



SYNBBS 3D BLOOD BRAIN BARRIER MODEL TECHNICAL MANUAL

For Kits and Chips: Catalog #s 402001, 402002, 402003, 402004,
402005, 402006, 102005-SB, 102015-SB, 108011-SB

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Introduction

SynVivo's SynBBB 3D blood brain barrier model recreates the *in vivo* microenvironment by emulating a histological slice of brain tissue cells in communication with endothelial cells across the blood brain barrier (BBB). Shear-induced endothelial cell tight junctions, which cannot be achieved in the Transwell® model, are easily achieved in the SynBBB model using physiological fluid flow. Formation of tight changes can be measured using biochemical or electrical analysis (assessing changes in electrical resistance) with the SynVivo Cell Impedance Analyzer. Interactions between brain tissue cells and endothelial cells are readily visualized in the SynBBB assay (**Figure 1**).

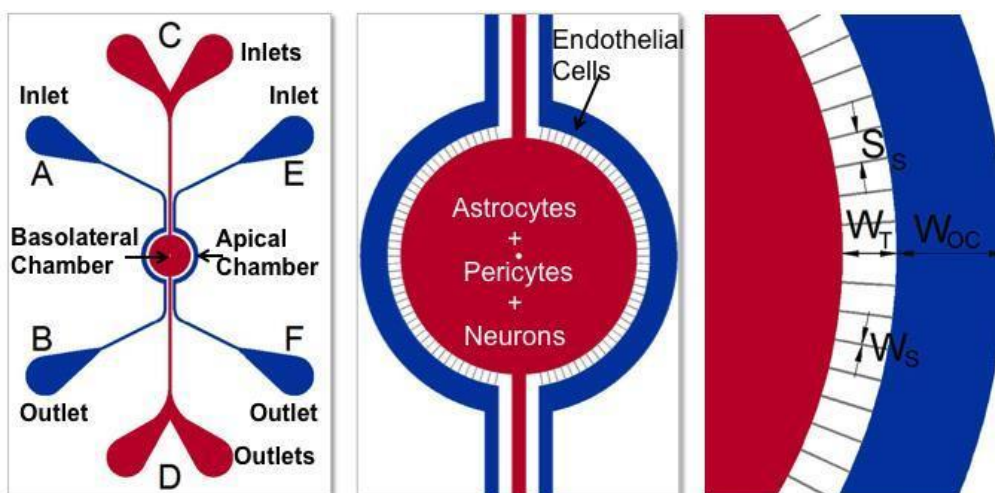


Figure 1. Schematic of the BBB Model. Apical chamber (outer channels) are for culture of vascular (endothelial cells) while basolateral chamber (central chamber) are for culture of brain tissue cells (astrocytes, pericytes, neurons). Porous architecture enables communication between the vascular and tissue cells.

The SynBBB system is a highly versatile platform for investigation of:

- Drug permeability: Evaluate real-time permeability of therapeutics and small molecules across the endothelium of the BBB.
- Inflammation: Understand the underlying mechanisms of inflammatory responses on the regulation of the BBB.
- Cell migration: Visualize and quantify in real-time migration of immune cells across the BBB.
- Omic changes: Perform genomic, proteomic and metabolic analysis on normal and dysfunctional BBB.
- Neurotoxicity: Analyze toxicity effects of chemical, biological and physical agents on the cells of the BBB.





For further information on how the SynBBB model has been used in research, see the following supporting information:

1. Yuan H., M. W. Gaber, T. McColgan, M. D. Naimark, M. F. Kiani, and T. E. Merchant. 2003. Radiation-induced permeability and leukocyte adhesion in the rat blood–brain barrier: modulation with anti-ICAM-1 antibodies. *Brain Res.* 969: 59-69.
2. Prabhakarandian, B., M. C. Shen, J. B. Nichols, I. R. Mills, M. Sidoryk-Wegrzynowicz, M. Aschner, and K. Pant. 2013. SyM-BBB: a microfluidic blood brain barrier model. *Lab Chip.* 13(6):1093-101.
3. Deosarkar, S. P., B. Prabhakarandian, B. Wang, J. B. Sheffield, B. Krynska, and M. F. Kiani. 2015. A novel dynamic neonatal blood-brain barrier on a chip. *PLoS One.* 10(11):e0142725.
4. Terrell-Hall, T. B., A. G. Ammer, J. I. Griffith, and P. R. Lockman. 2017. Permeability across a novel microfluidic blood-tumor barrier model. *Fluids Barriers CNS*, 14(1):3.
5. Terrell-Hall, T. B., M. I. Nounou, F. El-Amrawy, J. I. G. Griffith, and P. R. Lockman. 2017. Trastuzumab distribution in an in-vivo and in-vitro model of brain metastases of breast cancer. *Oncotarget.* 8(48):83734-83744.

Icons used in the Manual:

Many of the protocols will have special safety considerations or time constraints that are important to remember. **Table 1** lists the symbols that will be used to indicate these experimental considerations. Time sensitive assays and specific temperature conditions will be important for ensuring that the model is established successfully.

Table 1. Laboratory Safety Symbols and Icons for Experimental Conditions

<u>Icon</u>	<u>Safety/Experimental Condition</u>
	Temperature
	Time sensitive
	Hazard
	Special consideration

Tissue Culture

Principle:

This protocol describes how specific primary cells or cell lines used during training are maintained in tissue culture or dissociated for seeding.

Primary Cells or Cell Line(s):

- Human umbilical vascular endothelial cells (HUVECs; Lonza C2519A)
- Human astrocytes (HA; ScienCell 1800)
- Human brain vascular pericytes (HBVP; ScienCell 1020)

Reagents:

- 1X PBS without calcium or magnesium (Corning 21040CV)
- Poly-L-lysine (Sigma Aldrich P4707)
- Astrocyte medium (ScienCell 1801)
- Pericyte medium (ScienCell 1201)
- EGM-2 basal media (Lonza CC-3156)
- EGM-2 Supplement Kit (Lonza CC-4176)
- 1X TrpLE Express cell detachment solution, no phenol red (ThermoFisher Scientific 12604013)
 - Can be substituted with Trypsin-EDTA for Primary Cells (ATCC PCS-999-003); follow the manufacturer's guidelines
- HEPES buffered saline (Lonza CC-5022)

HUVEC Thawing and Cell Propagation Protocol:

The following protocols should be carried out within a laminar flow hood as much as possible to maintain sterility.

Adherent or suspension: Cells are adherent.

Growth Media: EBM-2 basal media (Lonza CC-3156)
EGM-2 Supplement Kit (Lonza CC-4176)

Flask coating required: Use human fibronectin (2 $\mu\text{g}/\text{cm}^2$) or 0.1% gelatin

Freeze Media: Complete endothelial medium with 5% (v/v) DMSO

Coating T-25 flasks with fibronectin:

1. Dilute 120 μ l of 1.06 mg/mL fibronectin in 5 ml of serum-free DMEM (final fibronectin concentration is approximately 2 μ g/cm²).
2. Pipette 2.5 mls of the fibronectin solution into each T-25 flask.
3. Incubate flask for 1 hour to overnight in a tissue culture incubator at 37°C, 5% CO₂.
4. Remove fibronectin solution from the T-25 flasks.
5. The flask is now coated and ready for seeding endothelial cells.

Thawing and seeding endothelial cells:

Note: This protocol will describe how to expand the HUVECs for both experimental purposes and for freezing down the cells. Depending on where the HUVECs are purchased from, follow the manufacturer's recommendations for preparing the freezing media and cryopreservation.

1. Prepare two T-25 flasks with the fibronectin coating.
2. Add 10 mL of endothelial cell media to each flask.
3. Remove vial of cells from liquid nitrogen storage.
4. Thaw vial in a 37°C water bath for 2 minutes, swirling the vial until only a small piece of ice remains in the vial.
5. Spray the vial with alcohol, and place it in the laminar flow hood.
6. Open the vial, and dispense half of the vial's volume into one T-25 flask.
7. Pipette the remaining half of the vial's volume into the second T-25 flask.
8. Add 1 mL of endothelial cell media into the vial to rinse, and dispense half of the solution into each of the cell culture flasks.
9. Incubate the cells overnight in a tissue culture incubator at 37°C, 5% CO₂.
10. Change the media the next day.
11. Change media every 1-2 days until the flask becomes confluent (70-80% coverage).
 - a. Do not allow the cells to reach 100% confluency. The tight junctions will be strong, and the cells will form a tissue. Dissociating this "tissue" into a single cell suspension will mean greater cell loss and extensive cell stress.
12. Split cells (1:2 or 1:3) into fibronectin-coated T-25 flasks.

- a. We use Gibco TrpLE Express to dissociate the cells, but always consult the manufacturer's guidelines for dissociation and quenching solutions.
 - b. Use complete cell media to wash cells from the flask.
13. Wash the cells twice with complete endothelial cell media prior to plating.
- a. Washing is critical, since these cells are very sensitive to cell signals that could indicate stress/dead/dying cells.
14. Change media every 1-2 days until the flasks become confluent (70-80%).
- a. Usually the cells will start to propagate a little faster, but the feeding schedule should remain the same.
15. Freeze down the contents from an entire T-25 flask into one cryovial.
- a. For a confluent T-25 flask, the cell numbers are around 750,000 cells or greater, depending on division rates and cell viability after dissociation.

Dissociating endothelial cells from the flask:

1. Flush all channels of the pre-coated SynVivo device with complete endothelial cell media.
 - a. This step will insure that the entire device is primed with endothelial cell media prior to seeding.
2. Remove and discard the endothelial cell media from the T-25 flask of endothelial cells.
3. Rinse the endothelial cells twice with room temperature HEPES buffered saline.
 - a. Gently rock the flask in between rinses.
 - b. Remove final rinse and discard.
4. Add 2 mL of room temperature TrypLE Express Cell Dissociation Reagent to the flask.
5. Incubate flask at 37°C for **3-5 minutes** for **HUVECs**.
 - a. These incubation times are cell type specific.
6. Add 3 mL of complete endothelial cell media to the flask to neutralize the dissociation reagent.
7. Gently wash the sides of the flask with complete endothelial cell media to remove the adherent cells.
8. Pellet the cells by centrifugation.



- a. Use 200 x g for 5 minutes at room temperature (do not use speeds greater than 1000 RPM).
9. Remove the media, leaving behind at least 100 µl to cover the cell pellet.
10. Wash the cells twice with 5 mL complete endothelial cell media, and pellet the cells by centrifugation.
 - a. Use 200 x g for 5 minutes at room temperature (do not use speeds greater than 1000 RPM).

Human Astrocytes or Pericytes: Thawing and Cell Propagation Protocol:

The following protocols should be carried out within a laminar flow hood as much as possible to maintain sterility.

Adherent or suspension: Cells are adherent.

Growth Media: Use specially formulated medias recommended by manufacturer

Flask coating required: Poly-L-lysine

Freeze Media: Follow guidelines provided by manufacturer for freezing primary cells.

Coating T75 flasks with poly-L-lysine:

1. Pipette 2 mLs of poly-L-lysine solution into each T75 flask.
2. Incubate flask for 1 hour in a tissue culture incubator at 37°C, 5% CO₂.
3. Remove the poly-L-lysine solution from the T75 flask and discard.
4. The flask is now coated and ready for seeding astrocytes or pericytes.

Thawing and seeding astrocytes or pericytes:

1. Prepare T75 flasks with the poly-L-lysine coating.
2. Add 15 mL of cell media to the flask.
3. Remove vial of cells from liquid nitrogen storage.
4. Thaw vial in a 37°C water bath for 2 minutes, swirling the vial until only a small piece of ice remains in the vial.
5. Spray the vial with alcohol, and place it in the laminar flow hood.

6. Open the vial, and dispense the entire contents into the prepared media flask.
7. Add 1 mL of cell media into the vial to rinse, and dispense the solution into the cell culture flask.
8. Incubate the cells overnight in a tissue culture incubator at 37°C, 5% CO₂.
9. Change the media the next day.
10. Change media according to the manufacturer's recommendations until the flask becomes confluent (70-80% coverage).

Dissociating astrocytes or pericytes from the flask:



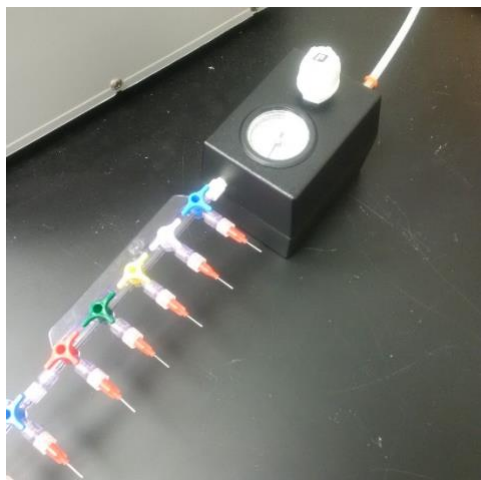
1. Flush the tissue chamber of the fibronectin-coated SynVivo device with complete cell media.
 - b. This step will insure that the tissue chamber is primed with fresh cell media prior to seeding.
2. Remove and discard the cell media from the T75 flask of astrocytes or pericytes.
3. Rinse the cells twice with room temperature HEPES buffered saline.
4. Gently rock the flask in between rinses.
5. Remove final rinse and discard.
6. Add 2 mL of room temperature TrypLE Express Cell Dissociation Reagent to the flask.
7. Incubate flask at 37°C for **3-5 minutes**.
8. Add 3 mL of complete cell media to the flask to neutralize the dissociation reagent.
9. Gently wash the sides of the flask to remove the adherent cells, and pipette cells into a 15-mL conical tube.
10. Pellet the cells by centrifugation.
11. Use 200 x g for 5 minutes at room temperature (do not use speeds greater than 1000 RPM).
12. Remove the media, leaving behind at least 100 µl to cover the cell pellet.
13. Wash the cells twice with 5 mL complete cell media, and pellet the cells by centrifugation (200 x g for 5 minutes at room temperature).
14. Seed devices as described in other sections.

Laboratory Equipment for Microfluidics Experiments

Principle:

In addition to the microfluidics devices, the following list describes the laboratory equipment needed to run microfluidics experiments using the SynVivo platform.

Pneumatic Primer with Multiple Port Manifold



The Pneumatic Primer (SynVivo 205001) is a pressure regulator that flows inert gas at a low pressure through the SynVivo devices. A multiple port manifold (SynVivo 207001) is connected to the primer and allows perfusion of multiple microfluidics devices at once.

Inert gas cylinder



The source of inert gas used with the Pneumatic Primer can be nitrogen or argon. Ultra-high purity gases are recommended for use in this system.

Inverted Microscope



An inverted microscope is used to examine the devices for trapped air, as well as for live imaging when the devices are being seeded with cells. Microscopes with a fluorescence capability can aid in imaging the devices after staining with fluorescent dyes or conjugated antibodies.

Syringe Pump for Seeding Cells



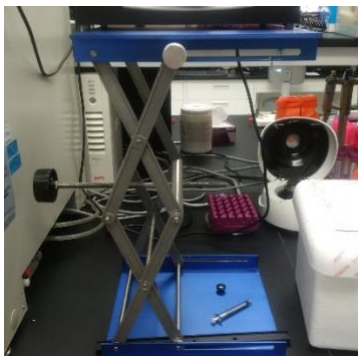
A syringe pump with an injector unit (PHD Ultra, Pump 11 Elite Nanomite is shown; Harvard Apparatus) is used to seed cells in the devices. The injector unit of the syringe pump is light enough to rest on a microscope stage for real-time viewing and imaging of cell seeding.

Syringe Pump for Media Changes or Constant Flow



A syringe pump (PhD Ultra is shown; Harvard Apparatus; SynVivo 301001 or 301002) is used to maintain media flow to cells inside the devices. A multi-syringe rack can provide space for multiple syringes and increase the number of microfluidics devices receiving media at one time.

Lab Jack



A lab jack with 12-inch x 12-inch plate (30.48 cm x 30.48 cm; Fisher Scientific S63083) is used to safely elevate the syringe pump beside a tissue culture incubator. The syringe pump should be higher than the shelf with the microfluidics device, so that fluid is not being flowed uphill against gravity.

Tissue Culture Incubator



A standard tissue culture incubator is used to incubate tissue culture or primary cells in the microfluidics devices.

Stainless Steel Scissors and Forceps



Both stainless steel forceps (ultra-fine; VWR 100494-698) and scissors (Fisher Scientific 19-062530) are used to cut and insert tubing into the microfluidics devices.

Setting Up Syringe Pumps Around an Incubator

Principle:

The following protocol describes some of the best methods to set up a syringe pump beside a tissue culture incubator. The important considerations to keep in mind include minimizing the length of the tubing between the syringe pump and the device, as well as making the syringe pump easily accessible to the user.

Equipment:

- SynVivo microfluidics device
- Tygon tubing (0.02 inch ID x 0.06 inch OD, or 0.05 cm ID x 0.15 cm OD; SynVivo 201005)
- Slide clamps (SynVivo 202001)
- 1 mL syringe with Luer-Lok tip (25 pack; SynVivo 203005)
- 24-gauge blunt-tipped needles (0.5 inches or 1.27 cm long; SynVivo 204003)
- Pneumatic Primer (SynVivo 205001)
- Forceps (ultra-fine; VWR 100494-698)
- Stainless steel scissors (Fisher Scientific 19-062530)
- Syringe pump (PhD Ultra; Harvard Apparatus; SynVivo 301001 or 301002)
- Lab jack with 12-inch x 12-inch plate (30.48 cm x 30.48 cm; Fisher Scientific S63083)
- Tissue culture incubator

Reagents:

- Cell culture media

Protocol:

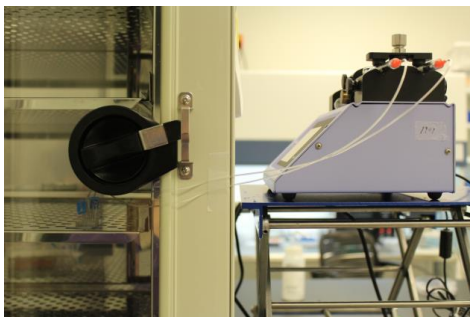


Figure 1. A syringe pump on a lab jack (right) is connected to a microfluidics device in a tissue culture incubator (left).

1. Place the syringe pump on the lab jack (Figure 1).

2. Set the height of the lab jack so that the syringe platform is higher than the microfluidics device (**Figure 2**).

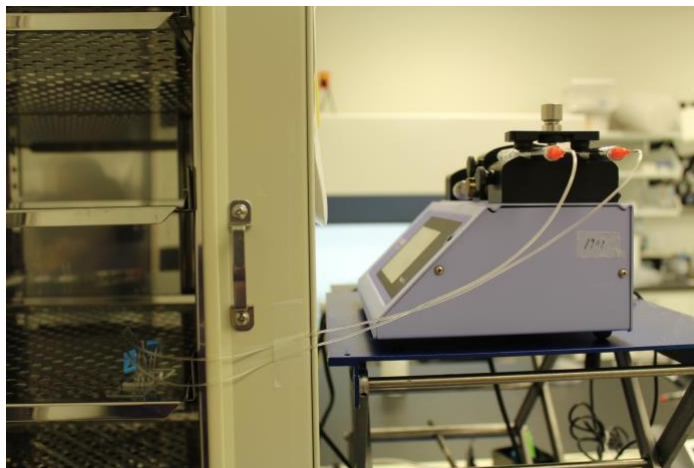


Figure 2. Use the lab jack to elevate the syringe pump so that the syringe platform is higher than the shelf with the microfluidics device.

3. Make sure that the tubing is long enough to reach the device, but not too long.
4. Use tape to secure the tubing to the incubator and prevent accidental snagging (**Figure 3**).

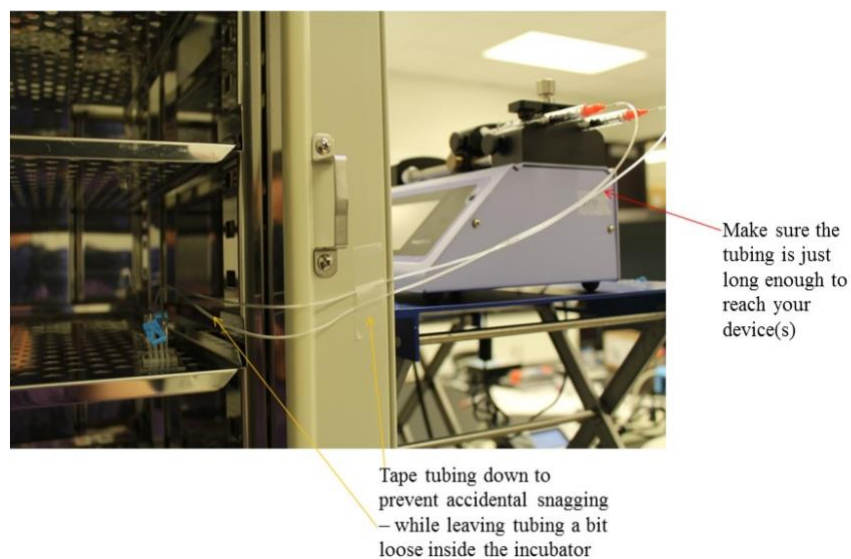


Figure 3. The length of tubing needs to reach from the syringe to the microfluidics device, without a significant amount of excess tubing. Tape down any lengths of tubing to prevent snagging.

5. Close the door of the tissue culture incubator on the tubing.
6. The gasket between the door of the incubator and the chamber will prevent the tubing from being crushed and will allow liquid to flow (**Figure 4**).

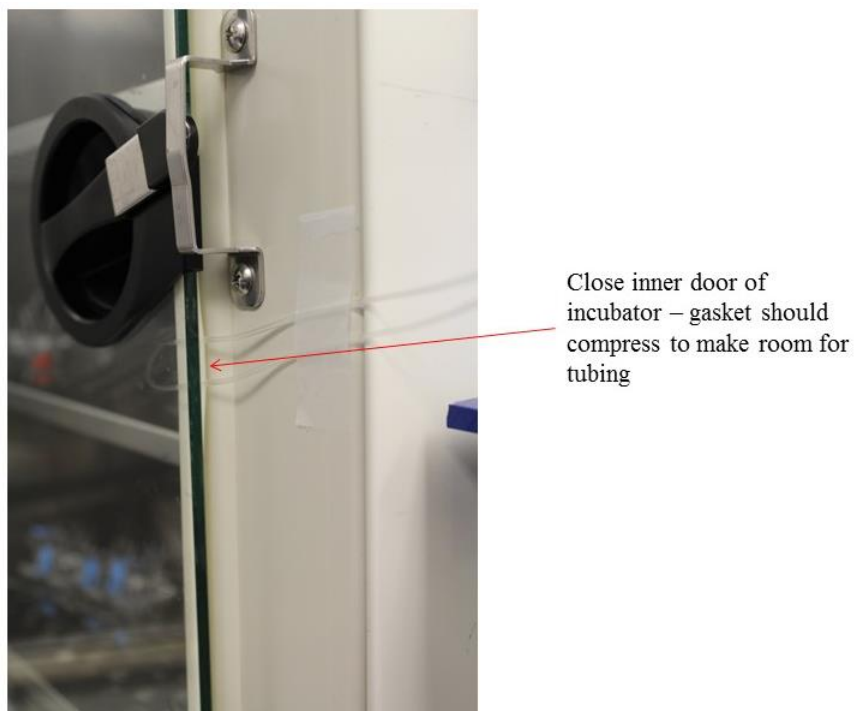


Figure 4. Close the door of the tissue culture incubator on the tubing. The tubing should be caught between the chamber gasket and the door of the incubator.

Setting up the Pneumatic Primer

Principle:

The Pneumatic Primer is a pressure regulator that flows inert gas at a low pressure through the SynVivo devices. The Pneumatic Primer consists of an inlet connector that connects the microfluidics device to the pressure regulator (**Figure 1A**). On the opposite side of the pressure regulator box, a metal male connector (**Figure 1B**) will join the pressure regulator to nylon tubing containing inert gas (**Figure 1C**; use ¼ inch or 0.635 cm outer diameter; 11/64 inch or 0.436 cm inner diameter nylon tubing).



Figure 1. A top view of the Pneumatic Primer, containing an inlet connector for the device (A), a metal male connector to the gas line (B), and nylon tubing that connects to the gas tank (C).

If the source of the inert gas is industrial grade, the gas may contain particles that could potentially contaminate the device during perfusion. The ¼-inch outer diameter, Push-in Serviceable Filter that comes with the Pneumatic Primer kit can be used to filter any particles from the flow of gas (**Figure 2A**). Three replaceable filters are also included with the Pneumatic Primer kit, and the 5-micron filters should be replaced every 3-6 months depending on usage.

