Establishing an *in vitro* angiogenesis model using Nunc cell culture inserts in a carrier plate system

Abstract

Developing an angiogenesis model to study endothelial cell behavior in a tumor microenvironment is a challenging task. To simulate the behavior of endothelial cells in the presence of tumor cells, co-culture systems are necessary. In this study, **Thermo Scientific™ Nunc™ cell culture inserts and a Nunc™ carrier plate system** were used to model interactions between tumor and endothelial cells *in vitro*. We successfully developed two assays related to angiogenesis, an endothelial migration assay and a model for ovarian tumor angiogenesis.

Why study angiogenesis?

Angiogenesis is a biological process by which new capillary blood vessels develop from preexisting ones. Under normal conditions, angiogenesis can play a vital role during fetal development, tissue repair, and wound healing. Under pathological conditions, it can play negative roles in processes like chronic inflammation, tumor growth, and cancer metastasis [1-3]. These events are tightly regulated by a complex mixture of proangiogenic and antiangiogenic factors in the vasculature, the inner workings of which can be better understood with the development of *in vitro* models.

What is a Nunc carrier plate system with cell culture inserts?

Each system consists of a multi-well cell culture plate, a carrier plate containing Nunc cell culture inserts with porous membranes, and a lid. They are designed to maintain cells in two different layers, enabling the study of specific cell functions, including ion and molecular transport, migration, and invasion, in various cell types. The insert membranes can have pore sizes of 0.4 µm, 3 µm, or 8 µm to suit a multitude of experiments. The adjustable Nunc carrier plate system can be used to increase or decrease the distance between the carrier plate and bottom well, providing flexibility to accommodate the volume of medium required for the specific application (Figures 1 and 2).

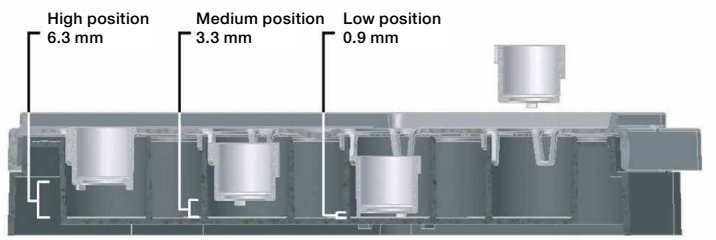

Figure 2. Different hanging height options available for Nunc cell culture inserts.

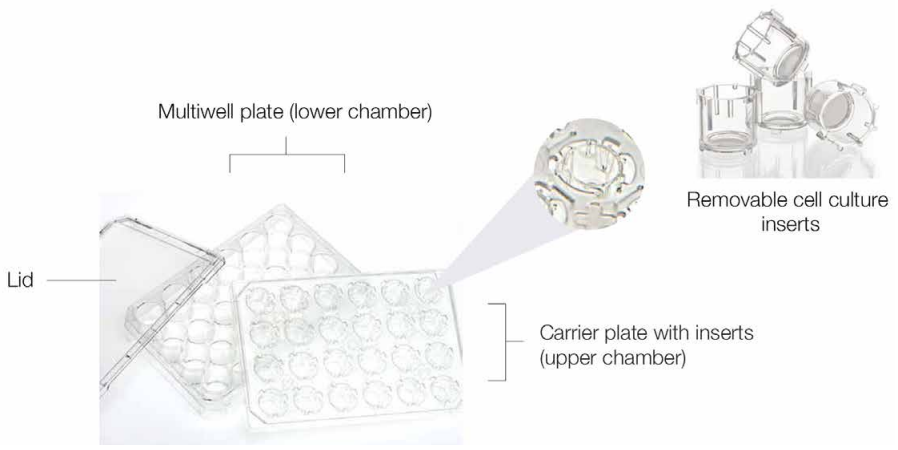

Figure 1. Components of a Nunc carrier plate system with Nunc cell culture inserts.

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Modeling endothelial migration and chemotaxis with Nunc cell culture inserts

Human umbilical vein endothelial cells (HUVECs) are a cell type commonly used to model angiogenesis *in vitro*. These cells expand in culture and can be easily modulated by exposing them to various angiogenic factors in cell culture to drive angiogenesis events, such as endothelial cell proliferation, migration towards chemotactic response, vascular sprouting, and endothelial tube formation. In vitro cell migration assays are often difficult to perform using monolayer culture vessels, which limits our ability to understand angiogenesis. Nunc cell culture inserts for carrier plate systems have a design optimized to create such *in vitro* migration assays. The Nunc Carrier Plate System consists of inserts with porous membranes through which cells can migrate or invade, resembling the endothelial barrier of vasculature. We demonstrate the migration of HUVECs in response to **proangiogenic factors bFGF and VEGF** using Nunc cell culture inserts.

