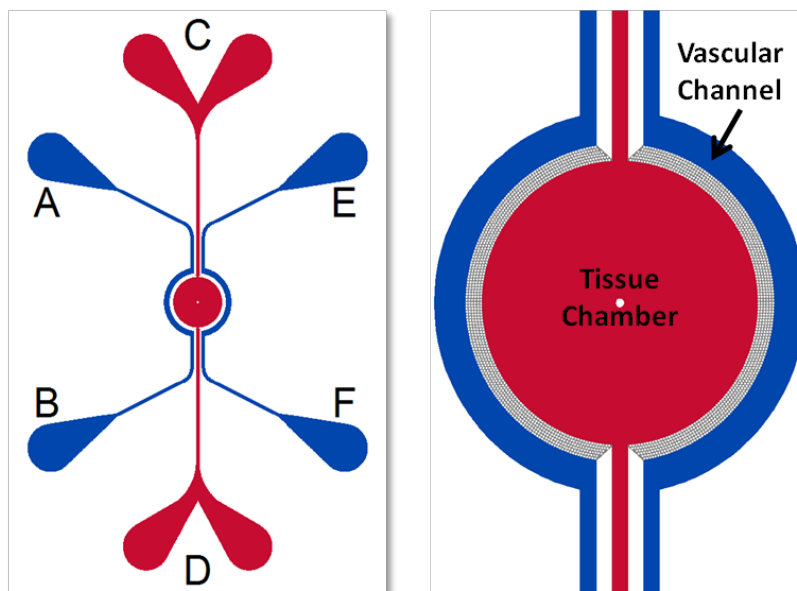


3D Toxicology Model using SynTox Idealized Network Kits and Chips – Technical Manual

Catalog #s 404001, 404002, 102016-STo



Schematic of the SynTox Model Chip. Vascular channels are for culture of endothelial cells) while the central chamber is for culture of tissue cells (smooth muscle cells, fibroblast, hepatocytes, cardiomyocytes, etc.). Porous architecture enables communication between the vascular and tissue cells.

Overview of the SynTox Model

SynTox is the only commercially available 3D toxicology model with real-time optical monitoring and multi-compartment, multi-cellular architecture and low reagent requirements. Other benefits of this platform are:

- Physiologically realistic morphological, fluidic and 3D cellular conditions
- Universal platform with architecture specific of desired organ
- Significant reduction in cost and time
- Robust and easy to use protocols
- Compatible with standard analytical instruments for both on chip and off chip assays including omic methodologies for systems biology and bioinformatic analysis

Material, Equipment and Cells Required

- SynTox Chips (Catalog # 102016-STo): Use Ports A, C and E as Inlets and B, D and F as Outlets
- SynVivo Pneumatic Primer Device (Catalog #205001)
- 1 mL BD plastic syringes or other 1 mL syringes (Catalog # 203004)
- 24 gauge blunt tip needles (Catalog # 204002)
- Tygon microbore tubing, 0.02" ID X 0.06" OD (Catalog # 201005)
- Clamps (Catalog # 202003)
- Forceps
- Syringe Pump capable of flow rates from 10nl/min to 10ul/min
- Fibronectin or any other extracellular matrix
- Endothelial Cells (primary or cell line)
- Tissue Cells (primary or cell line)

The following protocol should be carried out within a laminar flow hood to maintain sterility.

Culture of Endothelial Cells in the Apical Channels

Note: Most primary endothelial cells are usable only until passage 8. For endothelial cell lines, refer to vendor specifications for passage information. Most endothelial cell lines do not sustain shear stress which is required for formation of tight junctions.

A. Coating The Device with Endothelial Cell Culture Matrix (e.g. fibronectin). This process is performed using Pneumatic Primer (SynVivo Cat# 205001).