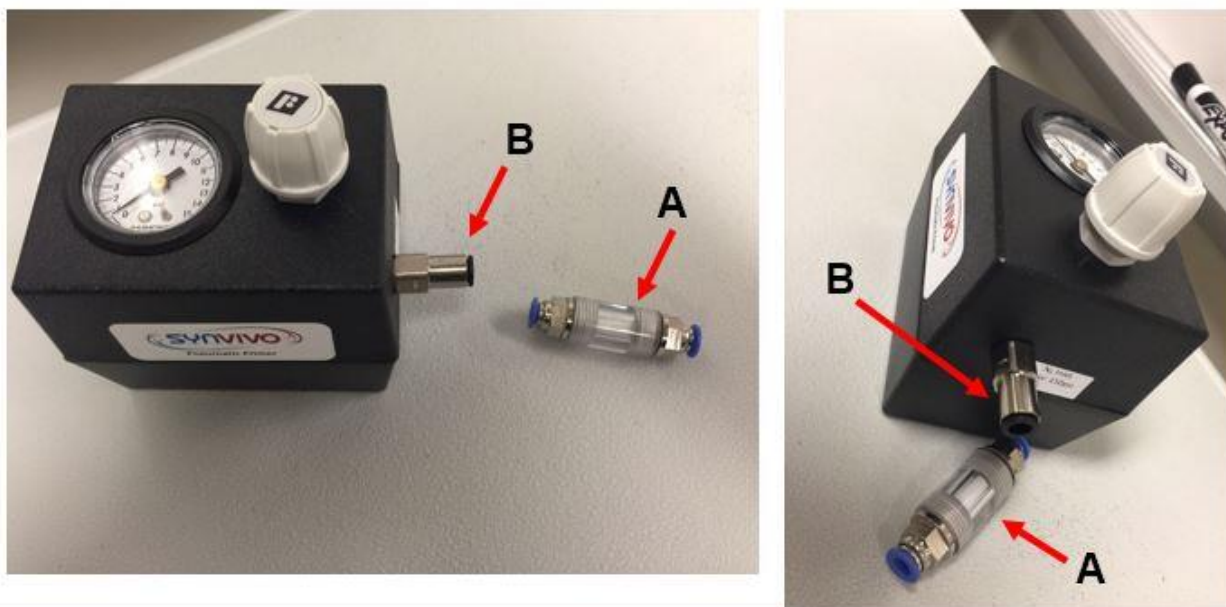


Figure 2. Visual comparison of the 1/4 inch (0.635 cm) outer diameter (OD) push-in serviceable filter (A), to the metal male connector on the Pneumatic Primer Box (B).

Equipment:

- Pneumatic Primer (SynVivo 205001)
 - Inlet connector
 - LEGRIS 1/4 inch (0.635 cm) metal male connector (Grainger 1PFG1)
 - 1/4 inch (0.635 cm) outer diameter Push-in Serviceable Filter (Industrial Specialties Mfg. PIF25-14-PP-5)
 - 3 porous polypropylene elements (5 micron; Industrial Specialties Mfg. PIF25-RF-PP-5)
- Multiple port manifold for the Pneumatic Primer (SynVivo 207001; optional)
- Nylon tubing (1/4 inch or 0.635 cm OD; 11/64 inch or 0.436 cm ID; Grainger 2VDL8)
- Gas cylinder, pressure regulator, and adapters for 1/4 inch (0.635 cm) OD nylon tubing
- SynVivo microfluidics device
- Tygon tubing (0.02 inch or 0.0508 ID; 0.06 inch or 0.1524 cm OD; SynVivo 201005)
- Slide clamps (SynVivo 202001)
- 24-gauge blunt-tipped needles (0.5 inch or 1.27 cm long; SynVivo 204003)

Protocol:

1. The user is responsible for purchasing the gas cylinder, the appropriate pressure regulator, tubing adapters to fit the specifications of the nylon tubing (1/4 inch or 0.635 cm OD; 11/64 inch or 0.436 cm ID), and the nylon tubing itself (**Figure 3A**).
 - a. For the **type of gas** to use as a gas supply, recommended sources include:
 - i. Inert gases such as argon.
 - ii. Nitrogen gas.
2. The gas pressure flowing from the tank and into the Pneumatic Primer will be set at 20 psi or 137.9 kPa (**Figure 3B**), but **do not turn on the gas until all connections are securely fastened**.
3. The serviceable filter can be installed in between the pressure regulator on the gas cylinder and the Pneumatic Primer (**Figure 3C**), using a piece of nylon tubing.
 - a. Connect one end of the nylon tubing to the pressure regulator, using adapters that are appropriate for the inner and outer diameters of the tubing.
 - b. Connect the other end of the nylon tubing to the filter.
 - i. Push in one of the blue connectors on the filter, and insert the nylon tubing into the blue connector.
 - ii. Release the blue connector, which will tighten around the nylon tubing and form a secure connection.
4. A second piece of nylon tubing joins the air filter to the Pneumatic Primer by the metal male connector attached to the Pneumatic Primer Box (**Figure 3D**).
 - a. Connect the nylon tubing to the air filter, as described in Step 3b.
5. Connect the nylon tubing to the Pneumatic Primer.
 - a. By pushing in on the plastic end of the male connector, the nylon tubing can then be inserted into the connector.
 - b. Releasing the plastic end will tighten the connection and secure the nylon tubing to the Pneumatic Primer box.

- With the knob of the Pneumatic Primer set at “0”, the gas can now be turned on and set at 20 psi or 137.9 kPa. Listen carefully for any gas leaks.

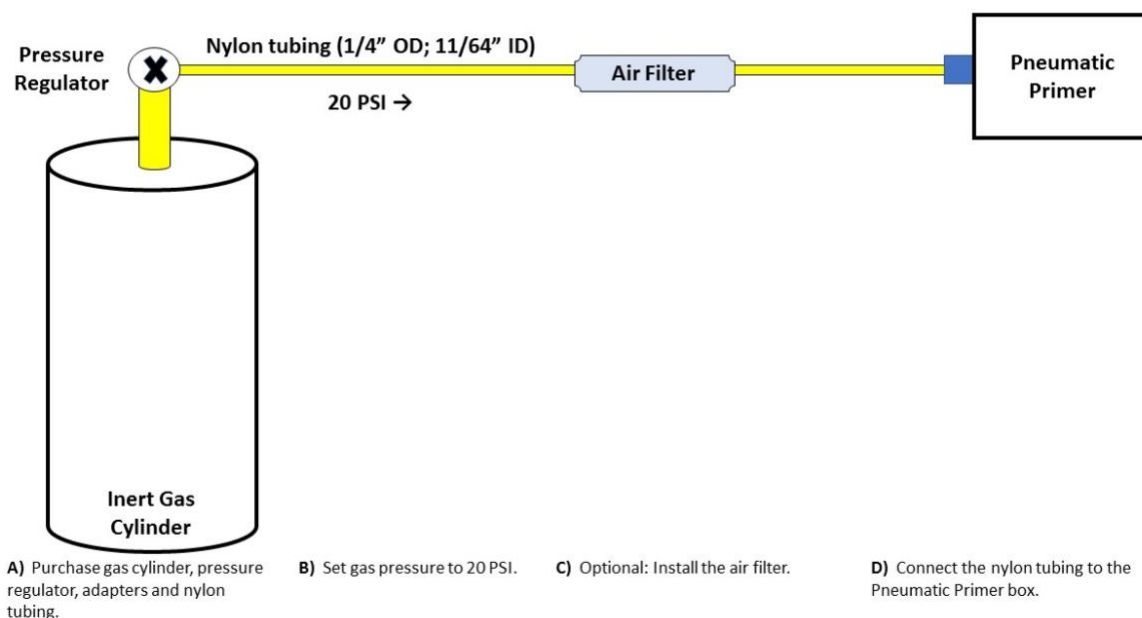


Figure 3. Schematic for connecting the Pneumatic Primer to a source of inert gas.

- Once the connections have been made between the gas cylinder and the Pneumatic Primer box, the microfluidics device can be connected to the white inlet connector (**Figure 4**).
- Unclamp one of the pieces of Tygon tubing that is connected to the microfluidics device.
- Slide a 24-gauge, blunt-tipped needle into the Tygon tubing, so that the needle is inside the tubing.
- Screw the red casing of the needle into the white inlet connector of the Pneumatic Primer box. Make sure the connection is secure.

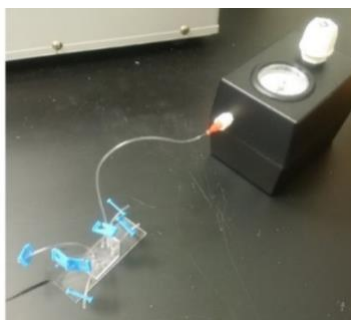


Figure 4. Schematic for connecting the microfluidics device to the Pneumatic Primer.

11. Turn the white knob on the Pneumatic Primer clockwise, until gas is flowing and the pressure on the gauge reads between 5-7 psi (34.47–48.26 kPa).
12. Listen carefully to make sure no gas is leaking out of any connections.
13. Proceed with priming as described in the SynVivo protocol.
14. Turn the white knob on the Pneumatic Primer counterclockwise to turn off the flow of gas.
15. If using the Multiple Port Manifold, attach the manifold to the Pneumatic Primer (**Figure 5A**).

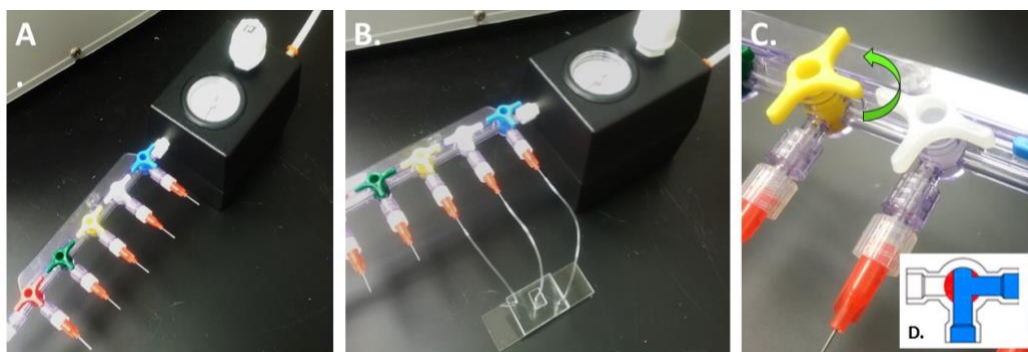


Figure 5. The Multiple Port Manifold is connected to a Pneumatic Primer (A). All 3 ports of a microvascular network device with a 3D tissue chamber are connected to the manifold (B). (C) Turn the 3-way valve counterclockwise on the T port, so that the flow of gas is directed into the last port of the device (D; inset graphic from <https://valveman.com/blog/understanding-tport-vs-lport-directional-flows/>).

16. Connect the devices to the manifold using the red needles (**Figure 5B**).

17. Turn the last 3-way valve that is connected to the device in a counterclockwise manner, so that the flow of gas is directed into the last port of the device (**Figure 5C**).
18. Turn the white knob on the Pneumatic Primer clockwise, until gas is flowing and the pressure on the gauge reads between 5-7 psi (34.47–48.26 kPa).
19. Listen carefully to make sure no gas is leaking out of any connections.
20. Proceed with priming as described in the SynVivo protocol.
21. Turn the white knob on the Pneumatic Primer counterclockwise to turn off the flow of gas.

Rinsing and Priming Devices

Principle:

Bubble-free filling of complex microvascular networks with aqueous media can be challenging when using a microfluidic device. Even with a hydrophilic surface, air can be easily trapped inside the channels when the device is first filled with liquid.

One method for removing air from microfluidic devices involves purging the system with an inert gas (usually nitrogen or argon) for a specific length of time (usually 15 minutes to 1 hour). The gas is under pressure (between 5-7 psi or 34-48 kPa), allowing the trapped air to escape through the pores in the PDMS. This method requires a source of inert gas, as well as a pressure regulator to control the flow of gas into the device (see protocol “Setting up the Pneumatic Primer” for more details).

Equipment:

- SynVivo microfluidics device
- Tygon tubing (0.02 inch ID x 0.06 inch OD, or 0.05 cm ID x 0.15 cm OD; SynVivo 201005)
- Blue slide clamps (SynVivo 202001)
- 1 mL syringe with Luer-Lok tip (25 pack; SynVivo 203005)
- 24-gauge blunt-tipped needles (0.5 inches or 1.27 cm long; SynVivo 204003)
- Pneumatic Primer (SynVivo 205001)
- Forceps (ultra-fine; VWR 100494-698)
- Stainless steel scissors (Fisher Scientific 19-062530)
- Multiple port manifold for the Pneumatic Primer (SynVivo 207001)
- Source of inert compressed gas

Reagents:

- 1X PBS without calcium or magnesium (Corning 21040CV)
- Sterile cell culture grade distilled water (ThermoFisher Scientific 1523001)

Protocol:

This protocol contains details on how to rinse and prime both radial and linear IMN styles of SynVivo devices. All steps should be carried out within a laminar flow hood as much as possible to maintain sterility.

IMN Radial Devices:

1. Place approximately 1-inch (2.54 cm) long segments of Tygon tubing into all the outlet ports (Outlets B, D, and F; **Figures 1A**

and 1B), and one inlet port (Inlet C; **Figures 1A and 1B**) of the radial IMN device.

- a. A total of 3 inlet ports will remain without tubing (**Figure 1C**).

A. Idealized Microvascular Network (IMN) Configuration

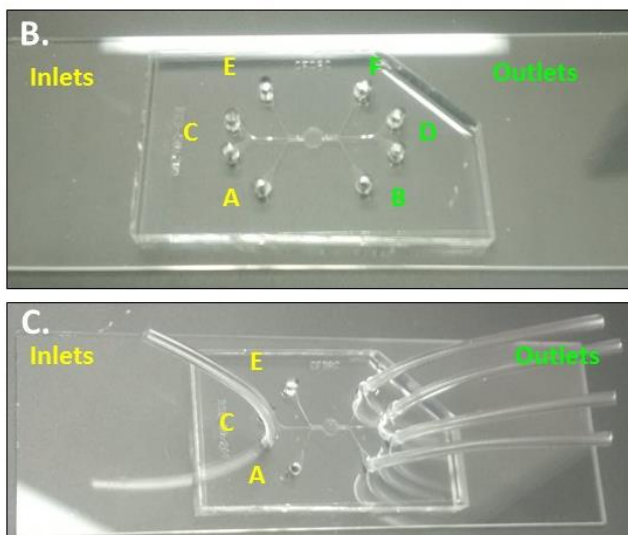
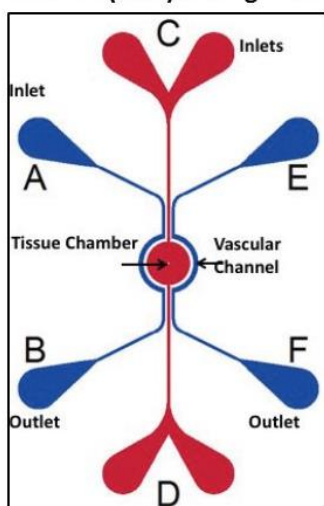


Figure 1. (A) Schematic of inlet and outlet ports on a SynTumor IMN radial device. (B) Actual SynTumor IMN radial device with inlet and outlet ports highlighted. (C) SMN radial device with Tygon tubing inserted into the outlet ports and 1 inlet port in preparation for rinsing the device.

2. Using a 1-mL syringe filled with 1X PBS or sterile water, attach approximately 3-5 inches (7.62–12.7 cm) of tubing to the syringe.
 - a. Perfuse the tubing with the liquid.

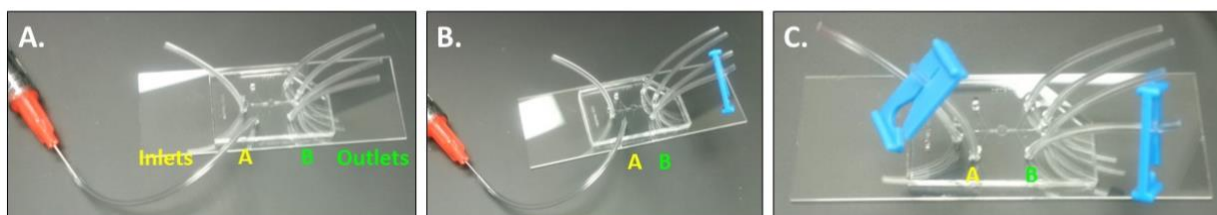


Figure 2. (A) Perfusing one channel in a SynVivo IMN radial device with PBS. (B) Clamping the outlet port B below the liquid line with a blue slide clamp. (C) Clamping the inlet port A with a blue slide clamp.

3. Place the liquid-filled tubing into the inlet port A (**Figure 2A**).

4. Fill the channel with 1X PBS or sterile water by pushing the solution through the channel until the tubing in outlet port B is filled.
5. Clamp the tubing below the liquid line in outlet port B using a blue slide clamp (**Figure 2B**).
6. Cut the tubing from inlet port A and clamp the tubing below the liquid line using a blue slide clamp (**Figure 2C**).
7. Place the liquid-filled tubing into the open inlet port C (**Figure 3A**).

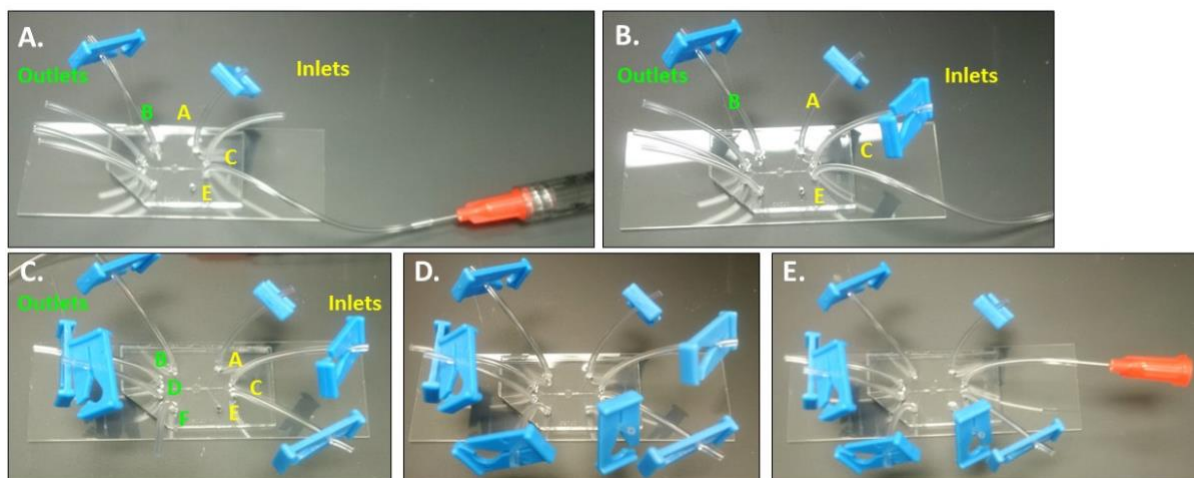


Figure 3. A SynVivo IMN radial device is perfused with 1X PBS (A-D), and a radial device is connected to a needle in preparation for the Pneumatic Primer (E).

8. Fill the empty tubing in adjacent hole of inlet port C with 1X PBS by pushing the solution through the channel until the tubing is filled.
9. Clamp the tubing below the liquid line using a blue slide clamp (**Figure 3B**).
10. Fill the empty tubing in the outlet ports D with 1X PBS by pushing the solution through the channels until both tubings are filled.
11. Clamp the tubes below the liquid line using blue slide clamps (**Figure 3C**).
12. Cut the tubing from inlet port C and clamp the tubing (**Figure 3C**).
13. Place the fluid-filled syringe in inlet port E.

14. Fill the channel with 1X PBS or sterile water by pushing the solution through the channel until the tubing in outlet port F is filled.
15. Clamp the tubing below the liquid line in outlet port F using a blue slide clamp (**Figure 3D**).
 - a. All channels are now filled with 1X PBS and clamped.
16. Unclamp one of the tubes from inlet port C and attach a needle (**Figure 3E**).
 - a. The device is now ready for the Pneumatic Primer.
17. Connect the device to the Pneumatic Primer by locking the bases of the 1 needle into the Luer Lok connector on the Pneumatic Primer (or the Multiport Manifold, if it is attached; **Figure 4A**).

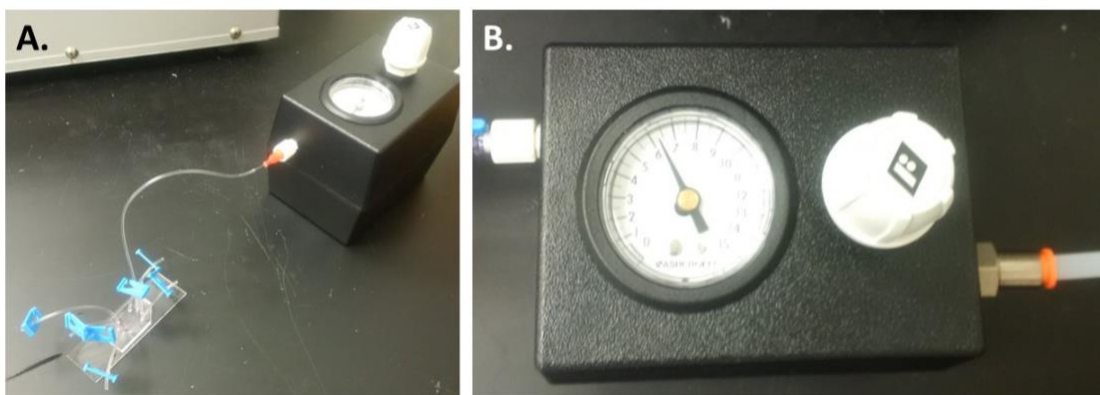


Figure 4. An IMN radial device is connected to a Pneumatic Primer (A), and inert gas is flowed into the device at 5–7 psi (34–48 kPa) (B).

18. Turn the knob on the controller box, and adjust the pressure to approximately 5–7 psi (34–48 kPa; **Figure 4B**).
19. Apply the pressure for approximately 5–20 minutes.
20. Turn off the pressure and unscrew the needle that connected the device to the Pneumatic Primer.
21. Clamp the tubing below the liquid line in inlet port C.
22. Check the device under the microscope to see if there are air bubbles trapped in the channels.
23. If air bubbles have not been removed, re-attach the devices to the Pneumatic primer and apply pressure for another 5–20 minutes.
 - a. Check the devices under the microscope to verify that the trapped air bubbles have been eliminated.

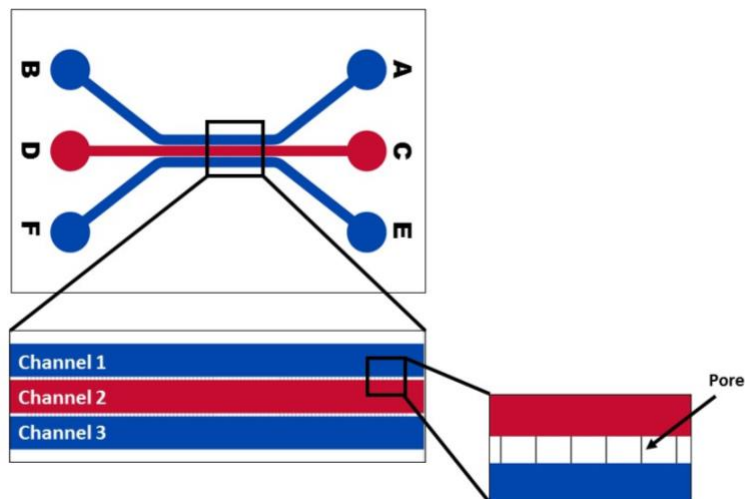
IMN Linear Devices:

Figure 5. A schematic of inlet and outlet ports A-F on a typical SynVivo linear device.

1. Place approximately 1-inch (2.54 cm) long segments of Tygon tubing into the outlet ports of the device (Ports B, D, F; **Figure 5**).
 - a. Leave 3 inlet ports free of tubing (Ports A, C, E; **Figure 6**).

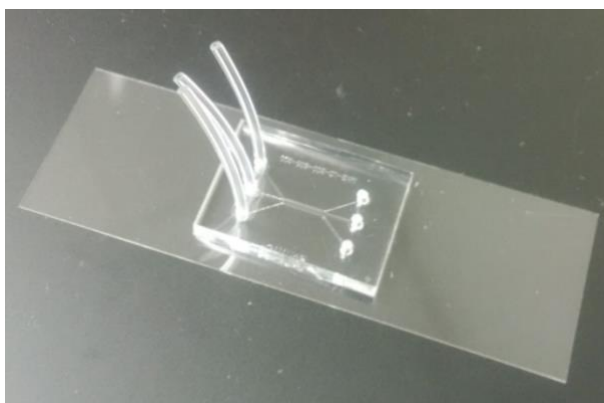


Figure 6. A SynVivo linear device with Tygon tubing in Ports B, D, F. Inlet Ports A, C and E are free of tubing.

2. Using a 1-mL syringe filled with 1X PBS or sterile water, attach approximately 3-5 inches (7.62–12.7 cm) of tubing to the syringe.

- a. Perfuse the tubing with the liquid.
3. Place the liquid-filled tubing into Port A.
4. Fill the channel with 1X PBS or sterile water by pushing the solution through the channel until the outlet tubing is filled (Port B; **Figure 7**).

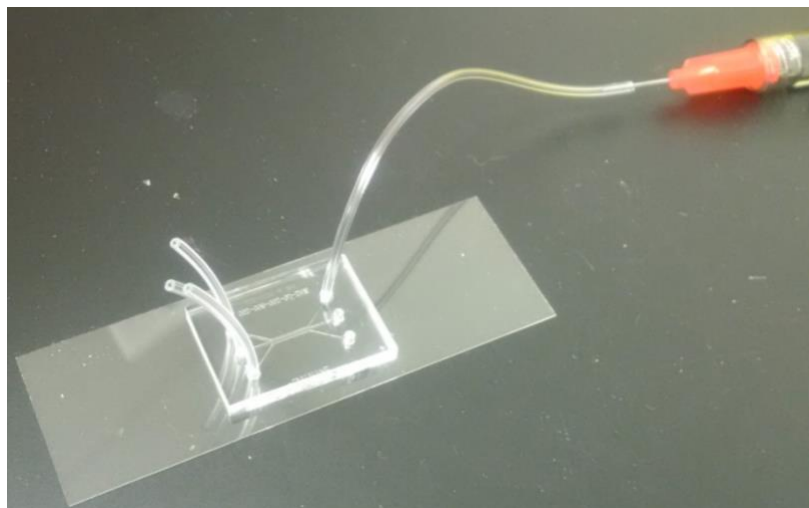


Figure 7. *Perfusing a SynVivo linear device with PBS.*

5. Clamp the tubing in Ports A and B below the liquid line using the blue slide clamps.
6. Repeat Steps 4 and 5 for Ports E and F.
7. For the remaining inlet port (Port C), place a piece of tubing into the port that is approximately 5 inches (12.7 cm) long.
 - a. Fill the channel with 1X PBS or sterile water until the tubing in the outlet port (Port D) is filled.
 - i. Clamp the tubing below the liquid line in Port D.
8. When the channel is filled, unlock the needle from the syringe, leaving the needle attached to the tubing in Port C.
 - a. Do not clamp the tubing coming out of Port C (where the needle is attached; **Figure 8**).