Methods

HUVECs in serum-free medium were seeded in cell culture inserts with 8 µm pores (upper chamber), and medium with chemoattractants bFGF and VEGF was added to the lower chamber (multi-well plate). After 24–48 hours, we assessed the migration of HUVECs from the top of the insert to the bottom of the porous membrane in response to bFGF and VEGF (Figures 3 and 4).

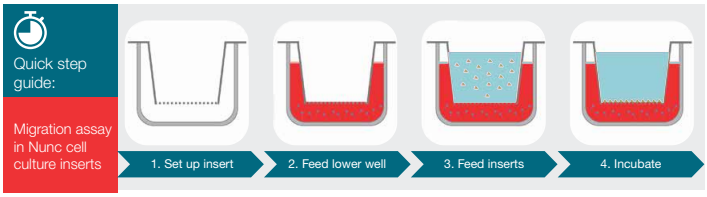

Figure 3. Performing an endothelial migration assay in Nunc cell culture inserts.

Preliminary work

- We first prepared the complete medium required to culture the HUVECs. We used **Gibco™ Medium 200 supplemented with Gibco™ Low Serum Growth Supplement (LSGS)** for the revival and culture of cells.
- The HUVECs were revived and cultured in **Thermo Scientific™ Nunc™ EasYFlask™ T25 flasks** and subcultured for at least two passages before starting the migration assay.

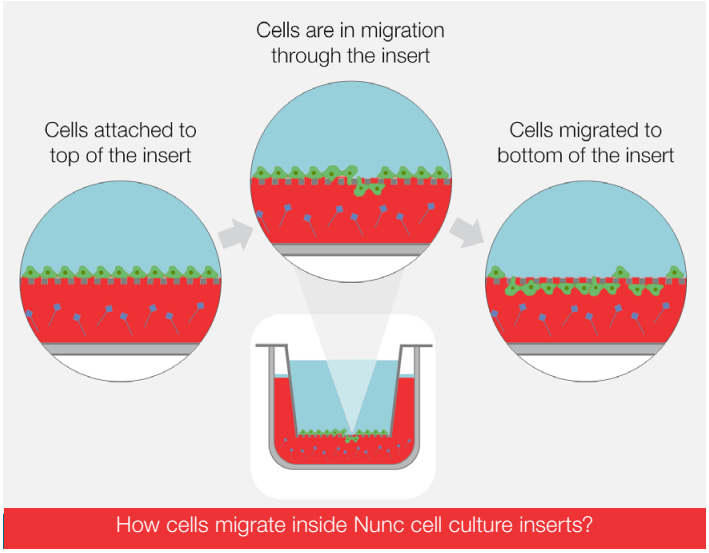

Figure 4. Migration assay using Nunc cell culture inserts.

Note: HUVECs are delicate in nature. Excessive pipetting or vigorous handling of cells can cause cell death. It is advised to handle the cells gently.

Endothelial migration assay

- We prepared five different media compositions to perform the migration assay. This included media with chemoattractants and positive and negative controls with and without supplement, respectively (Table 1).

Table 1. Different media compositions required to perform the endothelial cell migration assay.

Medium (serum-free)	Supplement	Chemoattractants	Condition
Medium 200	–	–	Negative control
Medium 200	FBS 10%	–	Positive control
Medium 200	–	bFGF (50 ng/mL)	Test 1
Medium 200	–	VEGF (10 ng/mL)	Test 2
Medium 200	–	bFGF and VEGF	Test 3

- Preparation of HUVECs: We removed the spent medium from the HUVECs cultured in the Nunc EasYFlask T25 flask and washed the cells with 5 mL 1X PBS. After the wash, we added 2 mL of Gibco™ TrypLE™ Express Enzyme to the flask and incubated for 5–7 minutes at 37°C in 5% CO_2 to dissociate the cells.

