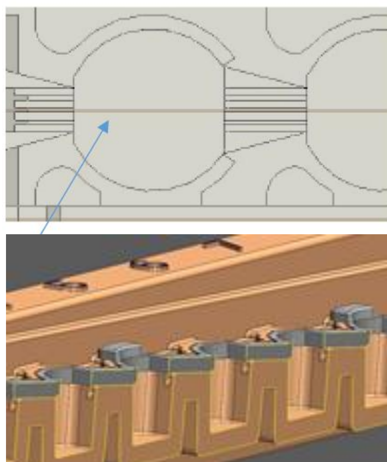
Basic Protocol for the SciFlow™1000 System
CellTox™ Green Assay for Membrane Integrity
Cell Viability in Real-Time Study (non-toxic)
Monolayer Culture (cell staining)

System Description:

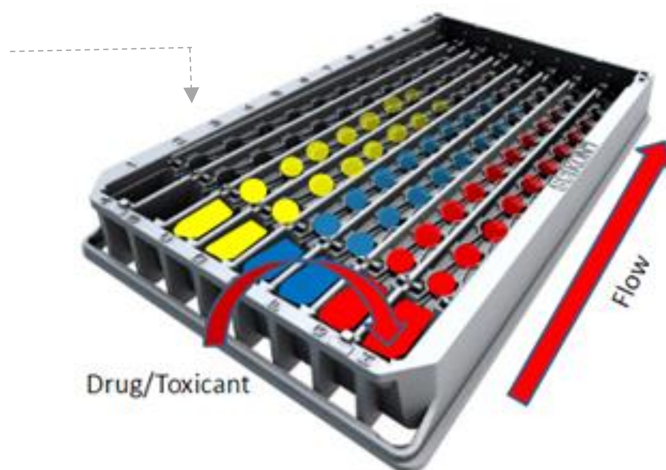
The SciFlow™ 1000 Fluidic Culture System is a benchtop tool for *in vitro* use to mimic cell, organ, and living systems. SciFlow operates like a shallow river bed with a series of compartments for cell culture. The design allows for isolated and stagnant culture during cell seeding then delivers real-time 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



Background: The CellTox™ 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™ 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™ 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™ Green Dye, a lysis solution and an assay buffer. The assay described here is a live cell assay and only uses the CellTox™ 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™ Green Express Cytotoxicity Assay (Promega, cat. # G8731)

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

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:

Data can be normalized against control cultures, such as vehicle only cultures within the same SciFlow system.

Signal/Image Accuracy:

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

Protocol:

Promega CellTox 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.

Cell Seeding in SciFlow 1000:

Seed your cells in traditional culture media, without CellTox^{TM} Green Dye.

CellTox^{TM} Green Dye Concentrations

After cells have attached, and cultures are ready for drug/compound exposure, the culture media is replaced to include culture media containing CellTox^{TM} Green Dye. Stock CellTox^{TM} Green is diluted between 1:500 (10ul to 5ml media) and 1:2000 (2.5ul to 5ml media) for use. We have been successful with dilutions of 1:1000 (5ul to 5ml media); optimize per cell type.

Volumes of Media in the SciFlow System:

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 12: 350 ul

If longer term experiments require additional feeding, then CellTox^{TM} Green should be diluted in fresh media, added into Well-1, to keep a constant concentration of CellTox^{TM} Green in all wells.

Example SciFlow Study; One SciFlow System

1. Determine the volume amount of CellTox^{TM} Green Dye reagent required.
    a. Assume a 1:1000 dilution (5ul to 5ml media)
    b. One SciFlow system requires one-time priming with 14.8ml
    c. Ten media changes correspond to 12mls of media
    d. Summation 14.8 + 12 = 26.8ml; rounding up to 30ml
    e. Need 30ul CellTox^{TM} Green Dye reagent placed into 30ml culture media
2. Day 0: Seed SciFlow using culture media **without** CellTox^{TM} Green Dye reagent
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. Add drug/toxicant into the dose well (well-1). This 150ul volume also contains CellTox™ Green Dye reagent.
      i. 150ul x 8 rows = 1.2ml media plus 1.2ul CellTox™ Green Dye reagent.
   d. Monitor fluorescence at any time (real time data acquisition)
4. Day 2 through Day 12: Once per day, remove 150ul from sink (well 12).
   a. Add drug/toxicant to dose well (well-1). This 150ul that also contains CellTox™ Green Dye reagent.
      i. 150ul x 8 rows = 1.2ml media plus 1.2ul CellTox™ Green Dye reagent.
   b. Monitor fluorescence at any time (real time data acquisition)
5. End protocol

<u>Protocol (Alternative using smaller amounts of CellTox™ Green reagent):</u>

In some cases, it may be desirable to retain the original media in SciFlow (i.e. don't remove media without CellTox™ Green). The following protocol uses a 10x stock of CellTox™ 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™ 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™ Green will have slightly different concentrations in the different wells due to different dilutions. CellTox™ 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)

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 (triplicate rows)		Cellular Model (e.g. HepaRG cells @ 40,000 cells/well)									
C												
D												
E	Vehicle control (triplicate)		Cellular Model (e.g. HepaRG cells @ 40,000 cells/well)									
F												
G												
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