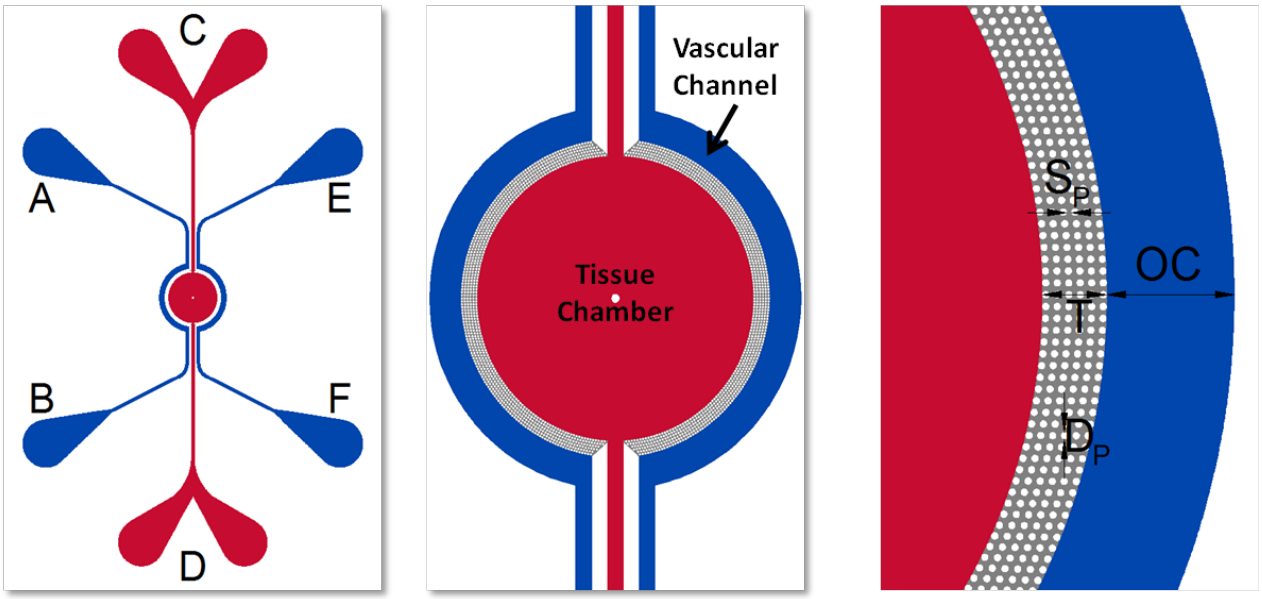
Rolling, Adhesion and Migration Assay Using SynRAM Idealized Network Kits and Chips – Technical Manual

Catalog #s 401002, 401001, 102008-SR



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

Overview of the SynRAM Assay

Immune cells play a key role in early response to tissue injury/infection resulting from physical, chemical or biological stimuli. Due to the significance of the immune cells and endothelium interactions, several in vitro models have been developed to study different aspects of the inflammation pathway including rolling, adhesion and migration. Flow chambers are used to study rolling and adhesion phenomena, and Transwell chambers are used for migration studies. However, current flow chamber designs are oversimplified, lack the scale and geometry of the microenvironment and cannot model transmigration. Similarly, Transwell chambers do not account for fluid shear and size/topology observed in vivo, the end point measurements of migration are not reproducible and do not provide real-time visualization.

SynVivo's SynRAM assay has overcome these limitations to provide an entirely new system for studying the entire inflammation pathway in a realistic and dynamic environment. By recapitulating a histological slice of co-cultured tissue and/or tumor cells with a lumen of endothelial cells, the SynVivo platform delivers a physiologically realistic model including flow and shear in a platform and enables real-time tracking of rolling, adhesion and migration processes. This assay has been successfully validated against in vivo studies showing excellent correlation with rolling velocities, adhesion patterns and migratory processes (Lamberti et al 2014, Soroush et al 2016).

Materials Needed

- SynRAM Chips (Catalog # 102008-SR): Use Ports A, C and E as Inlets and B, D and F as Outlets
- SynVivo Pneumatic Primer Device (Catalog #205001)
- 1 mL BD plastic syringes or other 1 mL syringes (Catalog # 203004)
- 24 gauge blunt tip needles (Catalog # 204002)
- Tygon microbore tubing, 0.02" ID X 0.06" OD (Catalog # 201005)
- Clamps (Catalog # 202003)
- Forceps
- Syringe Pump capable of flow rates from 10nl/min to 10ul/min
- Fibronectin
- Endothelial Cells
- Tissue Cells
- Immune Cells
- TNF-α or IL-1β
- Chemoattractant (e.g. fMLP, MCP-1, etc.)