- We neutralized the TrypLE enzyme with 5 mL of complete medium, and the cells were centrifuged at 200 x *g* for 5 minutes at room temperature. We discarded the supernatant and resuspended the cells in 1 mL of serum-free medium.
- We then counted the cells using the Invitrogen™ Countess™ 3 FL Automated Cell Counter. We calculated the required seeding density and prepared the cell suspension in serum-free medium. For the HUVEC migration assay, we prepared cell suspensions of 100,000–200,000 cells/mL for 24-well plates with cell culture inserts.

Selecting a multi-well plate for a Nunc cell culture insert system

Choosing the right multi-well plate and insert pore size is important for any experiment. Selection of a 6-well, 12-well, or 24-well plate should be dictated by the scale of your experiment.

Pore size: The suggested insert pore size varies according to the cell type and assay. Typically, larger cells require larger pores to migrate than smaller cells. We used Nunc cell culture inserts with 8 µm pores in a 24-well format to perform the HUVEC migration assay. The pore sizes and parameters of Nunc cell culture inserts in a 24-well multi-plate system are listed in Table 2.

Adjusting the insert hanging height: Before feeding the wells, we set the inserts to the appropriate hanging height. Nunc cell culture inserts can be adjusted to low, medium, and high positions (Figure 2). The advantage of hanging them higher is that the lower chamber can hold a larger medium volume, so longer incubation times are possible. Specifically, setting the insert at the highest position allows the lower chamber to be filled with up to 2 mL of medium. At the lowest position, it can hold up to 1 mL of medium. See Table 2 for hanging heights and suggested media working volumes. We kept the Nunc cell culture inserts at the lowest position for this migration assay, as we decided to incubate for 48 hours and proceed for downstream experiments. For prolonged incubation, other insert positions can be selected.

Filling the lower well: The first stage was to add media to each of the lower wells. Each well contained different chemoattractants, along with positive and negative controls.

- **Step 1:** We removed the inserts and the carrier plate from the multi-well plate (Figure 1).
- **Step 2:** We added 1 mL of each prepared medium to the lower chamber of the multi-well plate for the positive control, negative control, test 1, test 2, and test 3. See Table 1 for the media compositions.
- **Step 3:** We then gently placed the carrier plate with inserts back on the multi-well plate.

Filling the inserts: The second step was to fill the inserts with cells prepared in serum-free medium.

- We seeded 0.5 mL of HUVEC suspension (100,000 cells/cm^2) in each Nunc cell culture insert. **Note:** Avoid touching the bottom of the insert while pipetting, as it can damage the porous membrane.
- We closed the lid and incubated the plate at 37°C in 5% CO_2 for 24–48 hours. The incubation time depends on the migratory capacity of the cells and the concentration of the chemoattractants. Shorter incubation times may require a higher chemoattractant concentration. We recommend optimizing the concentration and incubation time for each cell type.

Table 2. Comparison of pore sizes in Nunc cell culture inserts and working volumes in a 24-well multi-plate system.

Pore size (µm)	Pore density (pores/cm^2)	Culture area of insert (cm^2)	Working volume of insert (mL)	Culture area of lower well (cm^2)	Working volume of lower well (mL)
0.4	<0.85 x 10^8	0.47	0.5	1.9	Low 1.0; medium 1.5; high 2.0
3.0	<1.7 x 10^6	0.47	0.5	1.9	Low 1.0; medium 1.5; high 2.0
8.0	<0.85 x 10^5	0.47	0.5	1.9	Low 1.0; medium 1.5; high 2.0

Visualizing the migrated cells using crystal violet stain
Migration of cells in Nunc cell culture inserts can be assessed using various methods, including simple staining with dyes or fluorescent reagents. Here, we used crystal violet staining as an example to evaluate HUVEC migration from the top of the insert to the bottom. Briefly, all the cells in the inserts were stained with crystal violet dye, after which the cells on the top of the inserts were removed with a cotton swab. Following the washing step, migrated cells at the bottom of the porous membrane were visualized using light microscopy.

Protocol: Wash > fix > stain > swab > image

1. First, we removed the cell culture plates from the incubator after 24–48 hours of seeding. We gently aspirated the media from both inserts and the lower wells of the plate.

2. We washed the inserts and lower wells once with 1X PBS. We gently added 1 mL of 1X PBS to the lower wells and 0.5 mL to the inserts. Note: Avoid vigorous pipetting. We removed the PBS from the inserts and lower wells. Then, we added 4% paraformaldehyde prepared in PBS to both inserts and lower wells (1 mL to the lower wells and 0.5 mL to the inserts). We then incubated the plate at room temperature for 15–20 minutes.

