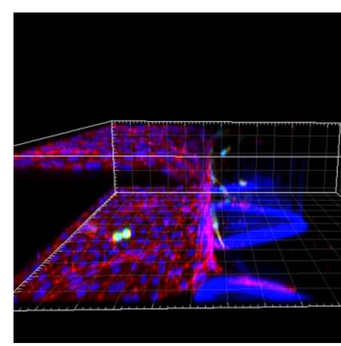
APPLICATION SPECIFIC PROTOCOL - EXTRAVASATION

Cancer cell extravasation is the process where cancer cells in blood circulation bind to adjacent endothelia and transmigrate through the endothelium into the secondary site. This complex process can be emulated *in vitro* by using AIM idenTx 3 chips or idenTx 9 plates. The idenTx 9 encompasses three individual idenTx 3 chips and a idenTx Holder, enabling 9 independent experiments on a single standard SBS-format plate. After establishing an endothelial monolayer in the media channel or a microvasculature network in the 3D hydrogel, cancer cells flowed into the apical side of the endothelium can extravasate into the basal side. idenTx 3 or idenTx 9 provide a more physiologically relevant microenvironment and offer greater control over the biochemical and biophysical factors that are critical in the events of extravasation. This protocol covers the techniques to perform cancer cell extravasation assay in idenTx 3 or idenTx 9.



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MONOLAYER FORMATION ⏱ TIMING 20 min

MATERIALS

Reagents

- 1X PBS (Life Technologies, Cat. No. 70011044)
- 0.25% trypsin with EDTA (Lonza, Cat. No. CC5012)
- Cell culture medium (Lonza, Cat. No. CC3202)

Others

- Collagen-filled and fibronectin-coated idenTx 3 or idenTx 9

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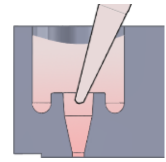
1. Trypsinize endothelial cells as per protocol and re-suspend the cells at 1.5 M cells/ml.
2. Add an additional 20 µl of medium into one of the ports at the media channel that is to be seeded with cells.

Reminder *Ports must be filled with medium before seeding cells into the media channels.*

3. Use a micropipette to withdraw 10 µl of endothelial cell suspension. Position the tip near the inlet of a media channel and inject the cell suspension. Wait for 2 min and then repeat the same procedure for the opposite connected inlet. In total, 20 µl of endothelial cell suspension is seeded per media channel. The additional 40 µl of fluid (20 µl of cell suspension and 20 µl of medium) creates a height difference between the two media channels thus generating interstitial flow across the gel. This helps the attachment of endothelial cells on the gel interface.



Position the pipette tip at media inlets while injecting cell suspension

! Critical *Do not insert the tip completely into the inlets to avoid introducing cells into the media channels at a high flow rate. High flows will not allow cells to settle along the channel, resulting in uneven distribution.*

! Critical *Lay chips (on idenTx holders or in humidified chambers) on a flat surface while seeding cells into idenTx 3 or idenTx 9. Inclination of the chips affects the cell distribution.*

4. Visual inspection under a microscope is recommended. If the cell distribution is not optimal for your application, adjust the concentration of the cell suspension and repeat the seeding steps.

? Troubleshooting (see Table 1 for troubleshooting advice)

5. (Optional) Change medium 2 h after the cells have been seeded to remove unattached cells.
6. Keep the chips in an incubator and change medium daily. Endothelial cells should form a confluent monolayer covering the channel in 1 to 2 d.

Reminder *Since a confluent monolayer is a prerequisite for this assay, we recommend keeping the culture for 2 d before proceeding to the next steps.*

? Troubleshooting (see Table 1 for troubleshooting advice)

SEEDING CANCER CELLS ⏱ TIMING 20 min

MATERIALS

Reagents

- 1X PBS (Life Technologies, Cat. No. 70011044)
- Trypsin (Life Technologies, Cat. No. 25300054)
- Cancer cell culture medium (Life Technologies, Cat. No. 10569010)
- Endothelial cell culture medium (Lonza, Cat. No. CC3202)

Others

- idenTx 3 or idenTx 9 with a complete endothelial monolayer

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7. Trypsinize cancer cells as per protocol and re-suspend the cells at 50 k cells/ml in cancer cell culture medium.

8. Remove endothelial cell culture medium from all 4 ports by carefully aspirating medium out from the troughs. To replace endothelial cell culture medium with cancer cell culture medium in a media channel, add 70 µl of cancer cell culture medium into one port and then add 50 µl to the opposite connected port. Repeat this for the other channel.