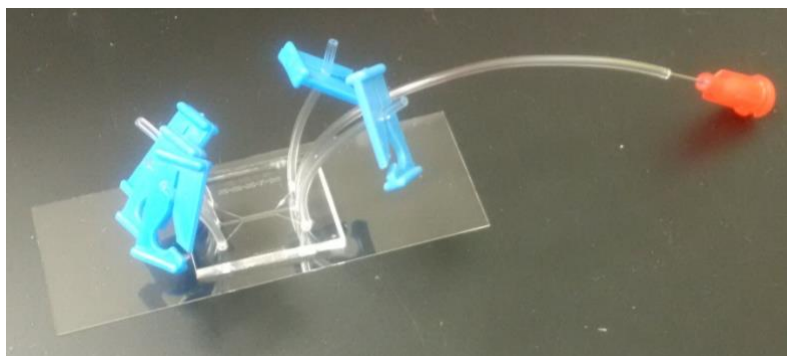


Figure 8. A SynVivo linear device perfused with PBS and clamped at all ports except Port C, which is attached to a needle. This device is ready to be connected to the Pneumatic Primer.

9. Connect the device to the Pneumatic Primer by locking the needle into the Luer Lok connector on the box.
10. Turn the knob on the controller box and adjust the pressure to approximately 5–7 psi (34–48 kPa).
 - a. Apply the pressure for approximately 5–20 minutes.
11. Turn off the pressure and unscrew the needle from the Pneumatic Primer.
12. Clamp the tubing below the liquid line in Port C.
13. Check the device under the microscope to see if there are air bubbles trapped in the channels.
14. If air bubbles have not been removed, re-attach the devices to the Pneumatic primer and apply pressure for another 5–20 minutes. Check the devices under the microscope to verify that the trapped air bubbles have been eliminated.

Results and Discussion

Check the devices carefully under the microscope to verify that the trapped air bubbles have been eliminated. Bubbles will appear as dark circles when the devices are imaged under phase contrast (**Figure 9**, yellow arrow).

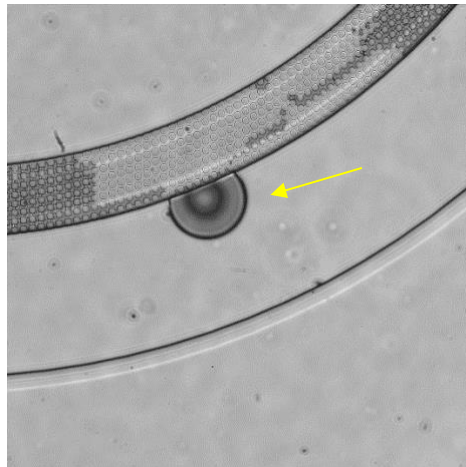


Figure 9. Example of a trapped air bubble in a SynVivo IMN radial device.

Establishing the Basement Membrane

Principle:

The extracellular matrix (ECM), or basement membrane, is a structural framework of proteins that provides a scaffold for cells comprising a tissue. Fibronectin is a major component of vascular endothelial networks, where it serves as a scaffold protein and plays a role in vascular morphogenesis.

Physical adsorption of proteins to polymers such as PDMS can occur through intermolecular interactions (van der Waals, hydrogen, hydrophobic or electrostatic bonds). Intermolecular forces are weak and influenced by environmental conditions (pH, ionic strength, temperature, surface condition). The rate and concentration of protein adsorbed onto PDMS will be dependent on protein concentration, time of incubation, temperature, and pH of the buffer solution. When the pH of the buffer is close to the isoelectric point (IEP) of the protein, the concentration of protein adsorbed to the PDMS will increase. Low incubation temperatures (4°C) will slow the adsorption rate and produce a thin protein film on the polymer surface. In contrast, higher incubation temperatures (37°C) increase the rate of adsorption and will produce a thicker protein coating.

The following protocols will describe how to create thin or thick coatings of ECM proteins in the channels of SynBBB devices.

Equipment:

- SynVivo microfluidics device
- Tygon tubing (0.02 inch ID x 0.06 inch OD, or 0.05 cm ID x 0.15 cm OD; SynVivo 201005)
- Slide clamps (SynVivo 202001)
- Forceps (ultra-fine; VWR 100494-698)
- Stainless steel scissors (Fisher Scientific 19-062530)
- 1 mL syringe with Luer-Lok tip (25 pack; SynVivo 203005)
- 24-gauge blunt-tipped needles (0.5 inches or 1.27 cm long; SynVivo 204003)
- Ice bucket with ice
- Tissue culture incubator
- Refrigerator
- Kimwipes (Fisher Scientific 06-666)
- Parafilm Laboratory Wrapping Film (Fisher Scientific 13-374-12)
- Disposable Petri dish (Corning 351058)

Reagents:

- 1X PBS without calcium or magnesium (Corning 21040CV)
- 10X PBS without calcium or magnesium (Corning 46013CM)
- Sterile cell culture grade distilled water (ThermoFisher Scientific 1523001)

- Serum-free tissue culture media (DMEM, RPMI, or basal medium without serum)
- 1.0N NaOH (Sigma Aldrich S2770-100ML)
- Corning Matrigel Matrix, GFR, phenol red free (VWR 47743-722)
- Fibronectin, human plasma (Calbiochem 341635)
 - Dilute in 1X PBS to a final concentration of 200 µg/mL (store at -20°C)
- Collagen, type IV, human placental (Sigma Aldrich C5533-5MG)
 - Dilute in glacial acetic acid to create a stock concentration of 1000 µg/mL (store at 4°C)

Protocol:

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

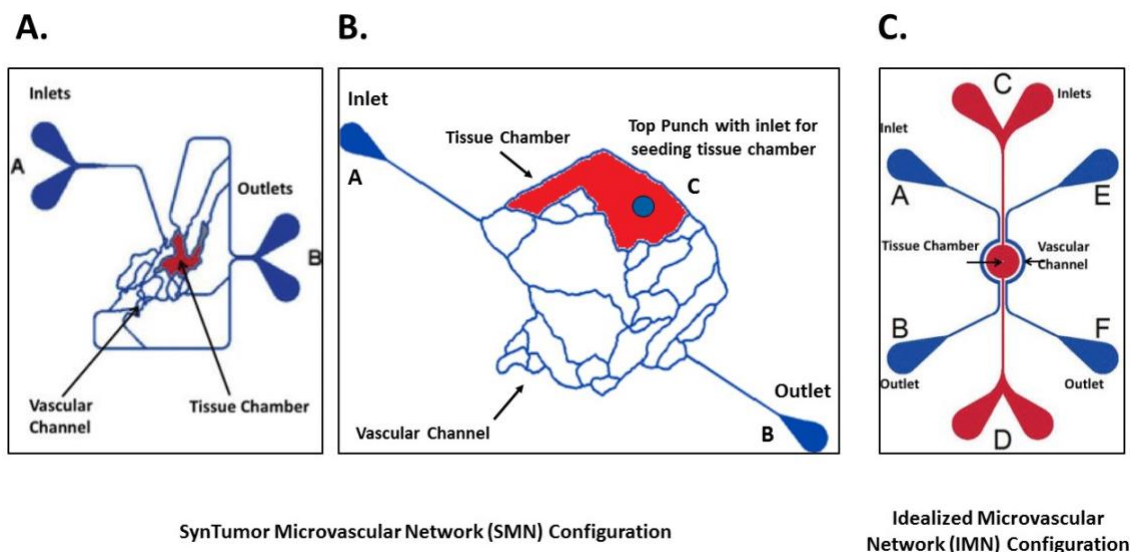


Figure 1. A schematic of inlet and outlet ports on 3 styles of SynVivo devices.

Type IV Collagen Coating:

1. Dilute the 1.0N NaOH with sterile water to create a 0.01N NaOH stock.
2. Dissolve the type IV collagen in glacial acetic acid to create a stock concentration of 1000 µg/mL.
3. On the day of coating, dilute the collagen stock concentration to 50 µg/mL as follows:

10X PBS 100 µl
 0.01 N NaOH 115 µl

Sterile water	735 μ l
Type IV collagen (1000 μ g/mL)	50 μ l
	1000 μ l

4. Using a syringe with approximately 3 inches (7.62 cm) of tubing attached, draw up the collagen solution into the syringe and tubing.
5. Unclamp one outlet port, depending on style of device (**Figure 1**). Leave the other outlet ports clamped.
6. Place a drop of liquid beside the tubing for the inlet port that corresponds to the unclamped outlet port. Remove the tubing and push out any bubbles using a needle.
7. Insert tubing with collagen into the inlet port and slowly inject the collagen until the outlet port tubing is filled (3–5 drops). This can be done by hand or by syringe pump (flow rate set at 5–10 μ l/min).
8. Clamp the outlet port's tubing.
9. Cut the inlet tubing (should be an equal length to the outlet port tubing).
10. Clamp the inlet tubing.
11. Repeat the process for the other channels.
12. Clamp all tubing of the device.
13. Place device at the following temperature:
 - a. **For a thin coat:** Place device on ice for 1 hour, or at 4°C overnight.
 - b. **For a thick coat:** Place device at 37°C for 1 hour.
14. Rinse the channels with cell media prior to seeding cells.
 - a. Check to see if there are air bubbles.
15. If the devices are not being used immediately, store the devices in a disposable Petri dish with a Kimwipe soaked in 1X PBS.
 - a. Seal the Petri dish with Parafilm and store at 4°C for 2–3 days.
 - b. Use the device within 3 days of coating.



Fibronectin Coating:

Avoid shaking thawed fibronectin, as this can cause clumps and misalignment of fibers.

1. Dilute fibronectin in sterile 1X PBS to a final concentration of **200 μ g/ml**.
2. Unclamp one outlet port. Leave the other chambers clamped.

3. Place a drop of liquid beside the tubing for the inlet port. Remove the tubing and push out any bubbles using a needle.
4. Using a 1-mL syringe with approximately 3 inches (7.62 cm) of tubing attached, draw up the fibronectin solution into tubing.
5. Place the liquid-filled tubing into the free inlet port.
6. Fill the device chamber with fibronectin.
7. Clamp the outlet tubing below the liquid line.
8. Cut the inlet tubing (should be an equal length to the outlet port tubing).
9. Clamp the inlet tubing.
10. Repeat process for the other channels.
11. Place device at the following temperature:
 - a. **For a thin coat:** Place device on ice for 1 hour, or at 4°C overnight.
 - b. **For a thick coat:** Place device at 37°C for 1 hour.
12. Rinse the channels with cell media.
 - a. Check to see if there are air bubbles.
13. Store the devices in a disposable Petri dish with a Kimwipe soaked in 1X PBS at 4°C overnight.
 - a. Use the device within 3 days of coating.



Matrigel Thin Coating:

Matrigel must be put on ice immediately and left on ice. Matrigel will polymerize above 4°C! Be sure that no serum is present in the device before introducing the Matrigel, because serum will prevent polymerization.

1. Place all materials on ice to cool: devices, forceps, syringe with needle and tubing.
 - a. Keep devices on ice through the entire process.
2. Dilute Matrigel 1:5 with chilled serum-free media.
 - a. Serum-free media can be any glucose-based tissue culture media (DMEM, RPMI), or cell-specific basal media without serum.
3. Place a drop of water around the device tubing to be removed.
4. Gently remove the tubing.
 - a. 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 will prevent air from entering the device.

5. Draw Matrigel into a small segment of tubing on the syringe.
 - a. Don't draw up the Matrigel into the needle or the barrel of the syringe.
 - b. It is recommended to only draw up what is necessary for one device (about 30 μ l). This is to prevent excess Matrigel from polymerizing in the syringe and excess tubing.
6. Quickly insert the tubing into the port and infuse by hand until 1-3 drops are seen in the outlet tubing.
7. Detach syringe from the tubing, clamp all tubing, and bury entire device in ice for 1 hour.
8. After 1 hour, wash the channels thoroughly with serum-free media.
 - a. Don't be too gentle – this step will help remove clumps.
9. Rinse the channels with cell media prior to seeding cells.
 - a. Check to see if there are air bubbles.
10. If the devices are not being used immediately, store the devices in a disposable Petri dish with a Kimwipe soaked in 1X PBS.
 - a. Seal the Petri dish with Parafilm and store at 4°C for 2–3 days.
11. Use the device within 3 days of coating.



Supporting Information:

1. Chumbimuni-Torres, K. Y., R. E. Coronado, A. M. Mfuh, C. Castro-Guerrero, M. F. Silva, G. R. Negrete, R. Bizios, and C. D. Garcia. 2011. Adsorption of proteins to thin-films of PDMS and its effect on the adhesion of human endothelial cells. *RSC Adv.* 1(4):706-14.
2. Sorokin, L. 2010. The impact of the extracellular matrix on inflammation. *Nat Rev Immunol.* 10(10):712-23.
3. Rhodes, J. M., and M. Simons. 2007. The extracellular matrix and blood vessel formation: not just a scaffold. *J Cell Mol Med.* 11(2):176-205.
4. Higuchi, A., Y. Takanashi, T. Ohno, T. Asakura, C. S. Cho, T. Akaike, and M. Hara. 2002. Production of interferon-beta by fibroblast cells on membranes prepared by extracellular matrix proteins. *Cytotechnology.* 39(3):131-37.

Seeding the Vascular Channel with Endothelial Cells

Principle:

The protocol describes seeding endothelial cells in devices that have been pre-coated with extracellular matrix proteins (see protocol “Establishing the Basement Membrane” for more information). Endothelial cells are seeded at a very high density to quickly form the vessel structure. The devices may be seeded with a syringe pump at a specific flow rate, or by hand. Viewing the devices under the microscope during seeding will help the user visualize the density of cells flowing through the devices.

Endothelial media formulations will be cell-type and manufacturer specific, so follow the manufacturer’s guidelines for cell seeding and propagation. This protocol describes seeding human umbilical vascular endothelial cells (HUVECs) into the vascular channels of SynBBB devices.

Equipment:

- SynVivo microfluidics device, previously coated with 200 µg/mL fibronectin
- Tygon tubing (0.02” ID x 0.06” OD; SynVivo 201005)
- Slide clamps (SynVivo 202001)
- 1 mL syringe with Luer-Lok tip (25 pack; SynVivo 203005)
- 24-gauge blunt-tipped needles (0.5” long; SynVivo 204003)
- Forceps
- Stainless steel scissors
- T75 flasks (Corning 430641)
- Syringe pump (Harvard Apparatus)
- Tissue culture incubator

Primary Cells:

- Human umbilical vascular endothelial cells (HUVECs; Lonza C2519A)

Reagents:

- 1X PBS without calcium or magnesium (Corning 21040CV)
- DMEM, high glucose (ThermoFisher Scientific 11965092)
- Fibronectin, human plasma (Calbiochem 341635)
 - Dilute in 1X PBS to a final concentration of 1.06 mg/mL (store at -20°C)
- Heparin (Stem Cell Technologies 07980)
- EGM-2 basal media (Lonza CC-3156)
- EGM-2 Supplement Kit (Lonza CC-4176)
- 1X TrpLE Express cell detachment solution, no phenol red (ThermoFisher Scientific 12604013)
 - Can be substituted with Trypsin-EDTA for Primary Cells (ATCC PCS-999-003)
- HEPES buffered saline (Lonza CC-5022)

Protocol:

Disclaimer: This protocol describes the seeding concentrations and conditions optimized for HUVECs. If a different endothelial cell line or primary cell is used, the conditions described in the protocol should be optimized for the specific cell type.

The following protocols should be carried out within a laminar flow hood as much as possible to maintain sterility.

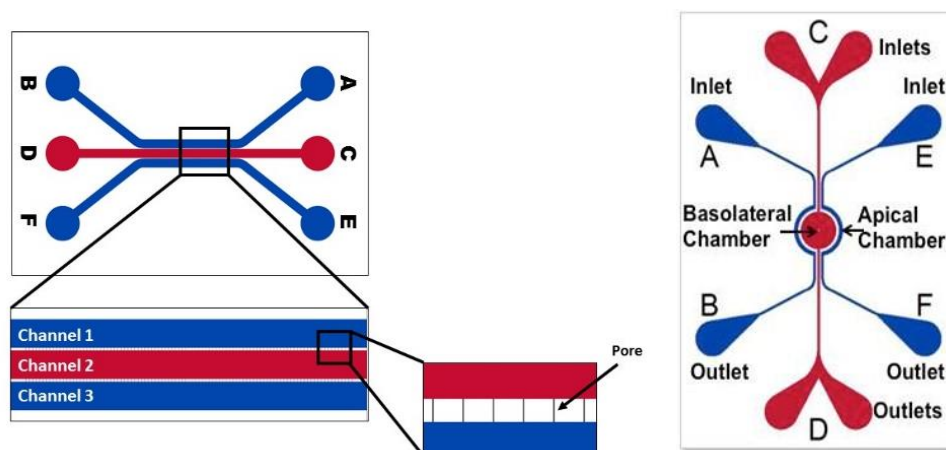


Figure 1. Schematics of linear and radial SynVivo IMN devices. Endothelial cells will be seeded into the vascular channels (highlighted in blue).

Dissociate endothelial cells:

1. Dissociate endothelial cells from the flask (see “Tissue Culture” or manufacturer’s protocols for further instructions).
2. Perform a cell count.

Seeding endothelial cells into the SynBBB devices:

1. Centrifuge the cells for 5 minutes at 200 x g at room temperature (no more than 1000 RPM).
2. Resuspend the cell pellet in the volume of complete endothelial cell media to achieve **5–7 x 10⁷ cells/ml**.
 - a. The cell solution should have the appearance of frosted glass, which will indicate a dense cell concentration.
3. Optional: Pre-coat a syringe and Tygon tubing with heparin (5 IU/mL) to prevent cell sticking to the tubing during seeding.
4. Unclamp one outlet port of the vascular channel. Leave the other chambers clamped.

5. Place a drop of liquid beside the tubing for the inlet port of one of the channels (**Figure 1**). Remove the tubing and push out any bubbles using a needle.
6. Draw back the plunger of a 1-mL syringe to the 0.3 mL mark.
7. If seeding by hand:
 - a. Load the Tygon tubing with the previously prepared cell suspension. Draw up enough cells to fill the tubing, but do not bring the cells into the syringe barrel.
 - b. Make sure that the tubing is free of air bubbles.
 - c. Push the cell mixture out until it is flush with the end of the tubing.
8. If seeding by a syringe pump:
 - a. Mount the syringe onto a syringe pump.
 - b. Load the Tygon tubing with the previously prepared cell suspension, using the withdraw button on the syringe pump (double arrows pointing to the left).
 - i. Draw up enough cells to fill the tubing, but do not bring the cells into the syringe barrel.
 - c. Make sure that the tubing is free of air bubbles.
 - d. Using the rapid infuse button on the syringe pump (double arrows pointing to the right), make sure the cell mixture is flush with the end of the tubing.
9. Insert the tubing into the inlet port - the drop of water will prevent air entering the device as the tubing is inserted.
10. Clean the fluid from the surface of the device.
11. Inject the cells into the device:
 - a. If using a syringe pump, begin the injection at a flow rate of 5 μ l/min.
 - b. If seeding by hand, gently push the plunger forward. Watch the fluid line inside the Tygon tubing carefully.
12. Watch the device as the cells are flowing. Once the vascular channel is filled with cells, stop the flow and clamp the outlet tubing.
 - a. Wiggle the clamped outlet tubing to distribute the cells evenly through the channel.
 - b. Only do this while the syringe is still connected to the inlet port.
13. Carefully and quickly cut the inlet tubing, keeping the length of the inlet tubing as small as possible.

14. Take an image of the cells (**Figure 2**). Cell density should be consistent across the whole channel.

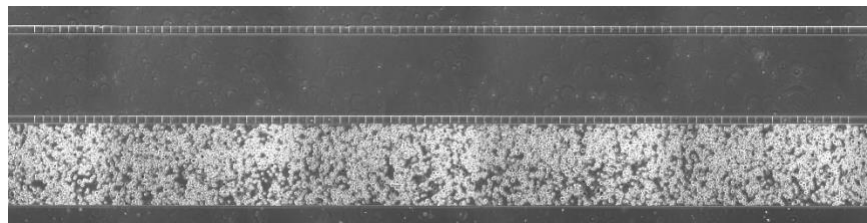


Figure 2. An example of a confluent HUVEC seeding density in a linear SynVivo device.

Incubating the device after cell seeding:



12. Place the device into a 37°C tissue culture incubator (5% CO₂).
13. Allow the cells to attach for at least 4 hours before changing media in the device.
14. After 4 hours, gently flush endothelial complete media through the endothelial cells to remove dead cells/debris.
15. Connect the devices to a syringe pump programmed with a media change program (see “Changing the Media in an Endothelial Monoculture”).

Changing the Media in an Endothelial Monoculture

Principle:

The goal of a media change is to slowly circulate nutrients and replace the volume of media within the channels. The flow of media is slow as the cells become established in the device, so that the shear stress on the cells is limited during early time points post-seeding.

Experimental planning is crucial to providing media to the endothelial cell monoculture in a timely manner (**Figure 1**). In general, devices are coated with ECM proteins the day before seeding. On the next day, endothelial cells are seeded before lunch and allowed to attach for 4 hours. HUVECs should be flushed with fresh media within 4 hours of seeding due to the high cell density and their high metabolic rate (waiting longer than this can result in cell death).

The devices are then connected to a syringe pump, and media is changed overnight for the next 16 hours at 4-hour intervals (“Media Change”). On the following morning, the devices can be imaged and reconnected to the syringe pump to ramp the flow rate up to a set point (see protocol “Endothelial Cells Under Flow”). The endothelial cells will be incubated at the set flow rate until treatment.

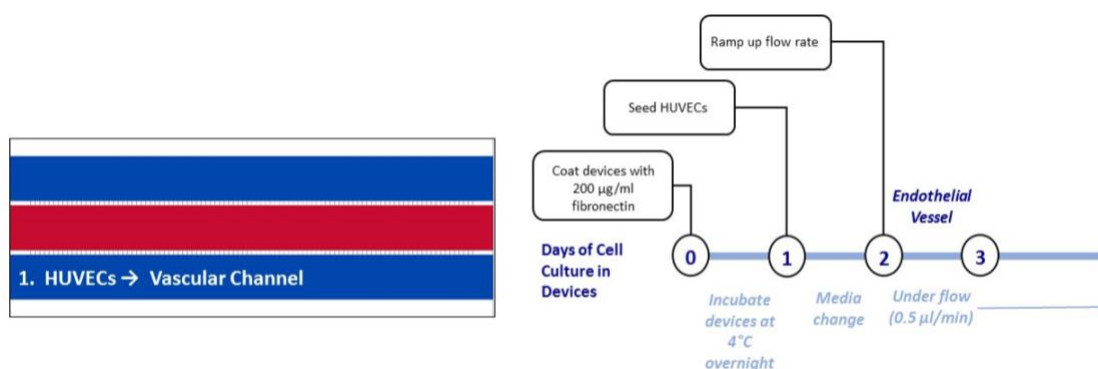


Figure 1. Proposed experimental timeline for establishing the endothelial cell monoculture in the SynVivo devices.

Equipment:

- SynVivo microfluidics device, previously coated with 200 µg/mL fibronectin and seeded with endothelial cells
- Tygon tubing (0.02 inch ID x 0.06 inch OD, or 0.05 cm ID x 0.15 cm OD; SynVivo 201005)
- Slide clamps (SynVivo 202001)
- 1 mL syringe with Luer-Lok tip (25 pack; SynVivo 203005)
- 24-gauge blunt-tipped needles (0.5 inches or 1.27 cm long; SynVivo 204003)

- Forceps (ultra-fine; VWR 100494-698)
- Stainless steel scissors (Fisher Scientific 19-062530)
- Syringe pump (PhD Ultra; Harvard Apparatus; SynVivo 301001 or 301002)
- Tissue culture incubator

Reagents:

- 1X PBS without calcium or magnesium (Corning 21040CV)
- EGM-2 basal media (Lonza CC-3156)
- EGM-2 Supplement Kit (Lonza CC-4176)

Protocol:

Disclaimer: This protocol describes the media change program optimized for HUVECs using an IMN device and a specific syringe pump (PhD Ultra from Harvard Apparatus). If a different endothelial cell line, style of SynVivo device (SMN vs. IMN), or syringe pump is used, the conditions described in the protocol should be optimized for those changes.

The programs listed below can be programmed using the workflow plan included in the user’s manual of the syringe pump from the Equipment List (**Figure 2**). Please refer to the manufacturer’s instructions for additional details.