3. After incubation, we gently washed the inserts and lower wells twice with 1X PBS. We added 2% crystal violet stain prepared in deionized water (diH_2O) to the inserts and lower wells of the plate, with 1 mL added to the lower wells and 0.5 mL to the inserts. We incubated the plates at room temperature for 10–15 minutes.

4. Post-incubation, we aspirated the crystal violet stain from the inserts and lower wells. We then washed the inserts and lower wells with diH_2O to remove excess stain. We gently added 1 mL of diH_2O to the lower wells and 0.5 mL to the inserts, and repeated the wash 3–4 times until the solution became visibly colorless. Note: Avoid excessive washing, as extra washes can remove the cells from inserts.

5. After the final wash, we removed the diH_2O from the inserts and gently swabbed the top layer of the porous membrane using a cotton swab to remove the non-migrated cells.

   Note: Do not apply excess force while swabbing, as it can damage the membrane itself.

6. Then, we again washed the inserts with 0.5 mL of diH_2O once and refilled both inserts (0.5 mL) and lower wells (0.5 mL) with diH_2O, then proceeded to image the cells.

7. We imaged the inserts using the **Invitrogen™ EVOS™ M7000 Imaging System**. Keeping the inserts hanging in the lowest position facilitated visualization of the bottom of the porous membrane. The migrated HUVECs stained with crystal violet dye were easily distinguished from the unstained cells. Images were captured under a bright-field 4x objective lens.

Note:

- The aforementioned volumes are for inserts in a 24-well multiplate system. Please refer to the suggested working volumes for other well formats.

- Use care when removing or adding solutions to Nunc cell culture inserts. Applying excessive force or vigorous pipetting directly onto the membrane can wash cells away from the inserts and cause damage to the membrane itself.

- If different hanging heights are used, adjust the inserts to the lowest height when imaging them. Imaging the inserts at other heights will result in poor image quality.

Results

Endothelial cells respond differently to a variety of growth factors in the vasculature. Basic FGF (bFGF) and VEGF are well-known proangiogenic factors that have been employed in several angiogenesis studies. The results outlined below show that HUVEC actively migrated from the top layer of the porous membrane to the bottom layer in response to bFGF and VEGF. bFGF induced better migration than VEGF. Combining bFGF and VEGF resulted in the highest percentage of migration among all the conditions tested (Figure 5).