! Critical *Pre-warm the cancer cell culture medium to 37 °C prior to changing the medium in idenTx 3 or idenTx 9. Cool medium may cause disruption of the endothelium and affect cancer cell extravasation.*

9. Use a micropipette to withdraw 40 µl of cancer cell suspension. Position the tip near the inlet of the endothelial cell populated channel and inject the cell suspension. The additional 40 µl of fluid creates a height difference between the two media channels thus generating an apical to basal interstitial flow. The flow drives the cancer cells to the side of endothelium that is in contact with the 3D collagen.

Reminder *This strategy focuses on getting cancer cells adhered onto the side of endothelium to study the subsequent extravasating events. To more accurately model the cancer cell rolling and adhesion to endothelium, a continuous flow has to be set up by connecting idenTx 3 or idenTx 9 to a syringe system through AIM luer connectors. For setting up shear flow in idenTx 3 or idenTx 9, please refer to the Application of Flow Protocol.*

? Troubleshooting (see Table 1 for troubleshooting advice)

10. Replace the cancer cell culture medium with endothelial cell culture medium after 1 h. About 1/3 of cancer cells that adhere to the side of endothelium would extravasate through the endothelium and enter into the collagen space within 24 h [1].

QUANTIFICATION OF EXTRAVASATION ⏱ TIMING Variable

In order to quantify the extent of cancer cell extravasation in idenTx 3 or idenTx 9, we recommend labelling the cells with appropriate fluorophores to visualize them. Bright field, phase contrast and epifluorescence microscopy are all compatible with idenTx 3 or idenTx 9 but three-dimensional imaging techniques such as confocal microscopy is preferred due to the nature of this assay. The following quantification method uses images taken from confocal microscopy as an illustrative example.

Number of Extravasated Cancer Cells

The number of extravasated cancer cells provides information on the extravasation efficiency of a particular cancer cell population.

11. We recommend using confocal images of endothelial cells and cancer cells that are specifically stained with distinct fluorophores for easy identification of one cell type from the other. Nuclear counterstain is also recommended for cell counting.

12. Use 3D image visualization and analysis software such as ImageJ (http://imagej.nih.gov/ij/) or Imaris (http://www.bitplane.com/imaris) to pre-process the images if necessary. Ensure that the region of interest (ROI) covers the endothelium and collagen as shown in Figure 1.

Reminder *ROIs are best determined during image acquisition to ensure partials of endothelial monolayer and collagen region are captured.*

13. Count the cancer cells that have passed through endothelium and entered into the 3D collagen gel. Each of these cells is an extravasated cell.

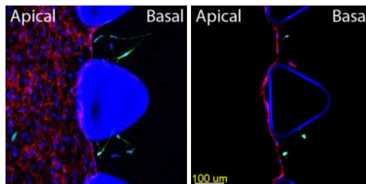

Figure 1 A projected image from a confocal stack (left) and one of its single focal plane confocal image (right) showing the apical and basal (collagen) side of an endothelium (red) where cancer cells (green) have extravasated into the 3D collagen space.

14. Count the total number of cancer cells in both apical and basal side of endothelium.

15. Calculate the extravasation rate by dividing the number of extravasated cells by the total number of cancer cells.

TROUBLESHOOTING

Table 1 Troubleshooting advice

Step	Problem	Possible Reason	Solution
4.	Cells do not distribute evenly	The interval between the injections of cell suspension is short thus the flow of cells in the channel may be disrupted	Wait for at least 2 min before seeding cells into the opposite connected inlet
4.	Too many cells in a channel	Concentration of cell suspension is too high	Flush out unattached cells with culture medium immediately and repeat the seeding steps with cell suspension that is less concentrated
4.	Too few cells in a channel	Concentration of cell suspension is too low	Increase the concentration of cell suspension or repeat the seeding steps (without modifying the concentration of cell suspension) until the target cell density is obtained
4.	Cells do not adhere to the gel interface	The pressure head applied is insufficient	Increase the volume of cell suspension
6.	Fail to form endothelial monolayer within 2d	Seeding density is too low	Increase the seeding density
			Wait for another 24 h
		Passage number of cell is too high	Use cells with earlier passage number
		Channel is not properly coated thus affecting cell attachment	Increase fibronectin incubation time
9.	Cancer cells fail to adhere to the side of endothelium	Seeding density is too low	Increase the seeding density
		Cancer cells flow through the EC-populated channels too fast	Inject cancer cell suspension gently into the EC-populated channels

1. Jeon, J.S., et al., *In Vitro Model of Tumor Cell Extravasation.* PLoS ONE, 2013. **8**(2).