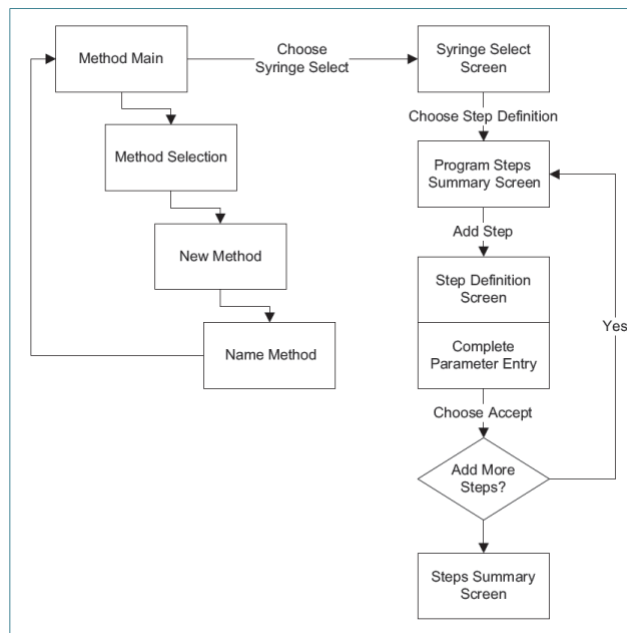


Figure 2. Workflow plan for programming a Harvard Apparatus syringe pump (Harvard Apparatus. 2012. “Pump 11 Elite Series Syringe Pump User’s Manual.” Document no. 5420-002, 10th revision. April 2012).

Program the pump with the media exchange program:

1. Name the new method “Media Change” (**Figure 2**).
2. Under the “Syringe Select” screen, set the diameter to 4.699 mm (SynVivo 203005; 1 mL syringes) by pressing the “Diameter” key and using the arrow keys to scroll through the numbers.
 - a. If a different 1 mL syringe is to be used, see the manufacturer’s information for more details regarding diameter information.
3. Choose the Step Definition button.
4. The first profile or step that you will choose for this program is a **Constant Rate**, which flows media at a specific rate (1 μ l/min for 30 minutes) for a total volume of 30 microliters (30 μ l) flowing through the device.
 - a. This step will wash away dead/dying cells and replenish fresh media through the vascular channel.
5. Enter the flow rate (1 μ l/min). Make sure the units are correct.
6. Choose “Accept” to confirm your settings.
7. Choose the Volume button to enter the volume (30 μ l).
 - a. Make sure the units are correct.
 - i. The volume is in microliters and NOT milliliters.
8. Choose “Accept” to confirm your settings.
9. Choose “Accept” again to return to the Step Definition screen.
10. From the Step Definition menu, the second profile or step that you will choose for this program is a **Delay**, where no fluid is flowed through the device.
 - a. This step provides the cells with 4 hours for growth and division (no media is flowing through the device at this step).
11. Enter the time (4:00:00 or 4 hours).
12. Choose “Accept” to confirm your settings.
13. Choose “Accept” again to return to the Step Definition screen.

14. From the Step Definition menu, the third profile or step that you will choose for this program is **Repeat**, where you will repeat the constant rate/delay steps for 99 times.
15. Choose the step that you will repeat from (“Constant Rate” step).
16. Choose “Accept” to confirm your settings.
17. Choose the number of repeats button to enter the number of times that the program will repeat (99 times).
18. Choose “Accept” to confirm your settings.
19. Choose “Accept” again to return to the Step Definition screen.
20. Program summary: Media Change
 - a. Step 1: Constant Rate
 - i. Mode: Infuse
 - ii. Set rate: 1 μ l/min
 - iii. Volume: 30 μ l
 - b. Step 2: Delay
 - i. Target time: 4:00:00 (4 hours)
 - c. Step 3: Repeat
 - i. From step: Constant Rate (Step 1)
 - ii. Number of Repeats: 99 (this keeps the pump running until you need to stop it).

Connecting the devices to a syringe pump:

An example of a device (inside the CO₂ incubator) connected to a syringe pump (outside the CO₂ incubator) is shown in **Figure 3**.

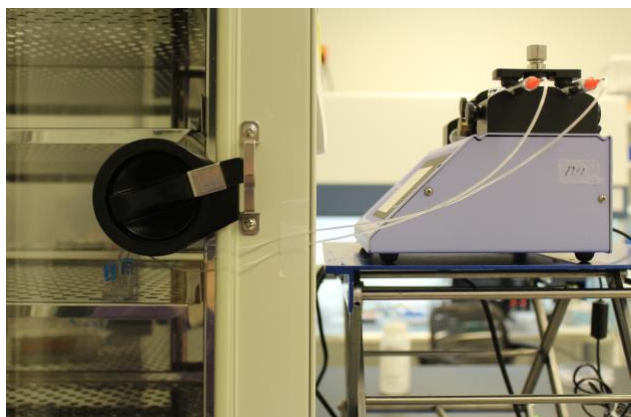


Figure 3. Connecting devices to a syringe pump for a media change program.

1. For each device, prepare a 1-mL syringe with complete endothelial cell media.
2. Attach a length of tubing to the syringe that is long enough to reach from the syringe pump to the device inside the tissue culture incubator (**Figure 3**).
3. Infuse the Tygon tubing with the endothelial cell media.
4. With the infused tubing inserted into a conical tube of endothelial cell media, withdraw enough media into the syringe barrel to bring it close to the 1-mL mark.
5. Place the filled syringe into the syringe pump. Tape the end of the tubing onto the pump, with the end of the tubing pointing up to the ceiling and not touching any surface.
6. Place a drop of water at the base of the inlet port tubing to be removed (vascular channel with seeded HUVECs), and gently remove the tubing.
7. Remove the clamp on the outlet port of the vascular channel. Keep all other ports clamped.
 - a. To capture the effluent during the media change, a 1.5-mL microcentrifuge tube can be placed under the outlet tubing to serve as a waste reservoir (**Figure 4**).

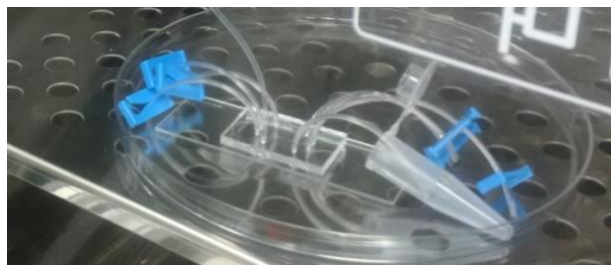


Figure 4. A microcentrifuge tube can serve as a waste reservoir to collect effluent during the media change.

8. Using the infuse arrow on the syringe pump, push the media up to the top of the tubing until a droplet forms at the top.
9. Insert the tubing into the inlet port. The drop of water will prevent air from entering the device as the tubing is inserted.
10. Clean the fluid from the surface of the device.
11. Start the syringe pump program.
12. Incubate the device overnight in a 37°C incubator, 5% CO₂.



Discussion:

When using a syringe pump for exchanging media, some useful features of the pump are the abilities to program delays, steady increases in flow rate, and infusions for specific times or volumes. The syringe pump protocol described here was designed for a PhD Ultra syringe pump by Harvard Apparatus (see specification sheet in Product Information).

The capabilities of your syringe pump may be different, so follow the manufacturer's instructions for programming the pump. The critical parameters to keep in mind are flow rate, increasing flow rate steadily over time, the size of syringe, the force that the pump uses to push against the syringe (50-100%), and the volume of media required to maintain the flow rate over a specific time.

Placing Endothelial Cells Under Flow

Principle:

A confluent endothelial layer will function as an effective cellular barrier. Endothelial vessels are anchored to surfaces by focal adhesions (complexes of cellular integrins and ECM proteins such as fibronectin). If endothelial cells lack focal adhesions, the cells will undergo apoptosis (see paper by Lu and Rounds in Supporting Information). Adjacent endothelial cells also form intercellular interactions or adherens (tight) junctions when the cells are confluent. These junctions are necessary for establishment of an intact vessel.

Standard tissue culture involves growth of cells under static conditions and in open wells, where the nutrient supply enters the cells through diffusion. In microfluidics devices, focal adhesions and tight junctions have been reported to form under static growth conditions (see Esch, et. al. in Supporting Information), but cell growth under shear stress leads to increased expression of proteins in focal adhesions and tight junctions.

The media flow rate is increased in this protocol to 0.5 $\mu\text{l}/\text{minute}$ for HUVECs. The flow rate is cell type dependent, and some endothelial cells may be able to sustain higher flow rates than those described in the protocol.

Equipment:

- SynVivo microfluidics device, previously coated with 200 $\mu\text{g}/\text{mL}$ fibronectin, seeded with endothelial cells
- Tygon tubing (0.02" ID x 0.06" OD; SynVivo 201005)
- Slide clamps (SynVivo 202001)
- 1 mL syringe with Luer-Lok tip (25 pack; SynVivo 203005)
- 24-gauge blunt-tipped needles (0.5" long; SynVivo 204003)
- Forceps (ultra-fine; VWR 100494-698)
- Stainless steel scissors (Fisher Scientific 19-062530)
- Syringe pump (PhD Ultra; Harvard Apparatus; SynVivo 301001 or 301002)
- Tissue culture incubator

Reagents:

- 1X PBS without calcium or magnesium (Corning 21040CV)
- EGM-2 basal media (Lonza CC-3156)
- EGM-2 Supplement Kit (Lonza CC-4176)

Protocol:

Disclaimer: This protocol describes the syringe pump program optimized for HUVECs in an IMN style of device. HUVECs can sustain flow rates between 0.1-1 $\mu\text{l}/\text{minute}$ in the

presence of complete endothelial media, but the flow rates may have to be changed based on the cell type, the style of SynVivo device used, or the treatments to be tested.

If a different endothelial cell line or primary cell is used, the conditions described in the protocol should be optimized for the specific cell type. Cell types may require lower flow rates, or longer periods of time needed to reach a specific flow rate.

Program the pump to ramp up to 0.5 μ l/minute (HUVEC):

14. Name the new method “Flow Step”.
15. Under the “Syringe Select” screen, set the diameter to 4.699 mm (SynVivo 203005; 1 mL syringes) by pressing the “Diameter” key and using the arrow keys to scroll through the numbers.
 - a. If a different 1 mL syringe is to be used, see the manufacturer’s information for more details regarding diameter information.
16. Choose the Step Definition button.
17. You will be programming a total of 7 steps, and each step will be at a **Constant Rate** for a specific period of time (**Table 1**).
 - a. Step 1: Constant Rate
 - i. Mode: Infuse
 - ii. Set rate: 0.02 μ l/min
 - iii. Time: 02:00:00
 - b. Step 2: Constant Rate
 - i. Mode: Infuse
 - ii. Set rate: 0.05 μ l/min
 - iii. Time: 02:00:00
 - c. Step 3: Constant Rate
 - i. Mode: Infuse
 - ii. Set rate: 0.075 μ l/min
 - iii. Time: 02:00:00
 - d. Step 4: Constant Rate
 - i. Mode: Infuse
 - ii. Set rate: 0.1 μ l/min
 - iii. Time: 02:00:00
 - e. Step 5: Constant Rate
 - i. Mode: Infuse

- ii. Set rate: 0.2 $\mu\text{l}/\text{min}$
- iii. Time: 03:00:00
- f. Step 6: Constant Rate
 - i. Mode: Infuse
 - ii. Set rate: 0.35 $\mu\text{l}/\text{min}$
 - iii. Time: 03:00:00
- g. Step 7: Constant Rate
 - i. Mode: Infuse
 - ii. Set rate: 0.5 $\mu\text{l}/\text{min}$
 - iii. Time: 24:00:00

Table 1: Programming an increase in the flow rate from 0.02 to 0.5 $\mu\text{L}/\text{min}$ (HUVEC)

STEP	Flow Rate ($\mu\text{l}/\text{min}$)	Time (h:m:s)	Direction
1	0.02	2:00:00	INFUSE
2	0.05	2:00:00	INFUSE
3	0.075	2:00:00	INFUSE
4	0.1	2:00:00	INFUSE
5	0.2	3:00:00	INFUSE
6	0.35	3:00:00	INFUSE
7	0.5	24:00:00	INFUSE

Connecting devices to a syringe pump:

- Program the syringe pump (see above).
- For each device, prepare a 1-mL syringe with complete astrocyte cell media.
 - Attach a length of tubing to the syringe that is long enough to reach from the syringe pump to the device inside the tissue culture incubator.
 - Infuse the Tygon tubing with complete cell media.
 - With the infused tubing inserted into a conical tube of cell media, withdraw enough media into the syringe barrel to bring it close to the 1-mL mark.

- Place the filled syringe into the syringe pump. Tape the end of the tubing onto the pump, with the end of the tubing pointing up to the ceiling and not touching any surface.
- Place a drop of water at the base of the inlet port tubing to be removed (vascular channel with seeded endothelial cells), and gently remove the tubing.
- Remove the clamp on the outlet port of the vascular channel. Keep all other ports clamped.
 - a. To capture the effluent during this period, a 1.5-mL microcentrifuge tube can be placed under the outlet tubing to serve as a waste reservoir (**Figure 1**).

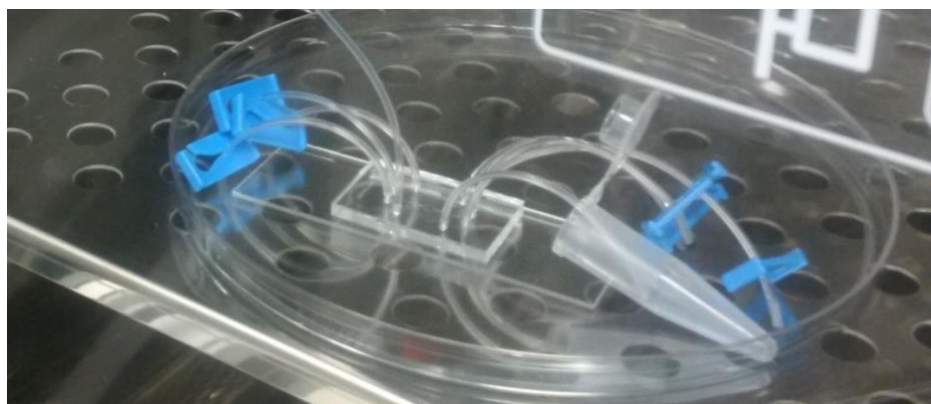


Figure 1. In a CO₂ incubator, media from the outlet tubing is being collected from an IMN SynTumor device using a microcentrifuge tube.

- Using the infuse arrow on the syringe pump, push the media up to the top of the tubing until a droplet forms at the top.
- Insert the tubing into the inlet port. The drop of water will prevent air from entering the device as the tubing is inserted.
- Clean the fluid from the surface of the device.
- Start the syringe pump program.
- Incubate the device in a 37°C incubator, 5% CO₂.

- Infuse media into the devices at a constant flow rate of 0.5 μ l/min for at least 10-16 hours before treating the endothelial cells with compounds.

Supporting Information:

1. White, C. R. and J. A. Frangos. 2007. The shear stress of it all: the cell membrane and mechanochemical transduction. *Phil Trans R Soc B.* 362:1459-67.
2. Lu, Q. and S. Rounds. 2012. Focal adhesion kinase and endothelial cell apoptosis. *Microvasc Res.* 83(1): 56-63.
3. Frangos, J. A., T. Y. Huang, and C. B. Clark. 1996. Steady shear and step changes in shear stimulate endothelium via independent mechanisms – superposition of transient and sustained nitric oxide production. *Biochem Biophys Res Comm.* 224:660-65.
4. Bao, X., L. Chuanyi, and J. A. Frangos. 1999. Temporal gradient in shear but not steady shear stress induces PDGF-A and MCP-1 expression in endothelial cells: role of NO, NF kappa B, and egr-1. *Arterioscler Thromb Vasc Biol.* 19(4): 996-1003.
5. Esch, M. B., D. J. Post, M. L. Shuler, and T. Stokol. 2011. Characterization of in vitro endothelial linings grown within microfluidic channels. *Tissue Eng Part A.* 17(23-24): 2965-71.

Seeding a 2D Cell Culture of Astrocytes and/or Pericytes in the Tissue Chamber

Principle:

Co-cultures of endothelial cells and astrocytes (with or without pericytes) establishes the blood brain barrier model in the microfluidics devices. Some groups seed the astrocytes first (see Deosarkar, et al. in Supporting Information), followed by the endothelium. The protocol performed during the training establishes the endothelium in the vascular channel first, followed by seeding the astrocytes in the tissue chamber. Both chambers should be coated with fibronectin prior to cell seeding.

One cell type can be seeded in the central tissue chamber, or a ratio of multiple cell types (such as astrocytes and pericytes) can be seeded together in the chamber. Both protocols are described below, including an optimized ratio of astrocytes to pericytes that has been used successfully in the SynBBB model.

Equipment:

- SynVivo microfluidics device, previously coated with 200 μ g/mL fibronectin
- Tygon tubing (0.02" ID x 0.06" OD; SynVivo 201005)
- Slide clamps (SynVivo 202001)
- 1 mL syringe with Luer-Lok tip (25 pack; SynVivo 203005)
- 24-gauge blunt-tipped needles (0.5" long; SynVivo 204003)
- Forceps
- Stainless steel scissors
- T75 flasks (Corning 430641)
- Syringe pump (Harvard Apparatus)
- Tissue culture incubator

Reagents:

- 1X PBS without calcium or magnesium (Corning 21040CV)
- Poly-L-lysine (Sigma Aldrich P4707)
- Human astrocytes (HA; ScienCell 1800)
- Human brain vascular pericytes (HBVP; ScienCell 1020)
- Astrocyte medium (ScienCell 1801)
- Pericyte medium (ScienCell 1201)
- 1X TrpLE Express cell detachment solution, no phenol red (ThermoFisher Scientific 12604013)
 - Can be substituted with Trypsin-EDTA for Primary Cells (ATCC PCS-999-003)
- HEPES buffered saline (Lonza CC-5022)

Protocol:

The following protocols should be carried out within a laminar flow hood as much as possible to maintain sterility.

If endothelial cells are not seeded in the vascular channel, flush all channels with cell-specific media before seeding the astrocytes or pericytes.

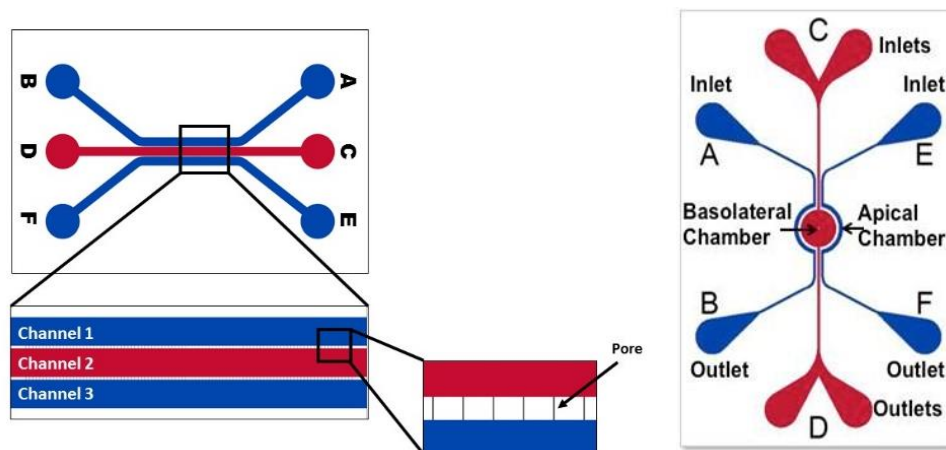


Figure 1. Schematics of linear and radial SynVivo IMN devices. Endothelial cells will be seeded into the vascular channels (highlighted in blue). Astrocytes and/or pericytes will be seeded into the tissue chambers (highlighted in red).

Dissociating astrocytes or pericytes from the flask:

1. Flush the tissue chamber of the fibronectin-coated SynVivo device with complete cell media.
 - a. This step will insure that the tissue chamber is primed with fresh cell media prior to seeding.
2. Remove and discard the cell media from the T75 flask of astrocytes or pericytes.
3. Rinse the cells twice with room temperature HEPES buffered saline.
 - a. Gently rock the flask in between rinses.
 - b. Remove final rinse and discard.
4. Add 2 mL of room temperature TrypLE Express Cell Dissociation Reagent to the flask.
5. Incubate flask at 37°C for **3-5 minutes**.
6. Add 3 mL of complete cell media to the flask to neutralize the dissociation reagent.



7. Gently wash the sides of the flask to remove the adherent cells, and pipette cells into a 15-mL conical tube.
8. Pellet the cells by centrifugation.
 - a. Use 200 x g for 5 minutes at room temperature (do not use speeds greater than 1000 RPM).
9. Remove the media, leaving behind at least 100 μ l to cover the cell pellet.
10. Wash the cells twice with 5 mL complete cell media, and pellet the cells by centrifugation.
 - a. Use 200 x g for 5 minutes at room temperature (do not use speeds greater than 1000 RPM).

Cell counting:

1. Resuspend cells in 1 mL of complete cell media.
2. Dilute cells 1/20 in complete cell media, and perform a manual cell count using a hemocytometer.
 - a. Example: 1/20 dilution is 5 μ l of cell suspension in 95 μ l of cell media or 1X PBS.
3. Calculate the volume of cell suspension needed to obtain 5×10^6 cells/mL for each cell type.

Seeding one cell type into the tissue chamber:

1. Centrifuge the cells for 5 minutes at 200 x g at room temperature (no more than 1000 RPM).
2. Resuspend the cell pellet in the volume of complete cell media to achieve 5×10^6 cells/mL.
3. Unclamp one outlet port of the tissue chamber. Leave the other chambers clamped.
4. Place a drop of liquid beside the tubing for the inlet port. Remove the tubing and push out any bubbles using a needle.
5. Prepare a 1-mL syringe with a 3–5-inch piece of Tygon tubing.
6. Draw the cell solution into the 1-mL syringe.
7. If seeding by hand:
 - a. Make sure that the tubing is free of air bubbles.
 - b. Push the cell mixture out until it is flush with the end of the tubing.
8. If seeding by a syringe pump:
 - a. Mount the syringe onto a syringe pump.

- b. Load the Tygon tubing with the previously prepared cell suspension, using the withdraw button on the syringe pump (double arrows pointing to the left).
 - c. Make sure that the tubing is free of air bubbles.
 - d. Using the rapid infuse button on the syringe pump (double arrows pointing to the right), make sure the cell mixture is flush with the end of the tubing.
9. Insert the tubing into the inlet port - the drop of water will prevent air entering the device as the tubing is inserted.
10. Clean the fluid from the surface of the device.
11. Inject the cells into the device:
 - a. If using a syringe pump, begin the injection at a flow rate of 5–8 $\mu\text{l}/\text{min}$.
 - b. If seeding by hand, gently push the plunger forward. Watch the fluid line inside the Tygon tubing carefully.
12. Watch the device as the cells are flowing. Once the tumor chamber is filled with cells, stop the flow and clamp the outlet tubing.
 - a. Wiggle the clamped outlet tubing to distribute the cells evenly through the channel.
 - b. Only do this while the syringe is still connected to the inlet port.
13. Carefully and quickly cut the inlet tubing off the syringe.
14. Incubate the device for 1 hour in a tissue culture incubator at 37°C (5% CO₂) to allow the cells to attach to the device.

Seeding two cell types in a ratio (astrocytes to pericytes):

1. Centrifuge the cells for 5 minutes at 200 x g at room temperature (no more than 1000 RPM).
2. Resuspend both astrocyte and pericyte cell pellets in the volume of complete cell media to achieve 5 x 10⁶ cells/mL each.
3. Mix astrocytes to pericytes in a ratio of 2:1 (typically 200 μl astrocytes to 100 μl pericytes should be adequate).
4. Inject the cells into the device at a flow rate of 5–8 $\mu\text{l}/\text{min}$.
5. Watch the device as the cells are flowing. Once the tumor chamber is filled with cells, stop the flow and clamp the outlet tubing.
6. Carefully and quickly cut the inlet tubing off the syringe.

7. Incubate the device for 1 hour in a tissue culture incubator at 37°C (5% CO₂) to allow the cells to attach to the device.

Results and Discussion

Once the astrocytes and/or pericytes have adhered to the tissue chamber (for 1 hour), the flow rate on the endothelium will be steadily increased to 0.1 μ l/minute. In models where the tissue chamber is seeded first (before the endothelium), the astrocytes can be allowed to attach overnight prior to seeding the endothelial cells (see Supporting Information).

Supporting Information:

Reference:

1. Deosarkar, S. P., B. Prabhakarandian, B. Wang, J. B. Sheffield, B. Krynska, and M. F. Kiani. 2015. A novel dynamic neonatal blood-brain barrier on a chip. *PLoS One*. 10(11):e0142725.

Leukocyte Adhesion Cascade and Cell Migration

Principle:

As stated in Grivennikov and Karin, most solid tumors contain inflammatory infiltrates, and chronic inflammation is associated with 20% of all cancers [1]. Tumor-associated inflammation leads to the release of proinflammatory cytokines, which draw circulating immune cells from the peripheral circulation to the site of tumorigenesis. In the leukocyte adhesion cascade (LAC), leukocytes are captured, roll, arrest and migrate through the vascular endothelial cells and toward the tumor by a series of adhesion receptor-ligand interactions [2].

Two published assays use the SMN/IMN devices to observe monocyte rolling, adhesion and migration [3-4]. The first protocol uses a chemotactic peptide such as N-formylmethionine-leucyl-phenylalanine (fMLP) in the tissue chamber, and endothelial cells in the vascular channels are activated with cytokines prior to injecting fluorescently-labeled monocytes. The second protocol contains tumor cells seeded in the tissue chamber, and fluorescently-labeled monocytes are injected into the vascular channels seeded with endothelial cells. The first protocol utilizes a peptide signal to draw immune cells into the tissue chamber, and the second protocol utilizes the proinflammatory signals released from tumor cells to induce immune cell migration toward the tumor.