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

A. Chemotaxis and Migration Assay

Cells are incubated in the vascular channel and chemoattractant is injected into the tissue chamber or the second vascular channel (if using the idealized network). Migration of immune cells across the engineered porous region into the tissue chamber is observed in real-time. The assay can be performed under static or flow conditions. Details are provided below

A.1. Priming The Device

It is very important to remove all air from the device, including within the barrier, for the assay to be carried out successfully. Prime the device with PBS or serum-free media using Pneumatic Primer (Cat# 205001).

1. Place approximately 1 inch long segments of Tygon tubing into the outlet ports of the device.
2. Draw PBS or serum-free media into a 1 mL syringe.
3. Using additional 1inch long segments of tubing, fill the device with liquid by inserting the primed tubing into the inlet and pushing the solution through until the outlet tubing is filled.
4. Do this for all but one inlet port. For this last port, use a tubing approximately 2-3 inch long. When the device is filled, unlock the needle from the syringe, leaving the needle attached to the tubing.
5. Clamp all tubing below the liquid line, except for the tubing with the needle attached.
6. Connect the device to the Pneumatic Primer by locking the needle into the LuerLock connector on the box.

Note: Multiple devices can be primed simultaneously using the multiple port manifold, SynVivo cat # 207001.

7. Turn the knob on the controller box and adjust the pressure to ~5-7 psi. Apply the pressure for ~5-20 minutes. Devices will take at least 15 minutes to completely fill.
8. Turn off the pressure and cut the Tygon tubing connected to the Pneumatic Primer.
9. Allow the device to incubate at 37° for a minimum of 1 hour before use.
10. Flush fresh media into device just before performing assay.

A.2. Polymerization of Chemoattractant (e.g. fMLP, MCP-1 etc.)

Once the device is air free, clamp the tubing for one vascular channel and the tissue chamber below the liquid line in the tubing (there should be no air in the tubing between the clamp and the port). Leave one vascular channel (inlet and outlet) unclamped.

1. Dilute chemoattractant to the desired concentration (10ng/ml -1000ng/ml) in serum free media followed by mixing with equal volume of cold Matrigel (Corning Life Sciences) placed in ice. For example, add 50uL of the 100ng/mL chemoattractant solution to 50uL of Matrigel for a final concentration of 50ng/mL. 100 µl total is enough for ~4 SynVivo® devices.

2. Place the device on a 30-35°C hotplate. Matrigel polymerizes quickly above 4°C; the hotplate will quicken polymerization. This can be done on the benchtop if a hotplate is not available.
3. Place a drop of water around the inlet tubing of the unclamped vascular channel and gently remove the tubing with fine-tip forceps.
4. Draw up a small amount of the Matrigel/chemoattractant mixture into a ~3" tubing attached to a syringe. Only draw up what is needed (about to the needle), leaving the stock on ice to avoid premature polymerization.
5. Insert the tubing into the open port, insuring no air is present in the tube, and slowly infuse the Matrigel mixture until 3-4 drops come out of outlet tubing. Do not infuse excessive amounts, as this can disrupt the gel in the channel. *Note: Infusion can be done carefully by hand or using a syringe pump at 5-10ul/min*
6. Cut the inlet tubing to the syringe. Place any remaining mixture back on ice. Leave the device on the hotplate or place in 37°C incubator for at least 10 minutes to allow full polymerization.

A.3. Introduction of Cells for Assay

1. Check the device using a microscope to ensure proper Matrigel polymerization and mark which vascular channel contains the Matrigel mixture.
2. Gently unclamp the vascular channel containing endothelial cells and one tubing on each side of the tissue chamber.
3. Place a drop of water around the inlet tubing for the tissue chamber and gently remove the unclamped tubing.
4. Infuse cell media into the tissue chamber, cutting the inlet tubing after 3-4 drops emerge from the outlet tubing.
5. Repeat media infusion for the vascular channel that does not contain the Matrigel mixture.
6. Suspend immune cells (e.g. neutrophils, monocytes, etc.) at ~5 x 10^6/ml.
7. Remove the inlet tubing for the vascular channel that does not contain Matrigel mixture.
8. Mount a syringe onto a syringe pump and draw up cell suspension into a syringe with tubing long enough to reach the device.
9. While observing on a microscope, attach cell-primed tubing to the inlet port of the device and infuse the cells into the media-only vascular channel.

   a. For flow experiments: Continue flowing between 0.25-5ul/min and image in real time or at specific interval using the inverted microscope.

   b. For static experiments: Flow cells at 5ul/min until desired density is seen in the channel. Clamp the inlet tubing (attached to the syringe) and stop the pump. It is important to leave the outlet tubing unclamped to avoid high channel pressure that can push cells through the porous barrier. Image the device in real time or place in incubator and image at specific intervals.