1. Place approximately 1 inch long segments of Tygon tubing into the outlet ports of the device.
2. Draw 200ug/ml human fibronectin into a 1 mL syringe.
3. Using additional 1inch long segments of tubing, fill the device with liquid by inserting the primed tubing into the inlet and pushing the solution through until the outlet tubing is filled.
4. Do this for all but one inlet port. For this last port, use a tubing approximately 2-3 inch long. When the device is filled, unlock the needle from the syringe, leaving the needle attached to the tubing.
5. Clamp all tubing below the liquid line, except for the tubing with the needle attached.
6. Connect the device to the Pneumatic Primer by locking the needle into the LuerLock connector on the box. *Note: Multiple devices can be primed simultaneously using the multiple port manifold, available from SynVivo (cat # 207001)*
7. Turn the knob on the controller box and adjust the pressure to ~5-7 psi. Apply the pressure for ~5-20 minutes. Tumor devices will take at least 15 minutes to completely fill.
8. Turn off the pressure and cut the Tygon tubing connected to the Pneumatic Primer.
9. Allow the device to incubate at 37° for a minimum of 1 hour before use.
10. Flush fresh media into device just before seeding endothelial cells.

B. Culture of Endothelial Cells

1. Prepare the endothelial cell suspension for seeding. Endothelial cells should be dissociated, centrifuged and concentrated to approximately $5-8 \times 10^6$ cells/ml in cell specific media.
2. Place a drop of water at the base of the inlet port tubing to be removed and gently remove the tubing from the port.
3. Remove the clamp on the outlet port. All channels should be clamped except for the channel being seeded.
4. Prepare a syringe and tubing with the previously prepared cell suspension and mount onto a syringe pump.
5. Ensure that the tubing is free of air bubbles and the cell mixture is flush with the end of the tubing

6. Insert the tubing into the port - the drop of water will prevent air entering the device as the tubing is inserted.
7. Clean the fluid from the surface of the device.
8. Begin the injection at 4-7 $\mu\text{L}/\text{min}$.
9. Watch the device as the cells are flowing. Once the vascular channel is filled with cells, stop the flow and clamp the outlet tubing.
10. Carefully and quickly cut the inlet tubing, keeping the length of all the tubing equal.
11. Allow the cells to attach for at least 4 hours before changing media.
 - a. Many endothelial cells can be incubated overnight before a media change is required.
12. Allow the cells to grow approximately 24 hours with at least 1 media change before running migration assay.
 - a. It should be noted that some endothelial cells may need up to 72 hours (with daily media changes) before they can withstand flow.
 - b. To set up a media change program using a syringe pump, use the following steps:
 - i. Program the pump to flush out the vascular channel without cells every 3 hours at $2\mu\text{L}/\text{min}$ for 3 minutes to refresh the media.
 1. Program summary: Media Change
 - a. Step 1: Constant Rate
 - i. Mode: Infuse
 - ii. Set rate: $2\mu\text{L}/\text{min}$
 - iii. Time: 0:03:00 (3 minutes).
 - b. Step 2: Pause
 - i. Mode: Pause
 - ii. Target time: 3:00:00 (3 hours)
 - c. Step 3: Repeat from Step 1
13. For complete confluent monolayers, introduce flow using programmable syringe pump with the flow rate climbing from $0.01\mu\text{L}/\text{min}$ to $1\mu\text{L}/\text{min}$ over a 24-hour period (Table 1).
 - i. The program example uses the “Step” feature of the pump, which infuses media at a user-defined flow rate over a specific period of time.

Table 1: Step programming to increase the flow rate from 0.01 to 1 $\mu\text{L}/\text{min}$ over 24 hours

SEQ	Flow Rate ($\mu\text{L}/\text{min}$)	Time (h:m:s)	Direction
1	0.01	2:00:00	INFUSE
2	0.025	2:00:00	INFUSE
3	0.05	2:00:00	INFUSE
4	0.075	2:00:00	INFUSE

5	0.1	2:00:00	INFUSE
6	0.2	2:00:00	INFUSE
7	0.3	2:00:00	INFUSE
8	0.4	2:00:00	INFUSE
9	0.5	2:00:00	INFUSE
10	0.6	2:00:00	INFUSE
11	0.8	2:00:00	INFUSE
12	1	2:00:00	INFUSE

Co-Culture of Endothelial Cells with Tissue Cells or Conditioned Media

Interactions between vascular and tissue cells are readily visualized in the SynTox model. Endothelial cells are cultured under flow in the vascular channel, and the tissue chamber is cultured with tissue cells.