Both protocols can use antibodies to block specific adhesion receptor-ligand interactions between immune cells and endothelial cells, which are required for immune cells to successfully infiltrate the tumor microenvironment [5]. The antibody blocking protocols are not included in this section, but see Supporting Information for further information regarding specific antibodies and concentrations used to perform the blocking.

Equipment:

- SynVivo microfluidics device, previously seeded with endothelial cells (HUVECs) and/or tumor cells
- Tygon tubing (0.02" ID x 0.06" OD; SynVivo 201005)
- Slide clamps (SynVivo 202001)
- 1 mL syringe with Luer-Lok tip (25 pack; SynVivo 203005)
- 24-gauge blunt-tipped needles (0.5" long; SynVivo 204003)
- Forceps (ultra-fine; VWR 100494-698)
- Stainless steel scissors (Fisher Scientific 19-062530)
- Kimwipes (Fisher Scientific 06-666A)

Reagents:

- EBM-2 basal media (Lonza CC-3156)
- EGM-2 Supplement Kit (Lonza CC-4176)
- TNF-alpha (R&D Systems 210-TA-020/CF)

- IFN-beta (R&D Systems 8499-IF-010/CF)
- Matrigel matrix (Corning 356234; aliquot and store at -20°C)
- DMEM or RPMI, no serum (e.g. ThermoFisher Scientific 11960077)
- 100X penicillin-streptomycin (ThermoFisher Scientific 15140122)
- L-glutamine (ThermoFisher Scientific 25030081)
- N-Formylmethionine-leucyl-phenylalanine (fMLP; Sigma Aldrich F3506)
- 1X PBS without calcium or magnesium (Corning 21040CV)
- CellTracker Green CMFDA Fluorescent Probes (Molecular Probes C2925)
- Human umbilical vascular endothelial cells (Lonza CC-2517)
- Human glioblastoma cells (U138-MG; ATCC HTB-16)
- Human monocytes (THP-1; ATCC TIB-202)

Protocol:

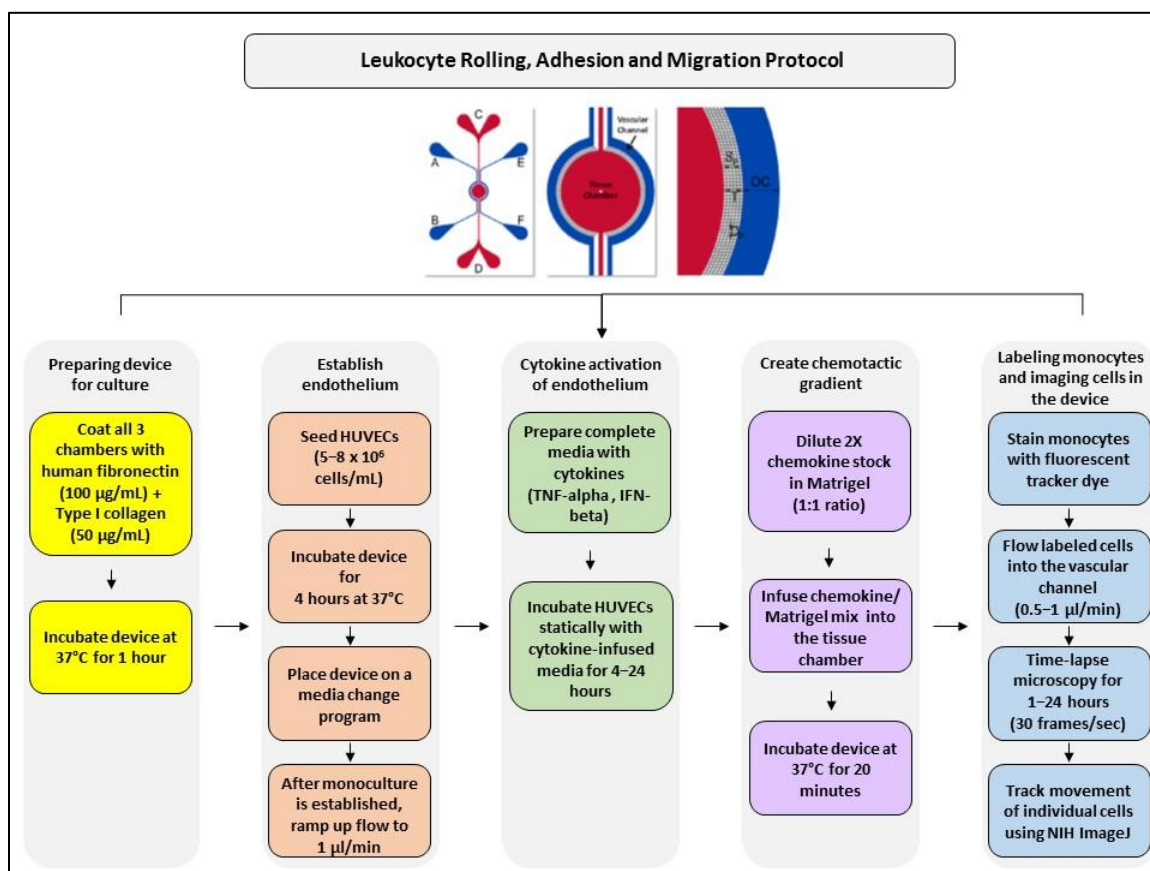


Figure 1. Protocol flow chart for studying leukocyte rolling, adhesion and migration in response to a chemotactic gradient. The device shown in the illustration is an idealized or IMN style of SynVivo device, but the experimental principles apply to both SMN and IMN style of devices.

Protocol 1: Chemotactic protein-induced cell migration

Treating endothelial cells statically with proinflammatory cytokines (receptor activation)

Note: HUVECs are seeded in the vascular channel and placed under flow conditions prior to this activation (**Figure 1**). The protocol described here exposes endothelial cells to low levels of cytokines over a long period of time (16–20 hours).

The literature describes endothelial cytokine treatments in tissue culture that are for shorter time periods (4 hours) using higher concentrations of cytokines. Take into account all experimental parameters when considering whether to do the low cytokine level/longer exposure route versus the higher cytokine level/shorter exposure period. If using higher concentrations of cytokines, test the exposure time in the devices first so the cells are not stressed to the point of inducing cell death and extensive loss of the vascular integrity.

1. Prepare a 1-mL syringe containing the following cytokines diluted in 1-mL total volume of HUVEC basal media with supplements:
 - a. TNF-alpha: 0.2 ng/mL
 - b. IFN-beta: 20 IU/mL
2. Place a 5-inch piece of Tygon tubing onto the syringe.
3. Make sure that the syringe barrel is free of air bubbles.
4. Push the media out until it is flush with the end of the tubing.
5. Insert the tubing into the inlet port - the drop of water will prevent air entering the device as the tubing is inserted.
6. Clean the fluid from the surface of the device.
7. Gently push the plunger forward until 3–4 drops of media come out of the outlet port.
 - a. This will replace the media inside the device with the cytokine-infused media.
8. Incubate the cells statically for 16-20 hours with the cytokine-infused media.

Creating a chemotactic gradient in the tissue chamber using Matrigel and fMLP

Note: A chemokine gradient is created by mixing chemotactic proteins with Matrigel. The chemokine slurry is injected into the tissue chamber of the SynVivo device.

1. Prepare a 1-mL syringe containing serum-free media.
2. Unclamp one outlet tubing.
3. Place a drop of water at the base of the inlet port tubing on the top punch over the tissue chamber, and gently remove the tubing.

4. Insert the tubing with the serum-free media into the inlet port.
5. Clean the fluid from the surface of the device.
6. Gently push the plunger forward until 3–4 drops come out of the outlet port.
 - a. There will be resistance if endothelial cells are seeded into vascular channels.
7. Clamp the outlet tubing.
8. Cut and clamp the inlet tubing.
9. Thaw Matrigel on ice.
10. Dilute the stock concentration of chemokine (1000 ng/mL) at a ratio of 1:5 in serum-free tissue culture media.
 - a. The final concentration of chemokine will be 200 ng/mL (2X the concentration that's required in the devices).
11. Dilute 2 μ M stock of fMLP in Matrigel (1:1, or 1 part fMLP to 1 part Matrigel).
 - a. The final concentration of chemokine will be 1 μ M, which is the target concentration for the experiment.
 - b. Gently mix the chemokine with the Matrigel until it is evenly disbursed. Do not introduce bubbles.
 - c. Keep the mixture on ice until ready to load the devices.
 - d. A volume of 50 μ l of Matrigel to 50 μ l of 2X chemokine works well for 1–2 devices.
12. Prepare an empty 1-mL syringe with tubing.
13. Unclamp one outlet tubing.
14. Place a drop of water at the base of the inlet port tubing on the top punch over the tissue chamber, and gently remove the tubing.
15. Draw Matrigel into a small segment of tubing on the syringe.
 - a. Don't draw up the Matrigel into the needle or the barrel of the syringe.
 - b. It is recommended to only draw up what is necessary for one device (about 20 μ l). This is to prevent excess Matrigel from polymerizing in the syringe and excess tubing.
16. Quickly insert the tubing into the port and infuse by hand until 1-3 drops are seen in the outlet tubing.
17. Clamp the outlet tubing.
18. Cut and clamp the inlet tubing.
19. Place the device in a 37°C tissue culture incubator for 20 minutes to allow the Matrigel to polymerize.



Fluorescent staining of monocytes and injecting cells into the device

Note: Human monocytes are stained with CellTracker fluorescent dyes for visualization.

1. Prepare serum-free media for staining (DMEM with high glucose, 1% penicillin-streptomycin, and 1% L-glutamine).
 - a. Warm the serum-free B cell media to 37°C in the tissue culture incubator.
 - b. If monocytes are currently in a media containing serum, wash the cells 3 times with 1X PBS without calcium and magnesium. Resuspend the cell pellet in serum-free media before proceeding.
2. Prepare the serum-free media with the CellTracker Fluorescent Probe.
 - a. Allow the vial of dye to warm to room temperature.
 - i. The dye is dissolved in DMSO at a final concentration of 10 mM.
 - ii. The final (recommended) working concentration is between 0.5–25 µM. We will be using a working concentration of 10 µM to stain the B cells.
 - b. Add 5 µl of CellTracker Fluorescent Probe to 5 mLs of pre-warmed, serum free DMEM with 1% L-glutamine and 1% penicillin-streptomycin.
 - c. Keep the media at 37°C until ready to use.
3. Pellet the monocytes, and gently re-suspend the cell pellet in the 5 mLs of pre-warmed, serum-free DMEM containing the CellTracker Dye.
4. Incubate the cells for 20 minutes in a 37°C incubator, 5% CO₂.
5. Pellet the monocytes and draw off the media.
6. Wash the monocytes 3 times with more prewarmed, serum-free media. This will remove the excess dye.
7. Count the stained cells.
 - a. A minimum of 1 x 10⁶ cells/mL should be rolled into each device.
8. Pellet the monocytes and draw off the media.
9. Re-suspend the cell pellet in enough pre-warmed, serum-free DMEM (without any dye) to yield 1 x 10⁶ cells/mL.
10. For each device, prepare a 1-mL syringe with 1 x 10⁶ stained monocytes/mL.

11. Place a drop of water at the base of the inlet port tubing to be removed from the vascular channel.
12. Gently remove the tubing.
13. Remove the clamp on the outlet port of the vascular channel.
Keep all other ports clamped.
14. Place the syringe of cells onto the remote head of a programmable syringe pump.
15. Prepare the microscope and set up the imaging capabilities for live capture over time:
 - a. Ideal frame capture rate: 30 frames/second
16. Start recording the live-cell capture.
17. Insert the tubing of the syringe into the inlet port - the drop of water will prevent air entering the device as the tubing is inserted.
18. Start the program on the syringe pump using the following program:
 - a. Step 1: Constant Rate
 - i. Mode: Infuse
 - ii. Start rate: 0.5–1 $\mu\text{l}/\text{min}$
 - iii. Target volume: 0.5 mL
19. Maintain the flow rate until you visualize cells rolling into the channel.
20. Adjust the flow rate depending on the cell speed and adherence rate. Do not exceed a flow rate of 2.0 $\mu\text{l}/\text{minute}$.
21. Image the migration of monocytes into the tissue chamber over 1–24 hours.

Protocol 2: Tumor-induced lymphocyte migration

1. Seed the tissue chamber of the SynVivo devices with cancer cells mixed with a 1:5 dilution of growth-factor reduced Matrigel in serum-free media. This procedure creates miniature tumor spheroids.
2. Allow the tumor cells to establish in the devices for a minimum of 24 hours before adding lymphocytes.
3. Follow the protocol for fluorescently staining monocytes, as described above.
4. Inject the fluorescently-labeled cells at a flow rate of 1 $\mu\text{l}/\text{min}$.
5. Image the migration of monocytes into the tissue chamber over **24 hours**.

- a. Image the cells every few hours or set up in stage-top incubator for regular imaging.
- b. Assay could take over 24 hours to see cell movement into center chamber.

Results and Discussion

Blocking antibodies can be used to significantly reduce the numbers of rolling, adhering, and migrating monocytes in the SMN devices. Monoclonal antibodies against E-selectin, ICAM-1, or phosphoinositide 3-kinase (PI3K) block adhesion receptor-ligand interactions between lymphocytes and endothelial cells, thereby halting the leukocyte adhesion cascade. Blocking antibodies are usually added after cytokine activation of endothelial cells (see protocol above) and before monocytes are injected into the device.

Supporting Information:

1. Grivennikov, S. I., and M. Karin. 2011. Inflammatory cytokines in cancer: tumour necrosis factor and interleukin 6 take the stage. *Ann Rheum Dis.* 70 (Suppl 1):i104-8.
2. Vestweber, D. 2015. How leukocytes cross the vascular endothelium. *Nat. Rev. Immunol.* 15:692-704.
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Permeability Assay for the SynBBB IMN Linear Device

Principle:

Vascular endothelium provides a semipermeable barrier that allows for fluid exchange [1], but exposure to specific drugs or inflammatory cytokines can induce vascular leakiness [2]. Unlike Transwell™ models, which are arranged in a top-to-bottom architecture, vascular leakiness can be assessed and quantified in real-time across the SynTox model due to its side-by-side architecture.

One method used to visualize microvessel permeability is to introduce a solution with a fluorescent tracer (e.g. FITC-dextran) into a vessel and observe its diffusion across the endothelial barrier using fluorescence microscopy [3]. The molecular weight of the fluorescent tracer can influence its permeability through specific barriers, e.g. 4 kDa FITC-dextran can pass through a healthy intestinal epithelial barrier but a 70 kDa molecule cannot [4].

If you want to visualize the diffusion rate, you can capture images every 30 seconds in a live cell imaging sequence. If you are conducting a toxicology screen to determine the impacts of a compound on permeability, the protocol used is an endpoint assay where an image is taken after FITC-dextran has been allowed to diffuse for 60 minutes. The analysis steps in this protocol were performed on an endpoint assay.

This protocol will describe how to visualize FITC-dextran diffusion across an endothelial microvessel and into a center chamber, as well as how to quantify microvessel permeability by measuring changes in relative fluorescence intensity across a barrier.

Equipment:

- SynVivo IMN linear device, previously seeded with endothelial cells (monoculture), or an endothelial-epithelial cell co-culture
- Tygon tubing (0.02" ID x 0.06" OD; SynVivo 201005)
- Slide clamps (SynVivo 202001)
- 1 mL syringe with Luer-Lok tip (25 pack; SynVivo 203005)
- 24-gauge blunt-tipped needles (0.5" long; SynVivo 204003)
- Forceps (ultra-fine; VWR 100494-698)
- Stainless steel scissors (Fisher Scientific NC0806723)
- Syringe pump (PhD Ultra; Harvard Apparatus; SynVivo 301001 or 301002)
- Tissue culture incubator
- Fluorescence microscope (Ex/Em 485/520 nm)

Reagents:

- 1X PBS without calcium or magnesium (Corning 21040CV)

- Serum-free tissue culture media (DMEM, RPMI, or basal medium without serum)
- 4kDa FITC-Dextran (Sigma Aldrich 46944)

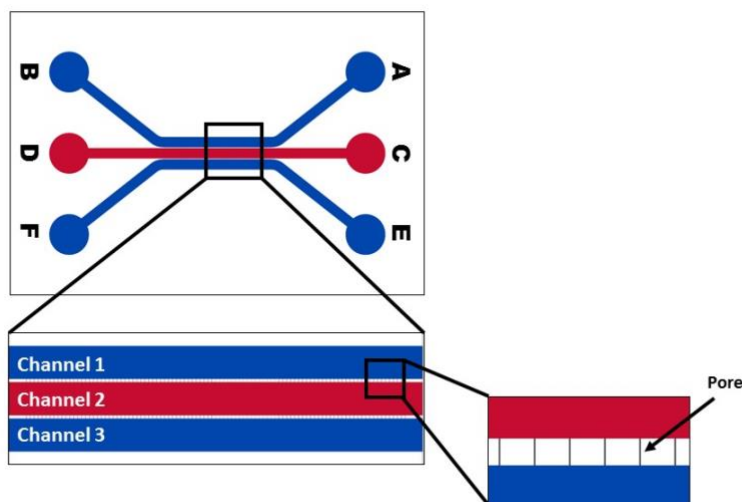
Protocol:

Figure 1. A schematic of inlet and outlet ports A-F on a SynVivo linear device.

Preparation of reagents:

6. Prepare a 10X stock solution (2.5 mg/mL) of 4 kDa FITC-Dextran in 1X PBS without calcium and magnesium.
 - a. Store solution in the dark at 4°C.
 - b. Solution is good for up to 4 months.
7. On the day of the experiment, prepare a 0.25 mg/mL solution of FITC-Dextran in serum-free media (50 μ l FITC in 450 μ l PBS).
 - a. Protect solution from light.
 - b. Immediately load the solution into a 1-mL syringe attached to a 3–5-inch length of Tygon tubing.
 - c. Prepare fresh on the day of experiment.
8. Prepare a 1-ml syringe with FITC-dextran solution.

Load the syringe pump with the syringe containing FITC-dextran:

11. Treat devices with compounds or controls prior to the beginning of this assay.
12. Carefully flush the endothelial channel (e.g. Channel 1; **Figure 1**) with fresh serum-free media prior to the start of the assay.
13. Attach a length of tubing to the syringe that is long enough to reach from the syringe pump to the device.
14. Infuse the Tygon tubing with the FITC-dextran solution.
15. Place the filled syringe into the syringe pump.
16. Tape the end of the tubing onto the pump, with the end of the tubing pointing up to the ceiling and not touching any surface.
17. Program the syringe pump to infuse at a flow rate of **0.1 $\mu\text{l}/\text{min}$** .
18. **Before connecting the devices to the syringe pump:** Take phase and fluorescence images (Ex/Em 485/520 nm).
 - a. These images will provide background fluorescence signals (if any treatments emit within the same range as FITC, this will be detected as well).

Infuse the center channel of each device with FITC-dextran:

1. Place a drop of liquid beside the inlet tubing of the center channel (**Figure 1**; Channel 2).
2. Gently remove the tubing and push out any bubbles using a needle.
3. Unclamp the outlet port for the center channel.
4. Insert the tubing of the syringe with FITC-dextran into the inlet port - the drop of water will prevent air entering the device as the tubing is inserted.
5. Clean the fluid from the surface of the device.
6. Infuse the fluorescent dye into the device at a flow rate of **0.1 $\mu\text{l}/\text{min}$ for 1 hour**.



Imaging the devices:

1. Take phase and fluorescence images of all devices after the 1-hour incubation period is completed (**Figure 2**).

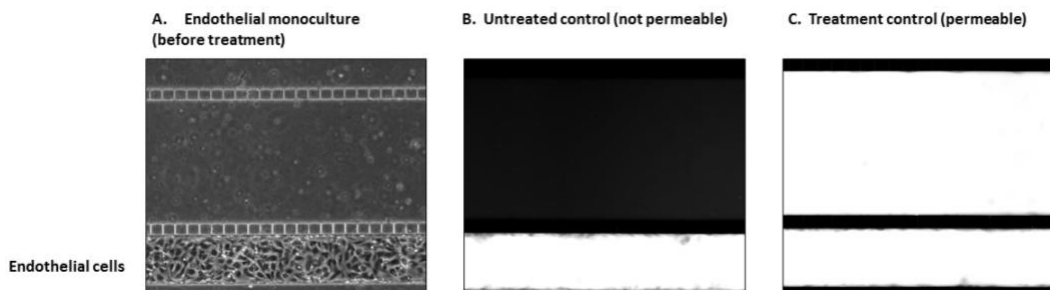


Figure 2. An endothelial monolayer is tested in a permeability assay. A phase contrast image of the monolayer before treatment (A) is shown. Endothelial microvessels were tested in a permeability assay, either as an untreated control (B) or with a proprietary compound that induces permeability (C).

2. Use an analysis program (i.e. ImageJ or Nikon Elements) to determine the relative fluorescence units.

Analysis using Nikon Elements software:

When permeability assays are performed in a Transwell™ assay system, media samples are removed from the bottom compartment and read in a fluorometer (Ex/Em 485/520 nm). The relative fluorescence units (RFU) can then be plotted as the increase/decrease in diffusion of FITC-dextran through the cell monolayers based on a treatment condition [5]. Other methods for quantitating the permeability across a monolayer in a microfluidics device have also been reported [6-9].

This protocol describes how to calculate the relative fluorescence units from images of a linear SynVivo device using Nikon Elements software. The results are plotted as a ratio of relative fluorescence units (R), measured from the FITC-dextran diffusing into the center channel (**Figure 1**, Channel 2; R_{TISSUE}) from the endothelial channel (**Figure 1**, Channels 1 or 3; R_{VASCULAR}).

1. In Nikon Elements, open the fluorescence image captured at Time 60 minutes (1-hour accumulation of FITC-dextran flowing through the vascular channel; **Figure 3**).



Figure 3. Fluorescence image of a permeability assay captured after a 1-hour infusion of 4 kDa FITC-dextran.

2. To measure the relative fluorescence units of the center channel (Channel 2; R_{TISSUE}), crop the image around Channel 2 only (**Figure 4**).
 - a. In Nikon Elements, go to this menu: Image > Crop
 - b. Draw a red square around the area of Channel 2.
 - c. Hit “Enter”. The image that remains will be the cropped image of Channel 2.
 - d. This cropped image is the first **Region of Interest (ROI)**.

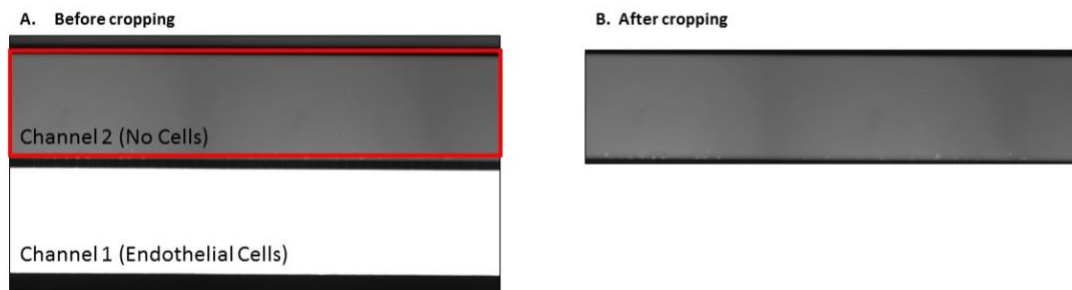


Figure 4. Outlining Channel 2 (red square; A) and using the “Crop” feature in Nikon Elements creates a Region of Interest (ROI; B).

3. Apply the Look Up Table (LUT) that eliminates any background fluorescence from the signal in the ROI.
 - a. The LUT is based on the fluorescence signal of the image captured at Time 0 (before FITC-dextran is flowed into the device).
 - b. It is created and saved as a separate file (“.lut”) in Nikon Elements.

- c. Under the “LUT” tab from the side menu (**Figure 5**), click on the disk icon and select “Load LUTs Setting....” From the drop-down menu.

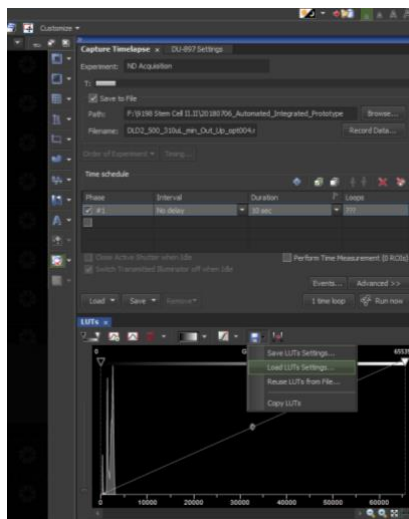


Figure 5. Loading the look up table (LUT) file.

- d. Select the LUT file and hit “Open” (**Figure 6**).

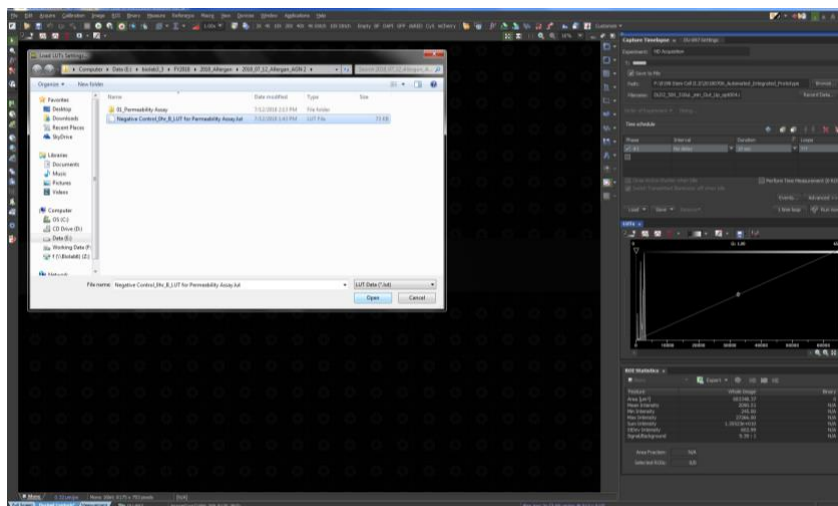


Figure 6. Opening the LUT file.