Note: If migration of the immune cells does not occur in the first 10 minutes, initiate migration by injecting 50 ng/mL of chemoattractant in serum free media into the tissue chamber until cells show signs of migration. Flush the chemoattractant out of the tissue chamber with serum free media before continuing the experiments.

B. Endothelial cells mediated Rolling, Adhesion and Migration Assay

Endothelial cells cultured in the vascular channel are activated with biological or chemical agents to produce an inflammatory response followed by real-time tracking of rolling, adhesion and migration of immune cells across the endothelium into the tissue chamber. *Note: Most primary endothelial cells are usable only until passage 8. For endothelial cell lines, refer to vendor specifications for passage information. Most endothelial cell lines do not sustain shear stress.*

B.1. Coating The Device with Endothelial Cell Culture Matrix (e.g. fibronectin). This process is performed using Pneumatic Primer (SynVivo Cat# 205001).

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

B.2. Culture of Endothelial Cells in the Device.

1. Prepare the endothelial cell suspension for seeding. Endothelial cells should be dissociated, centrifuged and concentrated to approximately 5-8x10^6cells/ml in cell specific media.
2. Place a drop of water at the base of the inlet port tubing to be removed and gently remove the tubing from the port.
3. Remove the clamp on the outlet port. All channels should be clamped except for the channel being seeded.
4. Prepare a syringe and tubing with the previously prepared cell suspension and mount onto a syringe pump.
5. Ensure that the tubing is free of air bubbles and the cell mixture is flush with the end of the tubing

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

Table 1: Step programming to increase the flow rate from 0.01 to 1 µL/min over 24 hours

SEQ	Flow Rate (µl/min)	Time (h:m:s)	Direction
1	0.01	2:00:00	INFUSE
2	0.025	2:00:00	INFUSE
3	0.05	2:00:00	INFUSE
4	0.075	2:00:00	INFUSE
5	0.1	2:00:00	INFUSE
6	0.2	2:00:00	INFUSE
7	0.3	2:00:00	INFUSE
8	0.4	2:00:00	INFUSE
9	0.5	2:00:00	INFUSE
10	0.6	2:00:00	INFUSE
11	0.8	2:00:00	INFUSE
12	1	2:00:00	INFUSE

B.3. Activation of Endothelial Cells to Initiate the Inflammation Response

1. Dilute inflammatory agent (e.g. TNF-α, IL-1β) in cell-specific media at desired concentration (typical range is from 10ng/ml to 100ng/ml).
2. Endothelial cells are activated with inflammatory agent for 4 hours under static conditions.
3. Selectin molecules, which are responsible for rolling peak between 2-6 hours, hence rolling, adhesion and migration assays should be performed under 6 hours following activation.
4. Integrins responsible for adhesion peak at 24 hour and last until 72 hour, hence adhesion and migration assays can be conducted until 72 hour if needed.

B.4. Polymerization of Chemoattractant (e.g. fMLP, MCP-1, etc.)

1. Once the device is air free, clamp the tubing for one vascular channel and the tissue chamber below the liquid line in the tubing (there should be no air in the tubing between the clamp and the port). Leave one vascular channel (inlet and outlet) unclamped.
2. Dilute chemoattractant to desired concentration (10ng/ml -1000ng/ml) in serum free media followed by mixing with equal volume of cold Matrigel (Corning Life Sciences) placed in ice. For example, add 50 uL of the 100 ng/mL chemoattractant solution to 50 uL of Matrigel for a final concentration of 50 ng/mL. 100 µl total is enough for ~4 SynVivo® devices.
3. Place the device on a 30-35°C hotplate. Matrigel polymerizes quickly above 4°C; the hotplate will quicken polymerization. This can be done on the benchtop if a hotplate is not available.
4. Place a drop of water around the inlet tubing of the unclamped vascular channel and gently remove the tubing with fine-tip forceps.