1. All tubing should be clamped before beginning this procedure.
2. Tissue cells should be dissociated, centrifuged and concentrated to approximately $1-3 \times 10^7$ cells/mL.
3. Place a drop of water at the base of the inlet port (C) tubing to be removed.
4. Gently remove the tubing - the water should cover the port once the tubing is removed, and should remain there until new Tygon tubing is inserted into the port. This step will prevent air from entering the device.
5. Remove the clamp on the outlet port (D).
6. Prepare a syringe and tubing with the cell suspension and mount onto a syringe pump.
7. Ensure that the tubing is free of air bubbles and allow a convex meniscus to form at the end of the tubing.
8. Insert the tubing into the port (C)—the meniscus and the drop of water will form a bridge to prevent air from entering the device as the tubing is inserted.
9. Clean the fluid from the surface of the device.
10. Begin the injection at 5 μ L/min.
11. Observe the device as the cells are flowing. Once the tissue chamber is filled with cells, clamp the outlet tubing (port D) and stop the flow.
12. Cut the inlet tubing approximately 1 inch above the device, and then clamp. This will help disperse the cells more evenly throughout the vascular chamber.
13. Allow tissue cells to attach for at least 4 hours before changing media.
14. Change media regularly until cells are confluent or time of experiments.
15. For conditioned media from tissue cells, repeat above process without cells.

Drug Permeability Assay

Unlike Transwell models that are arranged in top to bottom architecture small molecule transport can be assessed and quantified in real-time across the SynTox model due to its side-by-side architecture. A fluorescently-labeled drug molecule of interest is perfused through the vascular channels at physiological flow rate. Real-time videos are acquired and analyzed to calculate the rate of permeability into the tissue chamber. Different rates of permeability are observed across the endothelium due to tight junctions of endothelial cells. Permeability through the vascular channel into the tissue chamber is measured by observing diffusion of a fluorescently labeled molecule. The permeability is related to the rate of change of fluorescent intensity in the tissue chamber by the equation [1]:

$$P = (1 - H_{CT}) \frac{1}{I_{V0}} \frac{V}{S} \frac{dI_t}{dt} \quad (1)$$

H_{CT} is the hematocrit count (equal to 0 for in vitro measurements), I_{V0} is the fluorescent intensity in the apical channel containing the endothelial cells, V/S is the ratio of apical volume to surface area (in this case, calculated for a 200 μm wide and 100 μm height vascular channel), and I_t is the fluorescent intensity in the basolateral chamber.

Parameter	Description	Value
H_{CT}	Local Hematocrit	0 (in vitro)
I_{V0}	Apical Intensity	Calculated below
V/S	Ratio of Apical Volume to Surface Area	0.1 cm
dI_t/dt	Change in Basolateral Intensity over Time	Calculated below

A. Video Acquisition

1. Prepare a desired concentration fluorescent-tagged molecule (e.g. 0.1 mM FITC-Dextran (3kDa)) solution in PBS, and load the solution into a 1 cc syringe with a 24 gauge needle attachment with Tygon tubing. Set the syringe on the syringe pump and fully perfuse the tubing with the solution.
2. Place the cell-coated device onto the stage of an epifluorescence microscope. Place a drop of water around the inlet tubing, and remove tubing.
3. Set the camera exposure, and stage of the microscope to capture both the apical and basolateral channels of the device in one image.
4. Interface the perfused tubing containing the fluorescent small molecule to the device, and begin flow at 0.1 $\mu\text{L}/\text{min}$. Immediately begin to acquire images every 30 s for 1 hour. (Note: close the fluorescence shutter between image acquisitions in order to prevent photobleaching).
5. Save the image stack as an AVI.