- e. The LUT is now applied to the image.

- f. Save the cropped image of Channel 2 as a separate ND2 file.
4. Open a blank Excel spreadsheet.
5. Open the ND2 file of Channel 2 (with the LUT applied).
6. In the ROI tab of the side menu, hit the Excel icon that says “Export” (**Figure 7**).
 - a. The ROI values will be automatically downloaded into the open Excel spreadsheet.
 - b. This data is **R_{TISSUE}** for the captured images.

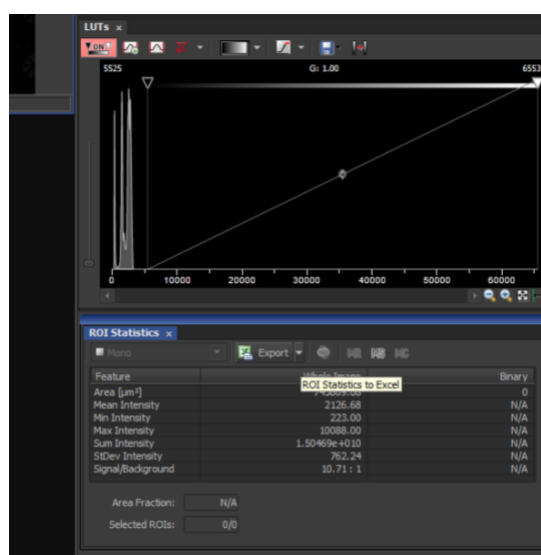


Figure 7. Exporting ROI statistics for Channel 2 to Excel (data is **R_{TISSUE}** for the captured images).

7. In the LUT tab of the side menu, hover the cursor over the right-hand number of the scale, until the cursor changes and you can type in the box.
8. Change the right-hand number of the LUT scale to 7500 (**Figure 8**).
 - a. This step will significantly magnify the fluorescence signal.

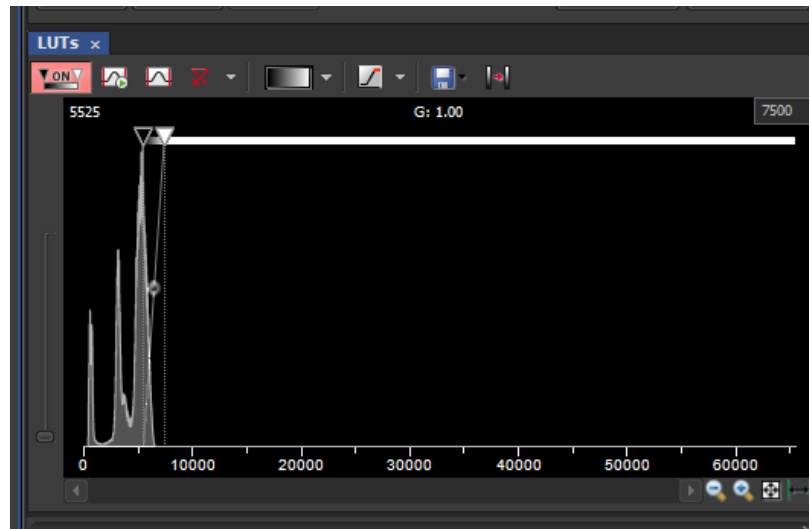


Figure 8. Magnifying the fluorescence signal by shortening the LUT range to 7500.

9. Examine the image of Channel 2 after magnifying the LUT signal.
 - a. If the negative control shows significant bleed through in one section of the device, but not across the whole chamber, the barrier is compromised. The data from the device should not be used (**Figure 9**).

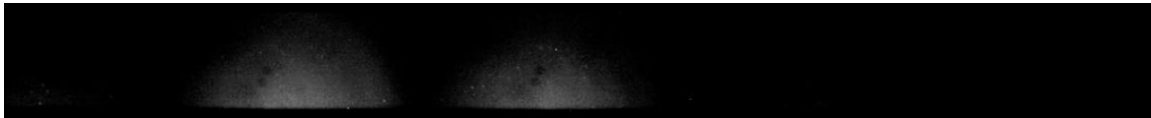


Figure 9. An example of a negative control device with broken barriers after the permeability assay. The enhanced fluorescent signal over a broad distance indicates 2 regions where the barriers were compromised.

10. To measure the relative fluorescence units of the vascular channel (Channel 1; R_{VASCULAR}), crop the image around Channel 1 only (**Figure 10**).
 - a. In Nikon Elements, go to this menu: Image > Crop
 - b. Draw a red square around the area of Channel 2.
 - c. Hit “Enter”. The image that remains will be the cropped image of Channel 2.
 - d. This cropped image is the second **Region of Interest (ROI)**.

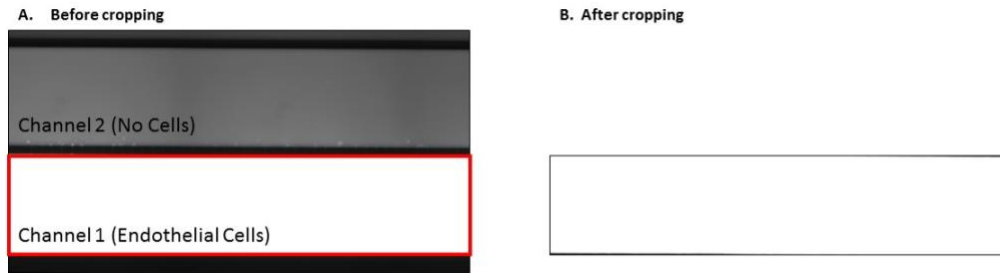


Figure 10. Outlining Channel 1 (red square; A) and using the “Crop” feature in Nikon Elements creates a Region of Interest (ROI; B).

11. Repeat all of the steps needed to quantitate and export the ROI values for Channel 1 into Excel.
 - a. This data is **R_{VASCULAR}** for the captured image.

Plotting the data:

The diffusion of FITC-dextran from the epithelial channel into the endothelial channel can be expressed in several ways, including as a ratio:

$$(R_{TISSUE} / R_{VASCULAR})$$

Or as a percent:

$$(R_{TISSUE} / R_{VASCULAR}) * 100$$

To plot the data, perform the following:

1. In the Excel spreadsheet with the ROI fluorescence intensity values, highlight the “ROI Mean Intensity” and the “ROI Sum Intensity” (**Figure 11**).

File Name	Time (ms)	Z Pos	X Pos	Y Pos	ROI ID	ROI Area	ROI Mean Intensity	ROI Min I	ROI Max I	ROI Sum Intensity	ROI StDev	ROI Signal	Binary Area	Binary Mean	Binary Min	Binary Max
Negative_Device7_Timethr_1	11832210	0	-1608.18	2063.1	N/A	675831.9	12513.23783	694	65535	80228223895	2484.195	73.38746	0	N/A	N/A	N/A
Negative_Device8_Timethr_1	11978817	0	12002.22	2400.8	N/A	723324.5	11585.31481	432	65535	79498661931	4085.899	126.7602	0	N/A	N/A	N/A
Negative_Device9_Timethr_1	12131169	0	25125.02	2875.9	N/A	742151.6	12419.28233	406	65535	87439546907	3940.176	129.2604	0	N/A	N/A	N/A

Figure 11. The raw values exported from Nikon Elements to Excel.

2. Copy and paste the mean intensity and sum intensity values into a new spreadsheet.
3. Calculate the ratio of Channel 2 mean intensity to the vascular channel mean intensity (Channel 1) by dividing $R_{TISSUE} / R_{VASCULAR}$ (in Cell E9, the equation would be “=C9/D9”). The ratio will likely be less than 1 (**Figure 12**).

7	8	ROI Mean Intensity			ROI Sum Intensity		
		Tissue Chamber	Vascular Chamber	Ratio (T/V)	Tissue Chamber	Vascular Chamber	Ratio (T/V)
9	Negative_Device 7	4349.761449	12513.23783	0.347612785	32009829259	80228223895	0.398984643
10	Negative_Device 8	1172.304451	11585.31481	0.10118883	8328057857	79498661931	0.104757208
11	Negative_Device 9	2945.186868	12419.28233	0.237146301	19313787403	87439546907	0.220881604

Figure 12. Calculating the ratio of intensity values.

4. Repeat this calculation for the sum intensity values.
5. To analyze the data, calculate the average (mean), standard deviation (Std Dev), standard error of the mean (SEM), and the Student’s t test (**Figure 13**).
 - a. For the standard error of the mean, use this equation in Excell:

$$=(STDEV(E11:E12))/(SQRT(COUNT(E11:E12)))$$

28	MEAN	STNDEV	SEM	T test (1-tailed; unequal variance)
30	Negative Control	0.169168	0.09613645	{RT(COUNT(E11:E12))}
31	Endotoxin	0.192892	0.027415012	0.393480594
32	TNF-alpha	0.265079	0.061904684	0.183123944
33	AGN Compound 2	0.529081	0.047857465	0.036659773

Figure 13. Use Excel to calculate the mean, standard deviation, standard error of the mean, and statistical significance using the Student’s t test.

6. Plot the average ratio of fluorescence intensity (TC/VC). Add error bars (either standard deviation or standard of the mean).
 - a. Plotting the data as a ratio can demonstrate the change in FITC-dextran diffusion into the endothelial channel as a result of a specific treatment (**Figure 14**).

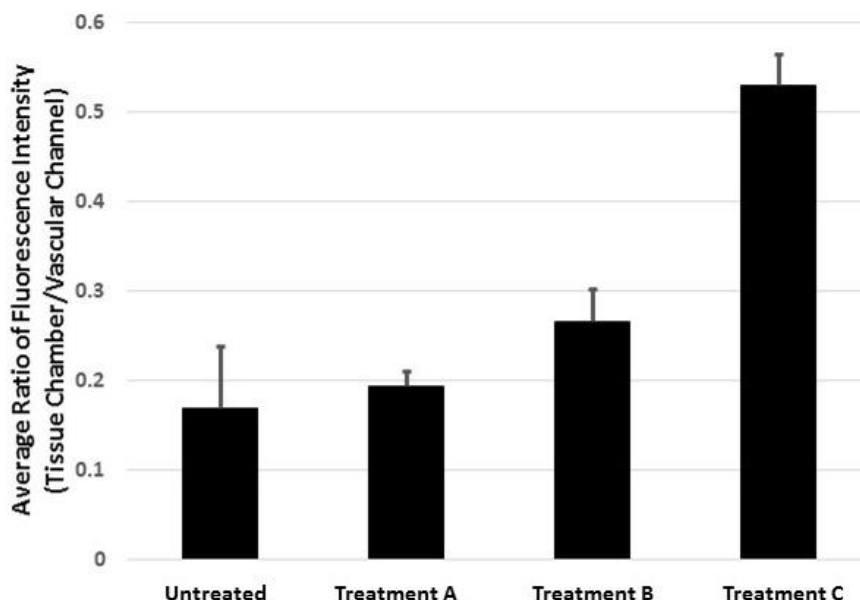


Figure 14. Example of plotted permeability results that show how treatments increase microvascular permeability compared to the untreated control.

Supporting Information:

6. Yuan, S. Y., and R. R. Rigor. Regulation of Endothelial Barrier Function. San Rafael (CA): Morgan & Claypool Life Sciences; 2010.
7. Pober, J. S., and W. C. Sessa. 2007. Evolving functions of endothelial cells in inflammation. *Nat Rev Immunol.* 7(10):803-15.
8. Woting A., and M. Blaut. 2018. Small intestinal permeability and gut-transit time determined with low and high molecular weight fluorescein isothiocyanate-dextrans in C3H mice. *Nutrients.* 10(6): 685.
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10. Tang, Y., F. Soroush, J. B. Sheffield, B. Wang, B. Prabhakarpandian, and M. F. Kiani. 2017. A biomimetic microfluidic tumor microenvironment platform mimicking the EPR effect for rapid screening of drug delivery systems. *Sci Rep.* 7:9359.
11. Yuan H., M. W. Gaber, T. McColgan, M. D. Naimark, M. F. Kiani, and T. E. Merchant. 2003. Radiation-induced permeability and leukocyte adhesion in the rat blood-brain barrier: modulation with anti-ICAM-1 antibodies. *Brain Res.* 969: 59-69.
12. Terrell-Hall, T. B., A. G. Ammer, J. I. Griffith, and P. R. Lockman. 2017. Permeability across a novel microfluidic blood-tumor barrier model. *Fluids Barriers CNS,* 14(1):3.

13. Terrell-Hall, T. B., M. I. Nounou, F. El-Amrawy, J. I. G. Griffith, and P. R. Lockman. 2017. Trastuzumab distribution in an in-vivo and in-vitro model of brain metastases of breast cancer. *Oncotarget*. 8(48):83734-83744.

Permeability Assay for the SynBBB IMN Radial Device

Principle:

Permeability through the vascular channel into the tissue chamber in SynBBB devices 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 (see Yuan et al. in Supporting Information):

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

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	0.01 cm
dI_t/dt	Change in Basolateral Intensity over Time	Calculated below

Equipment:

- SynVivo microfluidics device, previously coated with 200 $\mu\text{g}/\text{mL}$ fibronectin, seeded with endothelial cells and astrocytes/pericytes
- Tygon tubing (0.02" ID x 0.06" OD; SynVivo 201005)
- Slide clamps (SynVivo 202001)
- 1 mL syringe with Luer-Lok tip (25 pack; SynVivo 203005)
- 24-gauge blunt-tipped needles (0.5" long; SynVivo 204003)
- Forceps
- Stainless steel scissors
- Syringe pump (PhD Ultra; Harvard Apparatus; SynVivo 301001 or 301002)
- Tissue culture incubator

Reagents:

- 1X PBS without calcium or magnesium (Corning 21040CV)
- Serum-free tissue culture media (DMEM, RPMI, or basal medium without serum)
- 4kDa FITC-Dextran (Sigma Aldrich 46944)

Protocol:Preparation of reagents:

9. Prepare a 2.5 mg/mL stock of FITC-Dextran (4kDa) in 1X PBS without calcium and magnesium.
 - a. Store solution in the dark at 4°C.
 - b. Solution is good for up to 4 months.
10. On the day of the experiment, prepare a 0.25 mg/mL solution of FITC-Dextran in serum-free media (50 µl FITC in 450 µl PBS).
 - a. Protect solution from light.
 - b. Immediately load the solution into a 1-mL syringe attached to a 3–5-inch length of Tygon tubing.
11. Set the syringe on the syringe pump and fully perfuse the tubing with FITC-Dextran solution.

Video acquisition:

19. Capture a phase image of the device before beginning the assay.
20. Place the cell-coated device onto the stage of an epifluorescence microscope. Place a drop of water around the inlet tubing, and remove tubing.
21. Set the camera exposure, and stage of the microscope to capture both the apical and basolateral channels of the SynBBB device in one image.
22. Interface the perfused tubing containing FITC-dextran to the device, and begin flow at 0.1 µL/min.
23. Immediately begin to acquire images every 30 s for 1 hour (121 frames).
 - a. Close the fluorescence shutter between image acquisitions to prevent photobleaching.
24. Save the image stack as an AVI.

Results and Discussion

Video analysis using ImageJ:

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

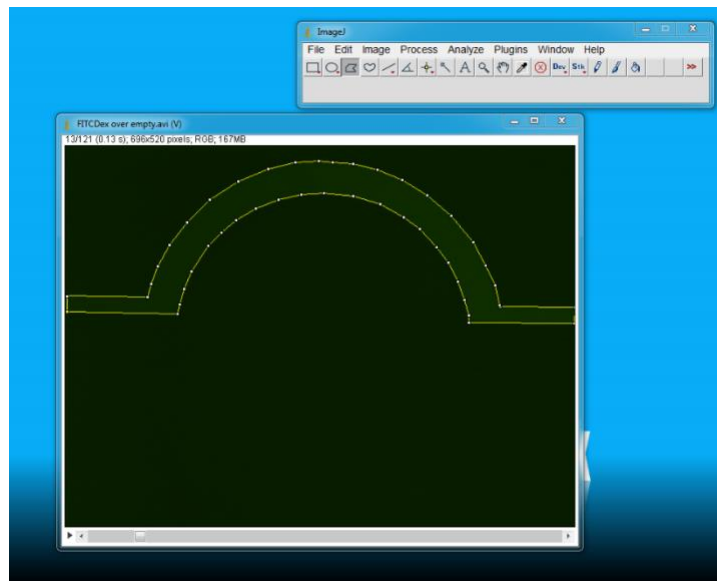
File→Import→AVI

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.

13. 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.



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

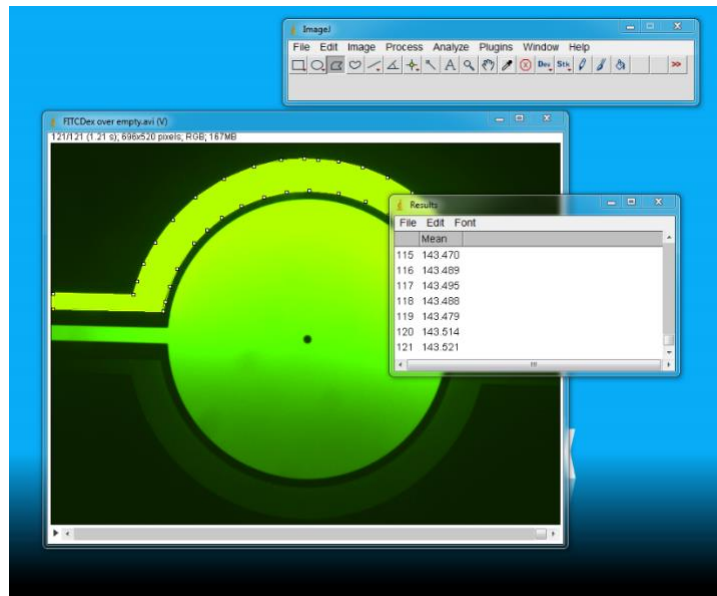
Analyze→Set Measurements
Select “Mean Gray Value”.

Under “Redirect to”, select the file name.

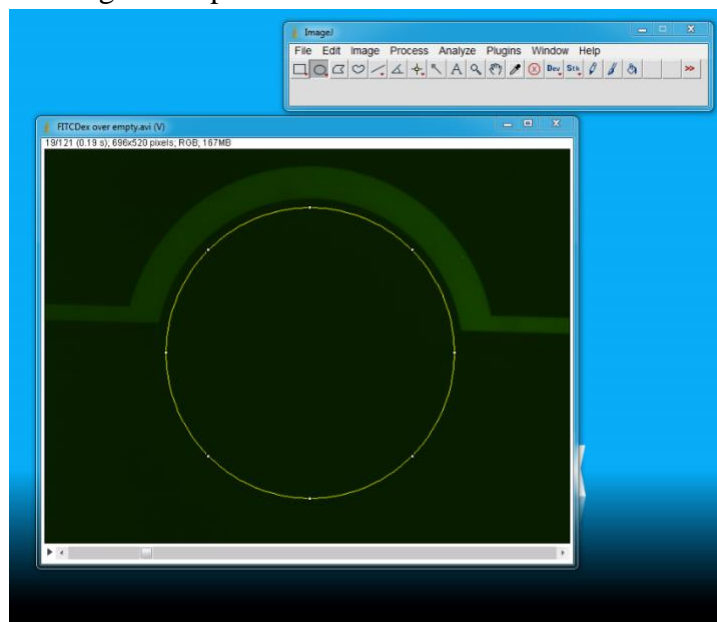
15. Analyze the mean gray value of the stack:

Plugins→Stacks→Measure Stack

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



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



17. Repeat gray value measurement of the stack:

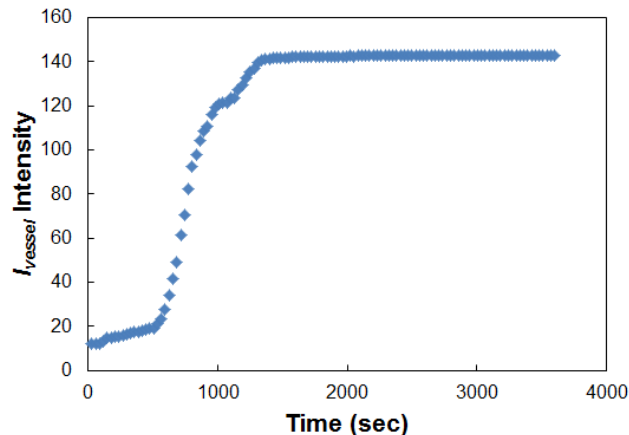
Plugins→Stacks→Measure Stack

Copy data from “Results” window to Excel for analysis.

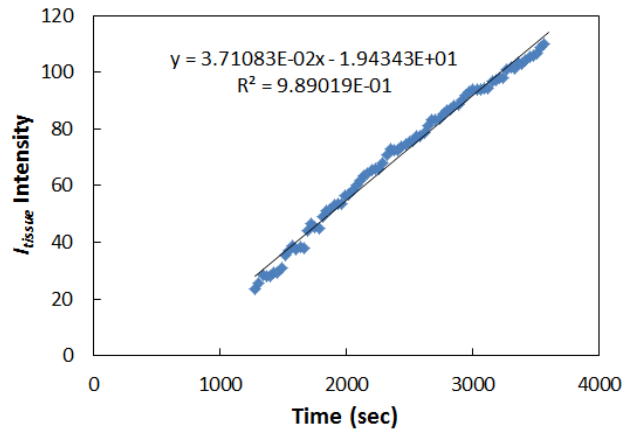
***I_{tissue}* will be generated from this data.**

18. 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).

19. 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.



20. Next, find the slope of *I_{tissue}* vs. time. Only use data in the time range specified by the *I₀* 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.



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

$$P = (1 - 0) \frac{1}{140.5} (0.01 \text{ cm})(3.7 \times 10^{-2} s) = 0.0121 \text{ cm/s}$$

Supporting Information:

Reference:

1. Yuan H., M. W. Gaber, T. McColgan, M. D. Naimark, M. F. Kiani, and T. E. Merchant. 2003. Radiation-induced permeability and leukocyte adhesion in the rat blood–brain barrier: modulation with anti-ICAM-1 antibodies. *Brain Res.* 969: 59-69.
2. Terrell-Hall, T. B., A. G. Ammer, J. I. Griffith, and P. R. Lockman. 2017. Permeability across a novel microfluidic blood-tumor barrier model. *Fluids Barriers CNS*, 14(1):3.
3. Terrell-Hall, T. B., M. I. Nounou, F. El-Amrawy, J. I. G. Griffith, and P. R. Lockman. 2017. Trastuzumab distribution in an in-vivo and in-vitro model of brain metastases of breast cancer. *Oncotarget.* 8(48):83734-83744.

Live/Dead Cell Staining in the Device

Principle:

Distinguishing between live and dead cells is a common strategy used in drug screening. The following protocol describes how to perform cell viability staining within the SynVivo devices using Calcein-AM/Ethidium homodimer-1 or PrestoBlue stains.

Equipment:

- SynVivo microfluidics device, previously seeded with endothelial cells and astrocytes/pericytes
- Tygon tubing (0.02" ID x 0.06" OD; SynVivo 201005)
- Slide clamps (SynVivo 202001)
- 1 mL syringe with Luer-Lok tip (25 pack; SynVivo 203005)
- 24-gauge blunt-tipped needles (0.5" long; SynVivo 204003)
- Forceps (ultra-fine; VWR 100494-698)
- Stainless steel scissors (Fisher Scientific 19-062530)
- Aluminum foil
- Kimwipes (Fisher Scientific 06-666A)

Reagents:

- Serum-free media such as DMEM or RPMI (or basal media without serum)
- 1X PBS without calcium or magnesium (Corning 21040CV)
- Molecular Probes Live/Dead Viability/Cytotoxicity Kit for Mammalian Cells (Fisher Scientific L3224)
- Molecular Probes PrestoBlue Cell Viability Reagent (ThermoFisher Scientific A13261)

Protocol:

Cell viability staining of cells in the SynVivo device using Calcein-AM and Ethidium homodimer-1:

1. Take a phase image of the cells to record cellular morphology and density prior to staining.
2. Prepare the stains:
 - a. In 997 μ l of serum-free media, add 1 μ l of Calcein-AM and 2 μ l of Ethidium Homodimer-1.
3. Flush all channels of the SynVivo device by hand with 1X PBS (without calcium or magnesium) to remove the growth media and any cellular debris.

- a. Gently push the plunger of a 1-mL syringe filled with PBS, until 3 drops come out of the outlet tubing for each channel.
4. Gently infuse all channels of the SynVivo device with the staining solution (inlet port to outlet ports).
 - a. The cells will quickly take up the stains.
 - b. Add the mixture quickly and gently, so that the stain is evenly distributed but the cells remain in the device.
 - c. Save any leftover stain.



5. Incubate the device **at 37°C for 10 minutes**.
6. Gently infuse all channels of the SynVivo device with the staining solution, but in the opposite direction (outlet ports to inlet ports).
 - a. This will saturate the entire device with stain.



7. Incubate the device **at 37°C for 10 minutes**.
8. Gently infuse all channels of the SynVivo device with serum-free media to wash away excess stain.
9. Image immediately. The Calcein-AM will begin to leach out of the cells in about 10 minutes.