5. Draw up a small amount of the Matrigel/chemoattractant mixture into a ~3" tubing attached to a syringe. Only draw up what is needed (about to the needle), leaving the stock on ice to avoid premature polymerization.
6. Insert the tubing into the open port, insuring no air is present in the tube, and slowly infuse the Matrigel mixture until 3-4 drops come out of outlet tubing. Do not infuse excessive amounts, as this can disrupt the gel in the channel. *Note: Infusion can be done carefully by hand or using a syringe pump at 5-10ul/min*
7. Cut the inlet tubing to the syringe. Place any remaining mixture back on ice. Leave the device on the hotplate or place in 37°C incubator for at least 10 minutes to allow full polymerization.

B.5. Introduction of Cells for Assay

1. Check the device using a microscope to ensure proper Matrigel polymerization and mark which vascular channel contains the Matrigel mixture.
2. Gently unclamp the vascular channel containing endothelial cells and one tubing on each side of the tissue chamber.
3. Place a drop of water around the inlet tubing for the tissue chamber and gently remove the unclamped tubing.
4. Infuse cell media into the tissue chamber, cutting the inlet tubing after 3-4 drops emerge from the outlet tubing.
5. Repeat media infusion for the vascular channel that does not contain the Matrigel mixture.
6. Suspend immune cells (e.g. neutrophils, monocytes, etc.) at ~5 x 10^6/ml.
7. Remove the inlet tubing for the vascular channel that does not contain Matrigel mixture.
8. Mount a syringe onto a syringe pump and draw up cell suspension into a syringe with tubing long enough to reach the device.
9. While observing on a microscope, attach cell-primed tubing to the inlet port of the device and infuse the cells into the media-only vascular channel. Continue flowing between 0.25-5ul/min and image in real time or at specific interval using the inverted microscope.
   Note: If migration of the immune cells does not occur in the first 10 minutes, initiate migration by injecting 50 ng/mL of chemoattractant in serum free media into the tissue chamber until cells show signs of migration. Flush the chemoattractant out of the tissue chamber with serum free media before continuing the experiments.

C. Vascular and tissue cells mediated Rolling, Adhesion and Migration assay

Endothelial cells are cultured in vascular channel while tissue chamber houses smooth muscle cells, epithelial cells, fibroblast or organ specific cells (e.g. astrocytes, hepatocytes). In response to a cellular signaling and inflammation activation, rolling, adhesion and migration across the endothelium can be observed and quantified in real-time.

Note: Follow steps outlined in section B.1 to B.2 for culturing of endothelial cells before proceeding further with the co-culture assays.

C.1. Culture of Tissue Cells in the Tissue Chamber

1. Prepare the tissue cell suspension for seeding. Cells should be dissociated, centrifuged and concentrated to approximately 1x107 – 5x107 cells/ml in cell specific media. Cell density will depend on the properties of each cell type, including size and tolerance of density.
2. Place a drop of water at the base of the inlet port tubing to be removed. The secondary port tubing on each side should remain clamped throughout device use.
3. Gently remove the tubing - the water should cover the port once the tubing is removed, and should remain there until new Tygon tubing is inserted into the port. This will prevent air from entering the device.
4. Remove the clamp on the outlet port. Leave all other tubing clamped.
5. Prepare a syringe and tubing with the previously prepared cell suspension and mount onto a syringe pump.
6. Ensure that the tubing is free of air bubbles and allow a convex meniscus to form at the end of the tubing.
7. Insert the tubing into the port - the meniscus and the drop of water will form a bridge to prevent air from entering the device as the tubing is inserted.
8. Clean the fluid from the surface of the device.
9. Begin the injection at 6-10ul/min.
10. Observe the device as the cells are flowing. Once the tissue chamber is filled with cells, clamp the inlet tubing and stop the flow. Light taping on top of the device with the forceps can help evenly distribute the cells within the chamber, if needed.
11. Carefully cut the inlet tubing to separate the device from the syringe.
12. Allow the cells to attach for at least 4 hours before changing media. Many cells can go overnight before media change is required.

C.2. Activation of Endothelial Cells to Initiate the Inflammation Response

Follow steps outlined in Section B.3 for activation of endothelial cells.

C.3. Polymerization of Chemoattractant (e.g. fMLP, MCP-1, etc.)

Follow steps outlined in Section B.4 for polymerization of chemoattractant.

C.4. Introduction of Cells for Assay

Follow steps outlined in Section B.5 for completion of experiments.

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