B. Video Analysis using ImageJ

1. Open ImageJ and import the acquired video as an AVI:

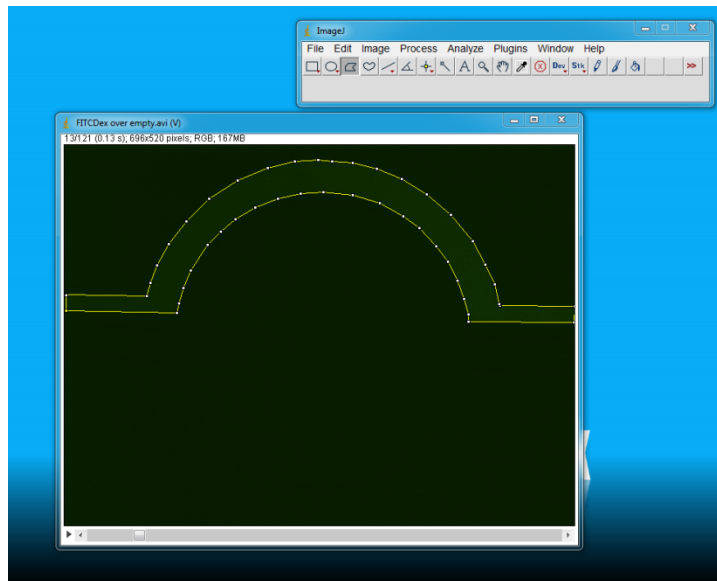
File→Import→AVI

Select video file

In the AVI Reader box, check “Use Virtual Stack” and click OK

The horizontal scroll bar will allow you to scroll through the stack

2. Using polygon selections tool, carefully outline the vascular channel geometry. You may need to scroll to a high contrast section to clearly see the outline.



3. Set the Measurements to measure the mean value of the selection:

Analyze→Set Measurements

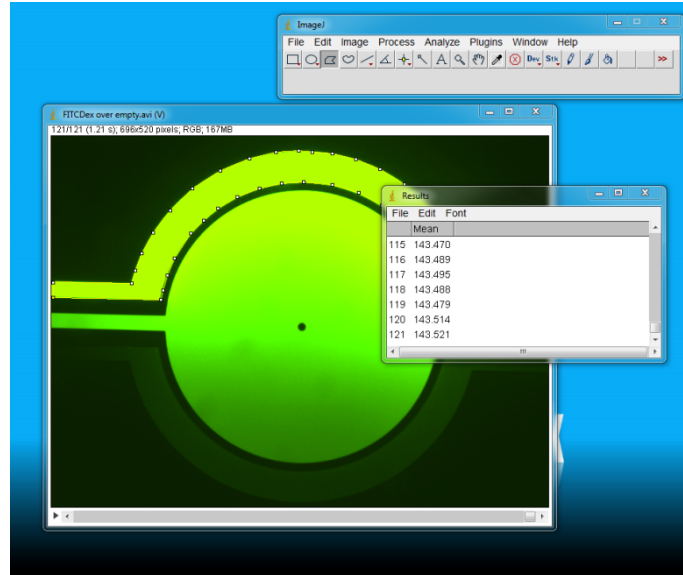
Select “Mean Gray Value”

Under “Redirect to”, select the file name

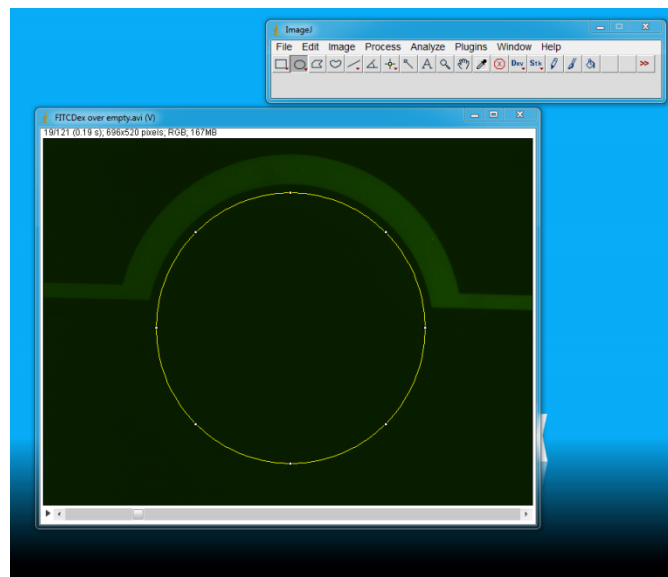
4. Analyze the mean gray value of the stack:

Plugins→Stacks→Measure Stack

Copy data from “Results” window to Excel for analysis. I_{vessel} will be generated from this data.