Cell viability staining of cells in the SynVivo device using PrestoBlue:

1. To correct for background fluorescence, a control device containing cell culture media alone (no cells) should be included in this protocol.
2. Take a phase image of the cells or the control device to record cellular morphology and density prior to staining.
3. Prepare the stains:
 - a. PrestoBlue stain is at a 10X concentration.
 - b. Dilute the stain to a 1X concentration in serum-free media.
4. Flush all channels of the SynVivo device by hand with 1X PBS (without calcium or magnesium) to remove the growth media and any cellular debris.

- a. Gently push the plunger of a 1-mL syringe filled with PBS, until 3 drops come out of the outlet tubing for each channel.
5. Gently infuse all channels of the SynVivo device with the staining solution (inlet port to outlet ports).
 - a. The cells will quickly take up the stains.
 - b. Add the mixture quickly and gently, so that the stain is evenly distributed but the cells remain in the device.
 - c. Save any leftover stain.
6. Incubate the device **at 37°C for 10 minutes**.
7. Image immediately.
 - a. PrestoBlue can be toxic to cells if left on the cells for longer periods of time. This toxicity may lead to a change in the fluorescence signal and can lead to false results.



Supporting Information:

1. Istivan, T. S., E. Pirogova, E. Gan, N. M. Almansour, P. J. Coloe, and I. Cosic. 2011. Biological effects of a de novo designed myxoma virus peptide analogue: evaluation of cytotoxicity on tumor cells. *PLoS One*. 6:e24809

Comment: This paper utilizes PrestoBlue and other cytotoxicity kits to fluorescently evaluate cell viability.

Fixing Cells in the Devices

Principle:

The following protocol describes how to fix cells in the devices after the permeability assay.

Equipment:

- SynVivo microfluidics device, previously seeded with endothelial cells (HUVECs) and/or tumor cells
- Tygon tubing (0.02" ID x 0.06" OD; SynVivo 201005)
- Slide clamps (SynVivo 202001)
- 1 mL syringe with Luer-Lok tip (25 pack; SynVivo 203005)
- 24-gauge blunt-tipped needles (0.5" long; SynVivo 204003)
- Forceps (ultra-fine; VWR 100494-698)
- Stainless steel scissors (Fisher Scientific 19-062530)
- Aluminum foil
- Kimwipes (Fisher Scientific 06-666A)
- Parafilm laboratory wrapping film (Fisher Scientific 13-374-12)
- Disposable Petri dishes (Corning 351007)
- Refrigerator

Reagents:

- 1X PBS without calcium or magnesium (Corning 21040CV)
- 4% formaldehyde (diluted from 16% stock; ThermoFisher Scientific 28908)

Protocol:

Fixing cells in the SynVivo device:

1. Take a phase image of the cells to record cellular morphology and density prior to staining.
2. Prepare 4% formaldehyde from a 16% stock solution of methanol-free formaldehyde.
 - a. Add 2.75 mL of 16% methanol-free formaldehyde to 8.25 mL of 1X PBS.
 - i. Prepare solution just before each assay.

3. Flush all channels of the SynVivo device by hand with 1X PBS (without calcium or magnesium) to remove any cellular debris.
 - a. Gently push the plunger of a 1-mL syringe filled with PBS, until 3–6 drops come out of the outlet tubing for each channel.
4. Gently infuse all channels of the SynVivo device with 4% paraformaldehyde (by hand).
 - a. Gently push the plunger of a 1-mL syringe filled with 4% paraformaldehyde.
 - b. Let 3 drops come out of the outlet tubing for each channel.
5. Incubate the device at **room temperature for 8–15 minutes**.
 - a. Cover the devices with aluminum foil during this incubation period.
6. Wash all channels of the SynVivo device by hand with 1X PBS (without calcium or magnesium).
 - a. Gently push the plunger of a 1-mL syringe filled with PBS, until 6 drops come out of the outlet tubing for each channel.
7. **The assay may be stopped at this point, if needed:**
 - a. Place devices in a sterile Petri dish.
 - b. Unclamp all tubing.
 - i. PDMS must be kept hydrated, or evaporation will lead to bubbles forming or drying out the cells.
 - ii. Unclamping the tubing allows the liquid to flow back into the device, which will hydrate the channels and chambers.
 - c. Put a PBS-soaked KimWipe in the Petri dish to keep the devices hydrated.
 - d. Seal the dish with the device inside using Parafilm.
 - e. Store device at 4°C overnight.



Antibody Staining and Imaging Cells in the Devices

Principle:

Antibody staining is a common way to distinguish between cells within a co-culture. Astrocytes can be stained with antibodies to glial fibrillary acidic protein (GFAP), and pericytes can be stained with antibodies to the alpha subunit of smooth muscle actin (α -SMA). Endothelial tight junctions can be stained with anti-VE-cadherin or anti-ZO1 (zonula occludens-1) antibodies.

Direct staining involves using primary antibodies conjugated to a fluorophore. Indirect staining uses a non-conjugated primary antibody, followed by staining with a conjugated secondary antibody. This protocol describes an indirect staining method, but either direct or indirect staining can be used effectively in the devices.

Equipment:

- SynVivo microfluidics device, previously seeded with endothelial cells (HUVECs) and/or tumor cells
- Tygon tubing (0.02" ID x 0.06" OD; SynVivo 201005)
- Slide clamps (SynVivo 202001)
- 1 mL syringe with Luer-Lok tip (25 pack; SynVivo 203005)
- 24-gauge blunt-tipped needles (0.5" long; SynVivo 204003)
- Forceps (ultra-fine; VWR 100494-698)
- Stainless steel scissors (Fisher Scientific 19-062530)
- Aluminum foil
- Kimwipes (Fisher Scientific 06-666A)
- Parafilm laboratory wrapping film (Fisher Scientific 13-374-12)
- Disposable Petri dishes (Corning 351007)
- 0.22 μ m pore Steriflip-GV centrifuge tube top filter unit (EMD Millipore SE1M179M6)
- Refrigerator

Reagents:

- 1X PBS without calcium or magnesium (Corning 21040CV)
- 4% formaldehyde (diluted from 16% stock; ThermoFisher Scientific 28908)
- 0.1% Triton X-100 (diluted from stock solution; VWR A16046-AE)
- Bovine serum albumin (Fraction V; Fisher Scientific BP1600-100)
- Sheep anti-human GFAP (R&D Systems AF2594)
- Mouse anti-human SMA (R&D Systems MAB2148F)
- Rabbit anti-human ZO1/TJP1 (Boster PB9234)
- AF 647 donkey anti-sheep IgG (Jackson ImmunoResearch 713-605-003)
- AF 488 donkey anti-mouse IgG (Jackson ImmunoResearch 715-545-150)

- AF 594 donkey anti-rabbit IgG (Jackson ImmunoResearch 711-585-152)
- NucBlue ReadyProbes reagent or Hoechst 33342 DNA dye (ThermoFisher Scientific R37605 or R37606)

Protocol:

Antibody staining should be carried out within a laminar flow hood. The antibodies and stains used in this assay are listed in **Table 1**. These antibodies are specific for a human model using hBMECs, as well as human astrocytes and pericytes. Working concentrations are listed as a starting point, but further optimization may be required depending on the cell types used in the SynBBB model.

Catalog Number	Host	Target	Isotype	Conjugate	Recommended Dilution
Boster PB9234	Rabbit	Human ZO1/TJP1	N/A	None	1:50
Jackson ImmunoResearch 711-585-152	Donkey	Rabbit IgG (H+L)	Polyclonal	AF 594	1:400
R&D Systems AF2594	Sheep	Human GFAP	IgG	None	1:20
Jackson ImmunoResearch 713-605-003	Donkey	Sheep IgG (H+L)	Polyclonal	AF 647	1:400
R&D Systems MAB2148F	Mouse	Human SMA	IgG	None	1:50
Jackson ImmunoResearch 715-545-150	Donkey	Mouse IgG (H+L)	Polyclonal	AF 488	1:400
ThermoFisher Scientific R37605 or R37606		Cellular DNA		Hoechst 33342 (UV)	2 drops per mL

Fix cells in the SynVivo device:

1. Fix the cells with 4% PFA prior to staining (see protocol “Fixing Cells in the Devices”).

Permeabilization and blocking:

1. Prepare 0.1% Triton X-100, and keep on ice until ready to use.
 - a. Dilute stock solution of Triton-X 100 in 1X PBS.

- b. If making 50 mL of 0.1% Triton X-100, add 50 μ l of the Triton X-100 stock solution to 49.95 mL of 1X PBS.
 - c. Store at 4°C.
 2. Prepare 1% BSA in 1X PBS, and keep on ice until ready to use.
 - a. Dissolve 1 gram of BSA into 100 mL of 1X PBS.
 - b. Sterile filter the solution using a 0.22-micron filter.
 - c. Prepare a new solution every two weeks.
 - d. Store at 4°C.
 3. **Permeabilization step:** Gently infuse all channels of the SynVivo device with 0.1% Triton X-100.
 - a. Let 3 drops come out of the outlet tubing for each channel that will be stained.
 4. Incubate the device at **room temperature for 15 minutes**.
 5. Wash the channel at least 2–3 times with 1X PBS.
 - a. This corresponds to a total of 6-10 drops of 1XPBS to push through the outlet tubing for each channel that was permeabilized.
 6. **Blocking step:** Gently infuse all channels of the SynVivo device with 1% BSA.
 - a. Gently push the plunger of a 1-mL syringe filled with 1% BSA.
 - b. Let 3 drops come out of the outlet tubing for each channel.
 7. Incubate the device at **room temperature** for 1 hour.
 8. Do not wash the device after blocking!!!



Antibody and DNA staining:

1. Dilute primary antibodies in 1% BSA according to vendor recommendations or previously optimized conditions (see **Table 1**).
2. Gently infuse all channels of the SynVivo device with primary antibodies diluted in 1% BSA (by hand).
 - a. Gently push the plunger of a 1-mL syringe filled with the antibody solution.
 - b. Let 3 drops come out of the outlet tubing for each channel.

3. Incubate the device at **room temperature** for 2–3 hours, OR at **4°C** overnight.
4. Wash all channels of the SynVivo device by hand with 1X PBS (without calcium or magnesium).
 - a. Gently push the plunger of a 1-mL syringe filled with PBS, until 6–10 drops come out of the outlet tubing for each channel.
 - i. The extra drops are equivalent to washing the device twice.
5. Dilute secondary antibodies in 1% BSA according to vendor recommendations or previously optimized conditions (see **Table 1** for examples).
6. Gently infuse all channels of the SynVivo device with secondary antibodies diluted in 1% BSA (by hand).
 - a. Gently push the plunger of a 1-mL syringe filled with the antibody solution.
 - b. Let 3 drops come out of the outlet tubing for each channel.
7. Incubate the device at **room temperature** for 45 minutes to 1 hour.
8. Wash all channels of the SynVivo device by hand with 1X PBS (without calcium or magnesium).
 - a. Gently push the plunger of a 1-mL syringe filled with PBS, until 6–10 drops come out of the outlet tubing for each channel.
 - i. The extra drops are equivalent to washing the device twice.
9. Gently infuse all channels of the SynVivo device with NucBlue DNA stain (by hand).
 - a. Gently push the plunger of a 1-mL syringe filled with the NucBlue solution.
 - b. Let 3 drops come out of the outlet tubing for each channel.
10. Incubate the device at **room temperature** for 5 minutes.



11. Wash all channels of the SynVivo device by hand with 1X PBS (without calcium or magnesium).
 - a. Gently push the plunger of a 1-mL syringe filled with PBS, until 6–10 drops come out of the outlet tubing for each channel.
 - i. The extra drops are equivalent to washing the device twice.

Imaging:

15. Proceed with imaging according to the specifications of your fluorescent microscope.

Results and Discussion

The quality of antibody staining is model and vendor-specific, and optimization of these conditions should be performed prior to establishing a co-culture in the SynVivo devices. Staining optimization usually begins in a tissue culture plate, but antibody staining in the devices may require further optimization to balance the signal to noise ratio. Please refer to the guidelines here, as well as the manufacturer's instructions for additional details.

Supporting Information:

1. Hol, E. M., and M. Pekny. 2015. Glial fibrillary acidic protein (GFAP) and the astrocyte intermediate filament system in diseases of the central nervous system. *Curr. Opin. Cell Biol.* 32:121-130.
2. Bazzoni, G., and E. Dejana. 2004. Endothelial cell-to-cell junctions: molecular organization and role in vascular homeostasis. *Physiol Rev.* 84:869-901.

Comment: There are several options for markers that stain endothelial cell tight junctions (CD31, VE-cadherin, etc.). This review highlights several of these markers. The tight junction protein ZO-1 was chosen for the laboratory exercise because of staining consistency and excellent signal-to-noise ratio for this cell type.

Circulating Media Through the Devices

Principle:

Syringe or peristaltic pumps apply pressure to a liquid that is at rest, and the fluid moves at a steady flow rate and programmed velocity. The elevation of the pump and the microfluidics device will determine whether the fluid pressure at a specific point will be affected when media is being recirculated in a closed loop.

If the pump is lower than the microfluidics device, the fluid is flowing upwards through the tubing and gaining elevation height as it moves. This results in a loss of fluid pressure in the tubing as the fluid rises, which will reduce the overall flow rate into the microfluidics device. When recirculating media through the device, it is important to keep the pump either on the same plane as the microfluidics device or slightly higher to prevent the loss of fluid pressure.

Equipment:

- SynVivo microfluidics device, previously seeded with endothelial cells (HUVECs) and/or astrocytes and pericytes
- Tygon tubing (0.02" ID x 0.06" OD; SynVivo 201005)
- Slide clamps (SynVivo 202001)
- 1 mL syringe with Luer-Lok tip (25 pack; SynVivo 203005)
- 24-gauge blunt-tipped needles (0.5" long; SynVivo 204003)
- Forceps (ultra-fine; VWR 100494-698)
- Stainless steel scissors (Fisher Scientific 19-062530)
- Peristaltic pump (Instech P720/37K)
- Peristaltic Pump Tubing (0.02 inch or 0.05 cm inner diameter; Instech FL-020C-FL)
- Syringe pump (PhD Ultra; Harvard Apparatus; SynVivo 301001 or 301002)
- Lab jack with 12-inch x 12-inch plate (30.48 cm x 30.48 cm; Fisher Scientific S63083)
- Tissue culture incubator

Reagents:

- Cell culture media

Protocol:

Media can be circulated through the devices in an open loop or a closed loop system. In this protocol, the open loop system uses a syringe pump, and the closed loop system uses a peristaltic pump.

An open loop system:

Note: An open loop system (**Figure 1**) is recommended if the media circulating through the device needs to be sampled over time.

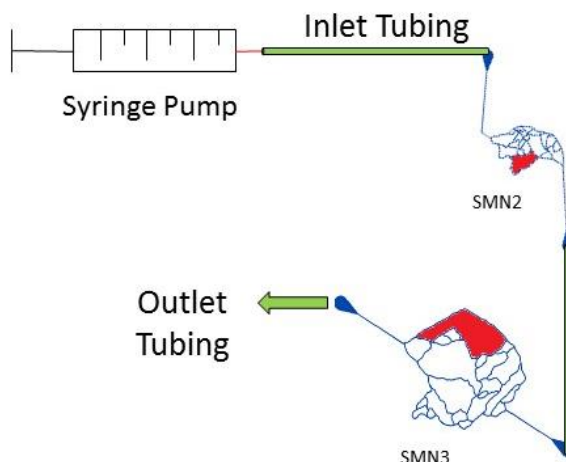


Figure 1. Microfluidic setup for an open-loop system using a syringe pump and two SynTumor devices connected in tandem.

1. Prime the device and establish cell cultures as described previously.
2. Connect the tubing from the syringe in the syringe pump to the inlet port of the first device.
3. The outlet and inlet ports of two devices can be connected to each other with Tygon tubing, or a customized chip can be designed with two networks connected on one chip.
4. Place the outlet tubing from the second device into an autosampler or a sterile collection tube.
5. Program the syringe pump according to the parameters needed for the experiment.
6. Start the flow of media through the devices.
7. Collect the media at specific time points.

A closed loop system:

Note: A closed loop system (**Figure 2**) is recommended to measure uptake kinetics of drugs or agents over time.

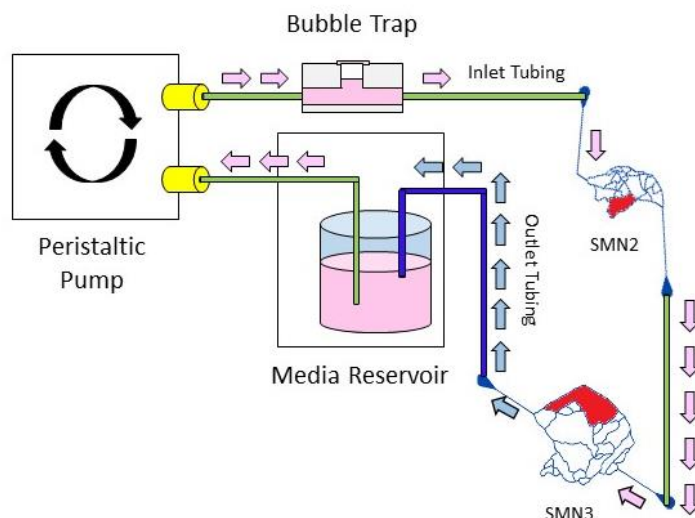


Figure 2. Microfluidic setup for a closed-loop system using a peristaltic pump and two SynTumor devices connected in tandem.

1. Prime the device and establish cell cultures as described previously.
2. Prime all tubing and connectors with the same media that will be in the media reservoir. Make sure all bubbles are eliminated.
3. Place the primed tubing in the peristaltic pump, according to the manufacturer's instructions.
4. Before connecting any microfluidics devices, run the peristaltic pump with the media to fully flush and remove air bubbles from the tubing prior to the start of the experiment.
 - a. Treat the peristaltic pump/tubing combination as an open loop system at this point.
 - b. Check to make sure that the amount of media that is coming out of the "outlet" tubing is accurate for the flow rate set on the peristaltic pump.
 - i. Make adjustments to the flow rate as necessary.
5. Connect the tubing from the peristaltic pump to the inlet port of the first device.
6. The outlet and inlet ports of two devices can be connected to each other with Tygon tubing, or a customized chip can be designed with two networks connected on one chip.
7. Place the outlet tubing from the second device into the media reservoir.
8. Start the flow of media through the devices.
9. Image the devices at specific time points.

Injecting Fluorescently-Labeled Particles into the Devices

Principle:

Fluorescently-labeled particles can be flowed into the microvascular networks of the SynBBB devices to observe which particles will cross the barriers and at what rates. These particles can be flowed into an empty (cell-free) device to observe the rate of the particles entering into the tissue chamber in an open system. Particles can also be flowed into a device containing endothelial cells to observe vessel leakiness in the presence of cytokines or tumor cells.

Equipment:

- SynVivo microfluidics device, either cell-free or previously seeded with endothelial cells and/or astrocytes and pericytes
- Tygon tubing (0.02" ID x 0.06" OD; SynVivo 201005)
- Slide clamps (SynVivo 202001)
- 1 mL syringe with Luer-Lok tip (25 pack; SynVivo 203005)
- 24-gauge blunt-tipped needles (0.5" long; SynVivo 204003)
- Forceps (ultra-fine; VWR 100494-698)
- Stainless steel scissors (Fisher Scientific 19-062530)
- Aluminum foil
- Kimwipes (Fisher Scientific 06-666A)

Reagents:

- Serum-free media such as DMEM or RPMI (or basal media without serum)
- 1X PBS without calcium or magnesium (Corning 21040CV)
- Fluoro-Max Green fluorescent particles, 3.0-micron in diameter (ThermoFisher Scientific G0300)
- Fluoro-Max Green fluorescent particles, 10-micron in diameter (ThermoFisher Scientific G1000B)

Protocol:

1. Vigorously shake the bottles with the particles to evenly mix the solution.
2. Prepare a solution of 2 particles of different sizes:
 - a. Add 100 μ l of each size of particle to 800 μ l of 1X PBS or serum-free media (total volume of 1 mL).
 - b. The goal is to achieve an overall solution with $1-5 \times 10^5$ particles/mL.
3. Perfuse the SynVivo device with 1X PBS or cell-free media.

4. Set up the microscope and filters for live imaging over time (between 5 minutes to 1 hour should be sufficient to observe particle entry into the tissue chamber).
5. Focus on an area containing tissue chamber, porous barrier, and vascular channel.
6. Inject the particles into the device at a flow rate of 5–10 $\mu\text{l}/\text{minute}$.
7. Image the perfusion of the particles into the tissue chamber over time.

Stabilization of Cell-Secreted Factors and Cellular DNA/RNA

Principle:

Gene expression analysis requires immediate stabilization of cellular DNA/RNA. Trypsinization can alter the genomic expression patterns of cells, leading to degradation of some RNA transcripts and/or upregulated expression of some genes.

To stabilize gene expression in the devices, an RNA stabilizing agent is infused into the device. The cells will dissociate from the PDMS in the presence of the stabilizing agent, so the cells can be flushed from the device and collected in an RNase/DNase-free collection tube. The cells can be archived by freezing the samples at -20°C.

The example shown in this protocol uses an IMN SynVivo device.

Equipment:

- Idealized SynVivo microfluidics device (IMN)
- Tygon tubing (0.02 inch ID x 0.06 inch OD, or 0.05 cm ID x 0.15 cm OD; SynVivo 201005)
- Slide clamps (SynVivo 202001)
- 1 mL syringe with Luer-Lok tip (25 pack; SynVivo 203005)
- 24-gauge blunt-tipped needles (0.5 inches or 1.27 cm long; SynVivo 204003)
- Forceps (ultra-fine; VWR 100494-698)
- Stainless steel scissors (Fisher Scientific 19-062530)
- Syringe pump (Harvard Apparatus; SynVivo 301001 or 301002)
- Tissue culture incubator
- 1.8-mL sterile Nunc cryovials, DNase and RNase-free (VWR 66021-986)

Reagents:

- Serum-free, cell culture basal media
- RNAprotect cell reagent (Qiagen 76526), or Allprotect (Qiagen 76405)
- 1X PBS, without calcium or magnesium

Protocol:

Flushing out and preserving cell-secreted factors from the channels (static conditions):

This protocol describes flushing out a channel seeded with cells that have incubated statically in media. The media accumulates cell-secreted factors (proteins, DNA, RNA) which will be preserved in a long-term storage buffer.

This protocol describes preservation of DNA/RNA, and RNAprotect is used as the preservation agent. If all cell-secreted factors need to be preserved (including protein), use a protein preservation solution such as Allprotect from Qiagen (catalog number 76405).

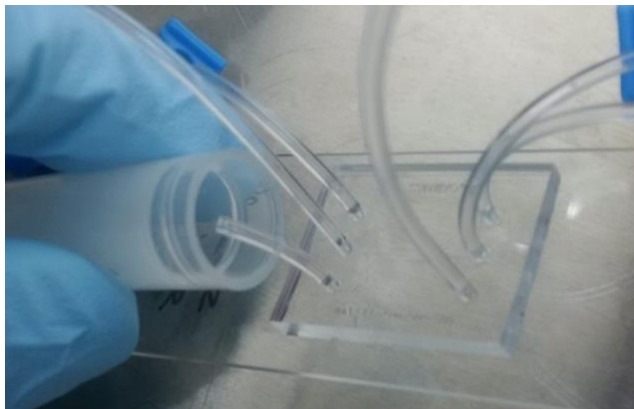


Figure 1. Collecting media that has incubated statically in one channel of the device.

1. Contact SynVivo Customer Support to obtain the void volume of the channel that will be flushed (support@synvivo.com).
 - a. This information is important for determining how much buffer is needed to flush the channel, as well as the amount of preservation agent needed.
2. Prime the device and establish cell cultures according to the parameters of the experiment.
3. Discontinue all media change programs, and allow the media to rest statically in the device for the time period designated by the experiment.
4. Prepare a 1-mL syringe with 1X PBS (without calcium or magnesium).
5. Identify the inlet and outlet ports connected to the channel that is to be flushed.
6. Unclamp and cut the outlet tubing in the device to a length of 0.5 inches (**Figure 1**).
7. Do not remove or dislodge the outlet tubing at this point. The contents of the channel should not be dislodged until the device is rinsed.
8. Place a drop of liquid beside the tubing for the inlet port that corresponds to the unclamped outlet port.
9. Remove the tubing and push out any bubbles using a needle.

10. Insert tubing with 1X PBS into the inlet port.
11. Tip the device so the outlet tubing is inside the mouth of a 1.8-mL Nunc cryovial (DNase/RNase free; **Figure 1**).
12. Slowly inject the 1XPBS until the channel is flushed (1–2 drops from the outlet tubing). This can be done by hand or by syringe pump (flow rate set at 5 μ l/min).
 - a. The void volume of the channel will determine what volume of PBS is required to completely flush out the channel.
13. After the collection is complete, add 5 volumes of RNAprotect to the effluent collected in the cryovial.
14. Store the material immediately at -20°C for long-term preservation of the sample.

Flushing out and preserving cells cultured in the channels (DNA/RNA preservation):

The preservation agent will lyse cells automatically, so dissociation reagents are not required to remove the cells before DNA/RNA preservation.