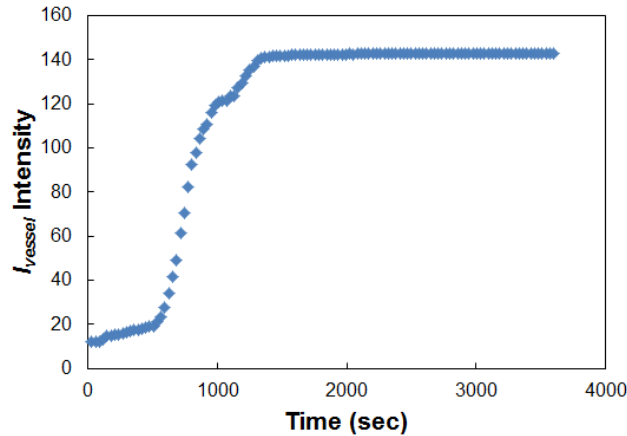


5. Deselect the vascular chamber, and select the tissue chamber using the elliptical selections tool.

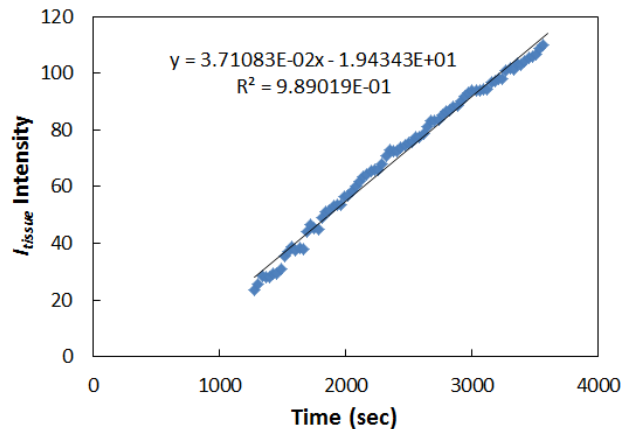


6. Repeat gray value measurement of the stack:
Plugins→Stacks→Measure Stack
Copy data from “Results” window to Excel for analysis. I_{tissue} will be generated from this data.
7. There should be three columns of data in Excel: the frame number, the I_{vessel} data and the I_{tissue} data. Calculate time in seconds by multiplying each frame number by 3600 and dividing by the number of frames (121).

8. Plot I_{vessel} vs. time in an Excel scatter plot. The plot should show an increase in I_{vessel} intensity, and then a plateau. Note the time point at the beginning of the plateau. The average gray value intensity of the plateau area will be I_{v0} in Equation [1]. In this example, $I_{v0} = 142.5$.



9. Next, find the slope of I_{tissue} vs. time. Only use data in the time range specified by the I_0 average above. Plot I_{tissue} data in the y-axis, and time in the x-axis. Display the trend line with its equation on the graph to determine the slope.



10. The slope of I_{tissue} is equal to I_t/dt in Equation [1] to calculate permeability in cm/s. From the example above, $I_t/dt = 3.7 \times 10^{-2} \text{ s}^{-1}$. For the given example, Equation 1 is calculated:

$$P = (1 - 0) \frac{1}{140.5} (0.1 \text{ cm}) (3.7 \times 10^{-2} \text{ s}) = 0.0121 \text{ cm/s}$$

Drug Toxicity Assay

The SynTox model allows drug toxicity assays under different modes of interaction; static, direct flow on tissue cells and vascular flow mediated tissue cell drug interactions, which are observed in vivo. Both bolus injection and continuous perfusion of drugs can be readily evaluated.

1. Prepare a desired concentration of the drug molecule solution into a 1 cc syringe with a 24 gauge needle attachment with Tygon tubing. Set the syringe on the syringe pump and fully perfuse the tubing with the solution.
2. Place the cell-coated device onto the stage of an epifluorescence microscope. Place a drop of water around the inlet tubing, and remove tubing.
3. Interface the perfused tubing containing the drug molecule to the inlet port of the device and begin flow at 0.1-5 $\mu\text{L}/\text{min}$. Switch to drug free media after 30 minutes of flow or use under continuous perfusion.
4. At the end of respective time points, perform Live/Dead assay or other off-chip and on-chip analysis for understanding the results of the experiments. For direct exposure, inject drug directly to tissue cells and compare the responses.

References

[1] Yuan H., Gaber M. W., McColgan T., Naimark M. D., Kiani M. F., Merchant T. E. Radiation-induced permeability and leukocyte adhesion in the rat blood–brain barrier: modulation with anti-ICAM-1 antibodies. Brain Res 969, 59-69 (2003).

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