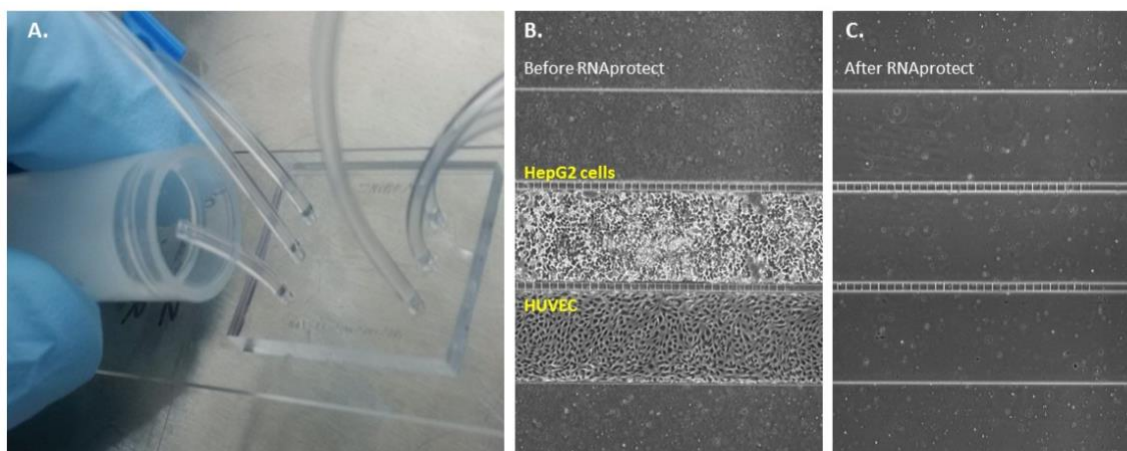


Figure 2. (A) Collecting cells from one channel of the device while flushing with an RNA preservation agent. (B) Appearance of cells in the device before flushing with the RNA preservation solution. (C) Appearance of cells in the device after flushing with the RNA preservation solution.

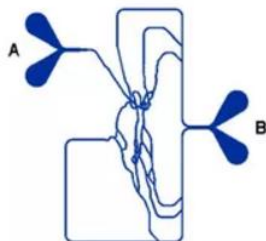
1. Prime the device and establish cell cultures according to the parameters of the experiment.

2. Flush the cells in the device with serum-free media to remove dead cells/debris immediately prior to cell removal.
3. Prepare a 1-mL syringe with RNAprotect.
4. Unclamp the outlet tubing.
5. Place a drop of PBS beside the tubing for the inlet port that corresponds to the unclamped outlet port.
6. Remove the tubing and push out any bubbles using a needle.
7. Insert tubing with RNAprotect into the inlet port.
8. Tip the device so the outlet tubing is inside the mouth of a 1.8-mL Nunc cryovial (DNase/RNase free; **Figure 2A**).
9. Slowly inject the RNAprotect until the channel is flushed (3–4 drops from the outlet tubing, or a minimum of 300 μ l).
 - a. This can be done by hand or by syringe pump (flow rate set at 5 μ l/min).
 - b. The cells will lyse, and the channel will look completely free of cells (compare **Figures 2B-C**).
10. After the collection is complete for one channel of the device, repeat the procedure for any other channels of the device containing cells.
11. Store the material immediately at -20°C for long-term preservation of the sample.

Microfluidic Chip Designs and Applications

Microvascular Networks

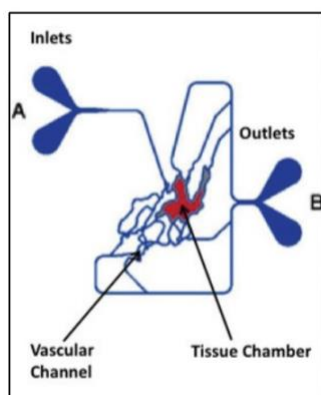
Device: SMN



- Vascular channel only
- No tissue chambers

Applications:

- Vasculature seeding and response to shear stress in a physiological architecture

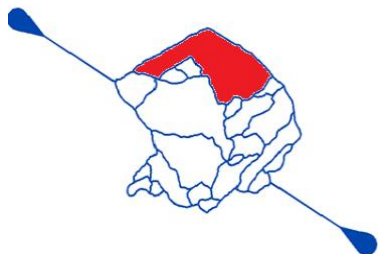


Device: SMN

- Vascular channel and tissue chambers
- Top punch over tissue chamber

Applications:

- 2D or 3D tissue (tumor or normal tissue) with surrounding vasculature
- Immune infiltration; permeability; drug delivery



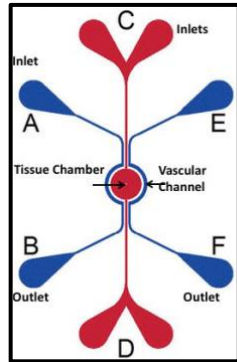
Device: SMN

- Vascular channel and tissue chambers
- Top punch over one tissue chamber
- Multiple tissue chambers

Applications:

- 2D or 3D tissue (tumor or normal tissue) with surrounding vasculature
- Immune infiltration; permeability; drug delivery; metastasis

Idealized Networks

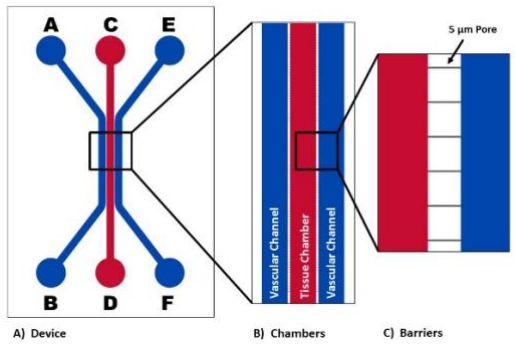


Device: IMN Radial

- Vascular channel and tissue chambers
- Barriers between tissue chamber and vascular channel can be pores or slits
- Radial design

Applications:

- 2D or 3D tissue (tumor or normal tissue) with surrounding vasculature
- Immune infiltration; permeability; drug delivery; metastasis



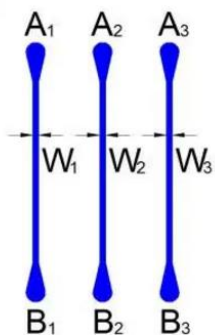
Device: IMN Linear

- Vascular channel and tissue chambers
- Barriers between tissue chamber and vascular channel can be pores or slits
- Consistent shear stress across entire channel (no bifurcations)
- Linear design

Applications:

- 2D or 3D tissue (tumor or normal tissue) with surrounding vasculature
- Immune infiltration; permeability; drug delivery; metastasis

Linear Channels



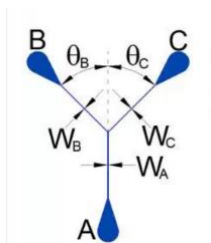
Device: Linear Channels

- Vascular channel
- No barriers
- Channel has fixed length and width

Applications:

- Measuring flow rates to determine the amount of shear stress that endothelial cells can handle
- Optimizing extracellular matrix-endothelial cell interactions
- Examining cell-to-particle adhesion rates

Bifurcation Channels



Device: Bifurcation Channels

- Vascular channel
- No barriers
- Channel has fixed length and width
- Angles can be precisely engineered

Applications:

- Examining cell-to-particle adhesion rates

Sterilization of Microfluidic Devices and Materials

Principle:

Sterilization and maintaining an aseptic environment within the microfluidics device is critical for reproducible results. SynVivo devices are plasma treated, which increases hydrophilicity of the PDMS and sterilizes the surfaces. The following table describes disinfection/sterilization procedures that can be applied to the devices and other materials purchased from SynVivo. For further questions or technical support, contact support@synvibio.com.

Table I. Sterilization and disinfectant solutions for the SynVivo products					
Item	Autoclavable	UV	Gamma Irradiation	70% Ethanol	Comments
SynVivo microfluidics devices	Not recommended	Yes ¹	Yes ²	Yes ³	<p>¹UV light does not penetrate deeply into the polymers, and the efficacy of the sterilization is doubtful.</p> <p>²Gamma irradiation is effective. SynVivo does not currently gamma irradiate its devices, so the customer would have to perform this action.</p> <p>³PDMS has minimum swelling in ethanol, and washing the devices with 70% ethanol for 10 minutes is an effective way to disinfect the device. Follow the disinfection procedure with rinsing the device with 1xPBS for 30 minutes at a 10 µl/min flow rate. Ethanol does not eliminate spores.</p>
Tygon tubing	Yes ¹	Not recommended	Yes ²	Yes ³	<p>¹Tygon tubing can be autoclaved on the solid setting. If the tubing turns white and sticky, the time of the autoclave cycle needs to be reduced.</p> <p>²Gamma irradiation is effective. Gamma irradiated tubing (medical</p>

					<p>grade) can be purchased from a variety of vendors.</p> <p>³Tubing can be rinsed with 70% ethanol to disinfect the tubing. Rinse the tubing thoroughly with 1X PBS after disinfection to remove the ethanol.</p>
Syringe needles	Yes ¹	Not recommended	Yes	Yes	¹ Autoclaving is an inexpensive and easy way to sterilize this product.
Syringes ¹					¹ Syringes are sold as a pre-sterilized product.
Clamps	Yes ¹	Not recommended ²	Yes	Yes	<p>¹Autoclaving is an inexpensive and easy way to sterilize this product.</p> <p>²The clamps can withstand UV light, but the clamps will become brittle over time and will crack.</p>

Cell Impedance Analyzer

Principle:

Impedance measures the oscillation of an AC current in response to cellular activity (cell morphology, locomotion, tight junction formation, loss of tight junctions, etc). Total impedance is composed of two parts: resistance (R) to the flow of current (how well are the cells and media conducting electrolytes through and across the tissue culture system); and capacitive reactance (C) to the flow of current (how well are the cell membranes acting as insulators, which indicates cellular health and function).

Because impedance is composed of two parts, the impedance measurement can be expressed in two ways. Impedance has a magnitude (in ohms [Ω] or kilohms [$k\Omega$]), which indicates the resistance of the system. The insulating properties of cell membranes create resistance (R) to the flow of electrons produced by a current. The Cell Impedance Analyzer generates an oscillating AC current at a specific frequency and measures the resistance to the flow of electrons. Changes in the alternating current (I) results in a potential (V), which is detected by the Cell Impedance Analyzer.

The instrument calculates the impedance (Z) based on Ohm's Law:

$$Z = V/I,$$

where Z can correlate to the AC equivalent of resistance (R) and corresponds to the magnitude of the impedance measurement.

Impedance can also be expressed as an angle, since the waveform of the voltage can either lead or lag behind the waveform of the oscillating AC current. The angle is known as the phase angle between the current and the voltage. The phase angle shows the capacitance (C) of the tissue culture system, which involves the insulating capacity of cell membranes and the barrier function of cell layers.

The following protocol describes how to set up the Cell Impedance Analyzer, how to connect it to a TEER (trans-endothelial electrical resistance) device, and how to take an impedance reading. The instrument readout expresses the impedance values as a magnitude (in kilohms, $k\Omega$) and as a phase angle (in degrees).

Contents of the package:

- SynVivo Cell Impedance Analyzer (**Figure 1**)
- Power cable
- Resistor wires
- Test leads with micrograbber clips and BNC connector (Pomona 3789)
- Four calibration test resistors (28, 49.9, 100 and 200 $k\Omega$)

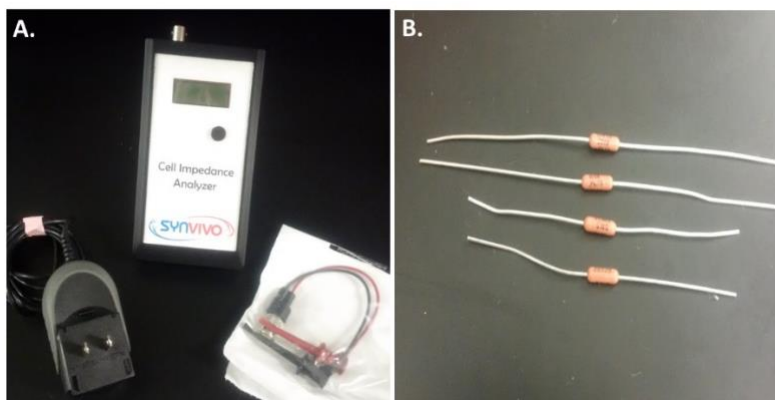


Figure 1. SynVivo cell impedance analyzer and components. (A) The impedance analyzer is shown with power cable and test leads. Depending on power source requirements, power cable options are available for 120 V or 220-240 V outlets. (B) Four calibration test resistors are included with the instrument.

Protocol:

Connecting the Test Leads

Fasten the BNC connector of the test leads to the silver connector on top of the impedance analyzer (**Figure 2**). Be sure to align the grooves on both sides. Gradually turn the BNC connector until it is securely fastened.

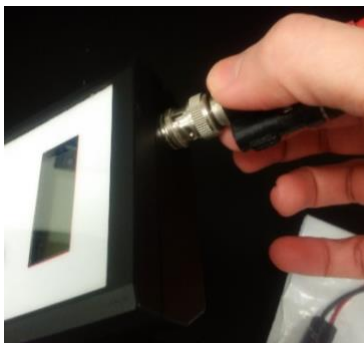


Figure 2. Connecting the test leads to the Cell Impedance Analyzer.

Connecting the Power Cable

Slide the power cable connector into the receiver port on the side of the impedance analyzer (**Figure 3**).



Figure 3. Connecting the power cable to the Cell Impedance Analyzer.

Turn ON the Power

Plug the power cable into an electrical outlet to turn on the power to the impedance analyzer. The instrument will perform a power-on self-test. During the self-test, the name of the instrument and firmware revision number will appear on the LCD (**Figure 4A**). When the self-test is completed, the measurement screen appears on the LCD (**Figure 4B**).

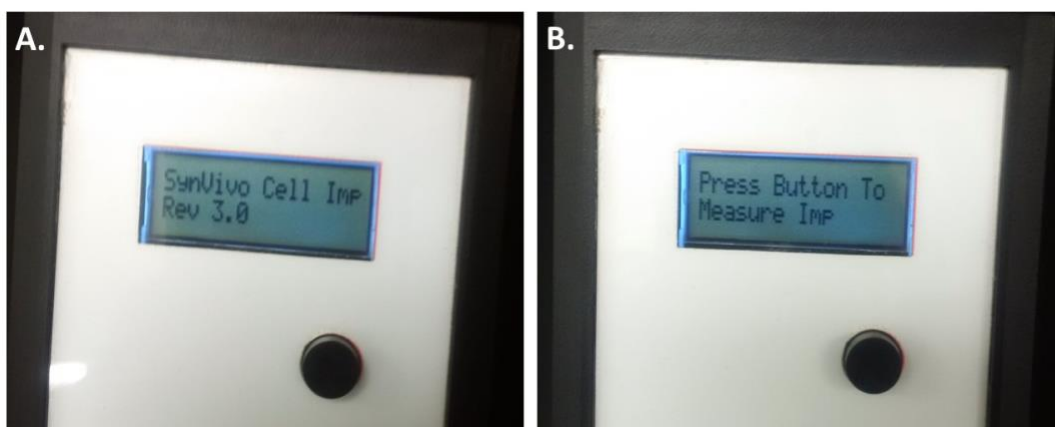


Figure 4. Self-test and measurement screen for the Cell Impedance Analyzer.

Setting the Frequency:

Impedance measurements can be measured at frequencies of 500 Hz, 1 kHz or 10 kHz. To select the measurement frequency, toggle the switch on the side of the instrument (**Figure 5**). The switch has a low, middle and high setting that corresponds to 500 Hz, 1 kHz or 10 kHz, respectively.

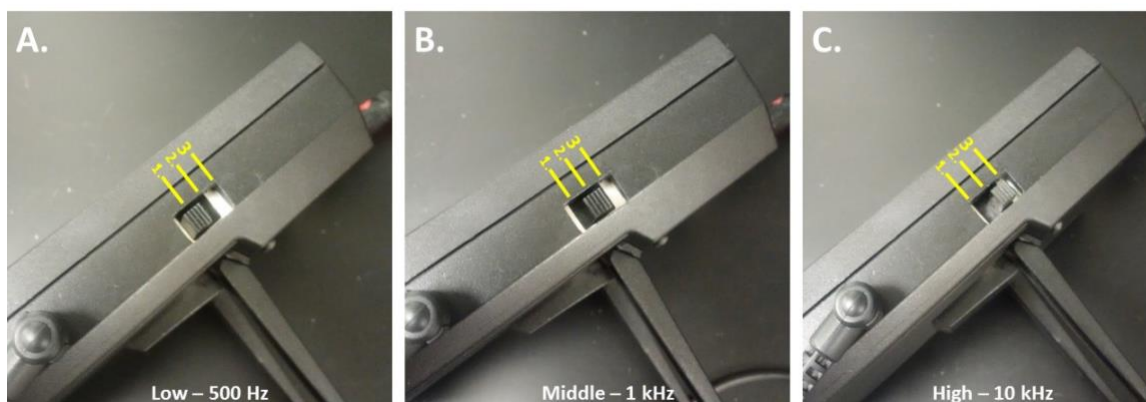


Figure 5. Setting the measurement frequency on the side of the Cell Impedance Analyzer. The toggle switch for the frequency is located above the power cord port. Three frequency settings are available (Positions 1–3): 500 Hz (Position 1), 1 kHz (Position 2), or 10 kHz (Position 3).

Calibration Test:

Resistance standards are used to insure the accuracy of the resistance measurements obtained from the Cell Impedance Analyzer. Four resistance standards are provided with the instrument (28, 49.9, 100 and 200 k Ω). The value of the calibration test resistor is printed on the resistor (**Figure 6**). Each resistor should be tested at all 3 available frequencies (500 Hz, 1 kHz, 10 kHz), and the impedance values should be recorded.



Figure 6. Reading the value and tolerance on the calibration test resistor. The value of the resistor is highlighted with a yellow arrow. This resistor is marked “200K”, indicating a resistor value of 200 k Ω .

Turn on the Cell Impedance Analyzer, and slide the frequency button to the lowest setting (500 Hz). Press the black button twice to see the “Freq Set At 500 Hz” screen. This insures that the correct frequency setting is being measured.

Connect the micrograbber clips on the test leads to one of the calibration test resistors (**Figure 7**). Resistors have no polarity, so it does not matter which test lead is connected to which resistor leg.



Figure 7. Connecting the test leads to the calibration test resistor.

Press the black button the Cell Impedance Analyzer to get the impedance reading. Record the value. Repeat the reading two more times and record the values. Slide the frequency button to the next setting (1 kHz), and press the black button twice to see the “Freq Set At 1 kHz” screen. Press the black button to get the impedance value, and record the value. Repeat the reading two more times and record the values. Follow the same steps for the 10 kHz setting.

Calculate the average impedance value at each frequency. An example of the impedance values obtained from the 200 kΩ calibration test resistor is shown (**Table 1**). The measurement accuracy should fall within ±5% of the calibration test resistor’s value.

Calibration Test Resistor	Frequency	Impedance Readings from Cell Impedance Analyzer (Z, kΩ)				Accuracy (%)
		Reading 1	Reading 2	Reading 3	Average	
200 kΩ	500 Hz	180.308	209.690	231.521	207.173	3.587
	1 kHz	189.055	220.843	192.611	200.836	0.418
	10 kHz	183.473	190.971	190.480	188.308	5.846

Table 1. Example impedance values obtained from a 200 kΩ calibration test resistor connected to the Cell Impedance Analyzer. The measurement accuracy should fall within ±5%.

Connecting the Cell Impedance Analyzer to the TEER Device:

Warm the TEER device to 37°C using a hot plate. Slide the resistance wires into the tubing of the TEER device (**Figure 8A and B**). Make sure that the electrode tips are in contact with the glass slide (**Figure 8C**).

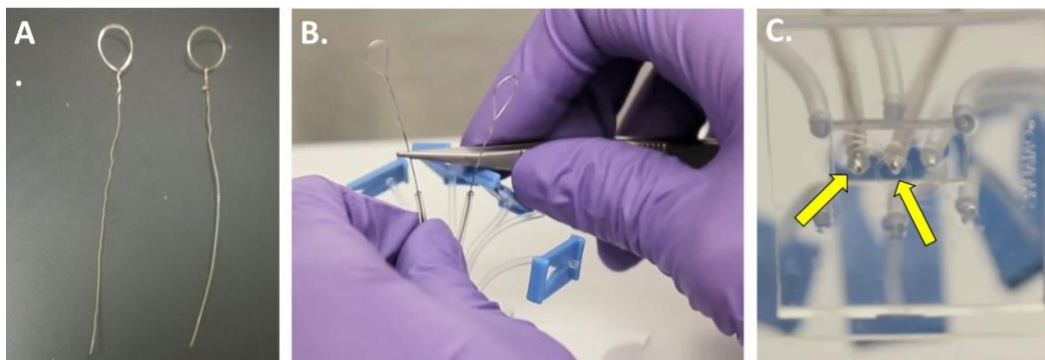


Figure 8. Sliding the resistance wires into the tubing of the TEER device. (A) Image of the resistance wires for the TEER device. (B) Two resistance wires are threaded into the tubing inserted in the TEER device. One resistance wire will be in the tissue chamber, and the second resistance wire will be located outside of the vascular channel. (C) The resistance wires must be in contact with the glass slide (yellow arrows).

Slide the frequency button to the lowest setting (500 Hz) and press the black button twice to see the “Freq Set At 500 Hz” screen.

Attach the micrograbber clips of the test leads to the resistance wires (**Figure 9A**). Press the black button on the front of the Cell Impedance Analyzer to measure the impedance (**Figure 9B**).

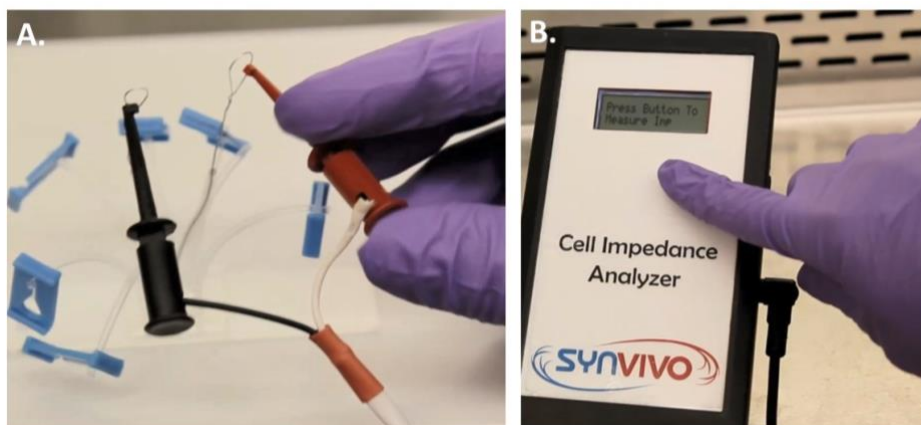


Figure 9. Connecting the TEER device to the Cell Impedance Analyzer. (A) Connect the clips on the test leads to the resistance wires in the TEER device. (B) Press the black button on the front of the Cell Impedance Analyzer to measure the impedance at the selected frequency.

The word “Measuring” will appear on the LCD (**Figure 10A**), followed by the magnitude of the impedance (Z) and the phase angle of the voltage (Phase; **Figure 10B**).

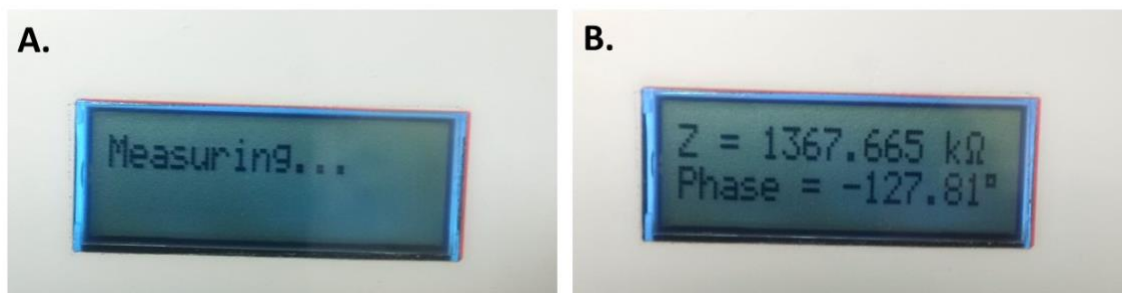


Figure 10. *Measuring the impedance. (A) “Measuring” appears on the LCD as the oscillating current is generated and the reading is taken. (B) The measurement for the resistance component of impedance (Z) is shown in kilohms. The phase angle, which indicates the capacitance component of impedance (Phase), is also shown in degrees.*

Record both the impedance and phase values. Repeat the reading two more times and record the values. Slide the frequency button to the next setting (1 kHz) and repeat the process. Perform the same steps at the highest frequency setting (10 kHz).

Results and Discussion

Temperature affects the conductivity of solutions and cells (1). It is important to keep the TEER device at 37°C while taking the impedance measurements, since this temperature increases the cell membrane capacitance and mimics physiological conditions (1–4). The device can be placed on a 37°C hot plate to perform the impedance measurements. After the device has acclimated to the temperature of the hot plate, the resistor wires can be inserted into the tubing of the device and the impedance measurements can be taken.

The Cell Impedance Analyzer can measure impedance values (Z , Phase) at 3 different frequencies (500 Hz, 1 kHz, and 10 kHz). As cells grow and divide, the ability of the cell membranes to act as a capacitor will increase. This increase in capacitance can be detected through shifts in the phase angle over time. The change in capacitance over time is cell line dependent (5), and biophysical properties of cells can be studied by measuring changes in the phase angle at different frequencies. Cell damage or death results in less capacity to store charge, and the phase angle moves closer to zero (6). Cell proliferation and tight junction formation produces larger phase angles, which points to a healthier cell membrane.

Supporting Documentation:

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