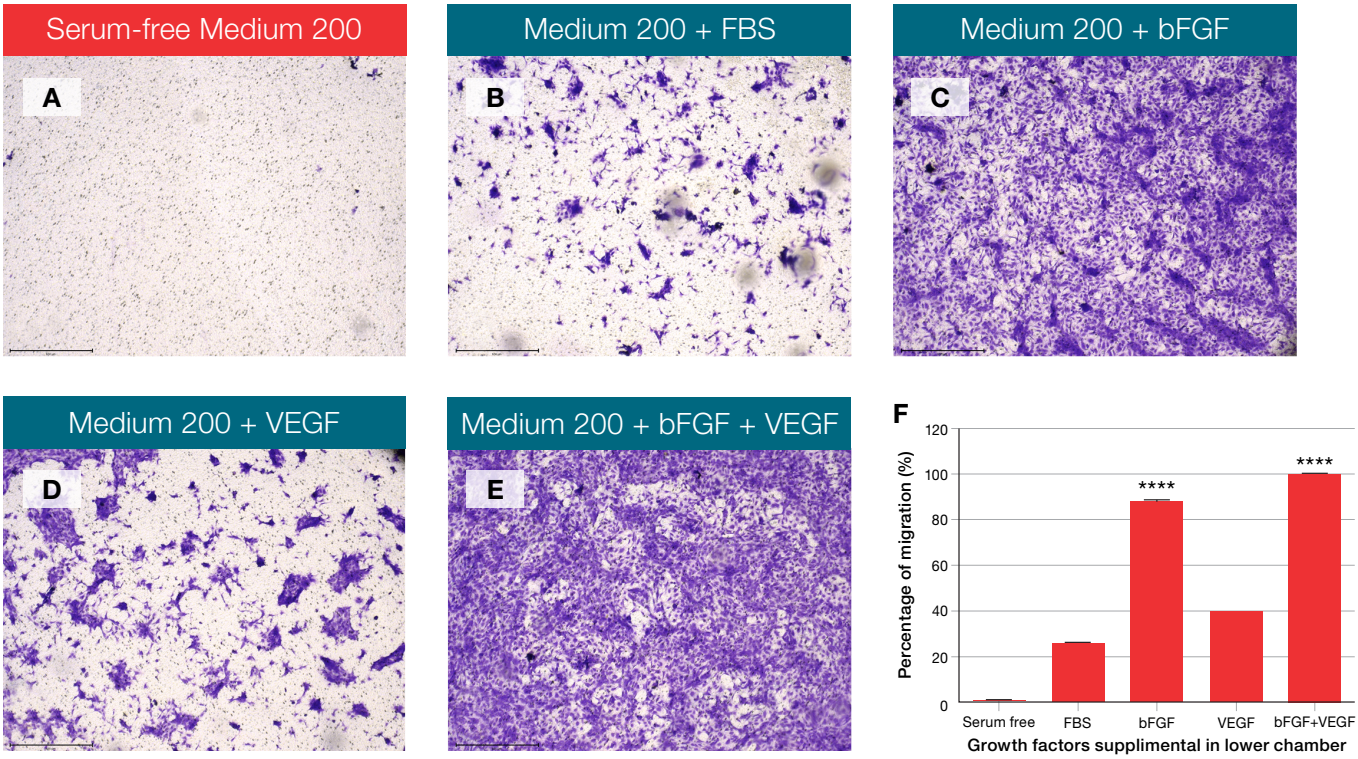

Figure 5. HUVEC migration assay performed using Nunc cell culture inserts in a 24-well plate multi-well system. HUVECs were seeded at 50,000 cells per insert with different chemoattractants. **(A)** Medium 200 alone, **(B)** FBS, **(C)** bFGF, **(D)** VEGF, and **(E)** bFGF and VEGF were added to the lower wells, incubated for 48 hours, and assessed for migration using crystal violet stain. The images in **A–E** show cells stained with crystal violet stain that migrated from the top of the insert to the bottom of the membrane. **(F)** Comparison of migration percentage between different chemoattractants used. Migration percentage was calculated using the Plot Profile feature of ImageJ software. All conditions were normalized to the bFGF and VEGF condition, which was set to 100%. Statistical significance shown between serum-free condition and bFGF or bFGF+VEGF condition with P value of <0.0001 (****) by unpaired t-test. Images were captured using an Invitrogen EVOS M7000 microscope with 4x objective lens. Scale bars represent 650 µm.

Tumor angiogenesis model with Nunc cell culture inserts

In addition to supporting the development of new blood vessels for tissue growth or would healing, angiogenesis is also an important hallmark of cancer. It is commonly associated with tumor growth and metastasis in several cancers [1]. Different pathways guide tumor angiogenesis that benefit cancer cells by providing the oxygen and nutrients required for the growth and spread of cancer. The tumor microenvironment is composed of cancer cells, endothelial cells, and a variety of other cell types. Robust cell culture models and plate systems are necessary to understand interactions between the cells during cancer development. Evidence reported in the literature shows that release of proangiogenic factors by cancer cells is an initial step in triggering angiogenesis, which leads to endothelial tube formation that supplies oxygen and nutrients to cancer cells [1, 8-10]. To better understand this process, establishing co-culture models to study cross-talk between cancer cells and endothelial cells is necessary. Nunc cell culture inserts in a carrier plate system enable this by allowing two cell types to grow physically separated in a single culture vessel. Here, we demonstrated one such model using an **ovarian cancer cell line (SKOV3)** and HUVECs to show tumor angiogenesis. Ovarian cancer cells are known to stimulate endothelial cells by releasing VEGF in the surrounding environment, which triggers tube formation in endothelial cells and ultimately leads to tumor vascularization [7,8].

Methods

HUVECs were seeded on **Gibco™ Geltrex™ matrix**–coated lower wells of the multi-well plate, and SKOV3 cells were seeded on Nunc cell culture inserts with 8 µm pores under serum-free conditions. After 24 hours of incubation, the HUVECs were stained with fluorescent probes to visualize endothelial tube formation (Figures 6 and 7).

Preliminary work

Cells and media: The HUVECs were cultured using Gibco Medium 200 supplemented with Gibco LSGS. SKOV3 cells were cultured using DMEM supplemented with 10% FBS and 1% penicillin-streptomycin in Nunc EasYFlask T25 flasks. Both cell types were subcultured for at least two passages before proceeding to the experiment.

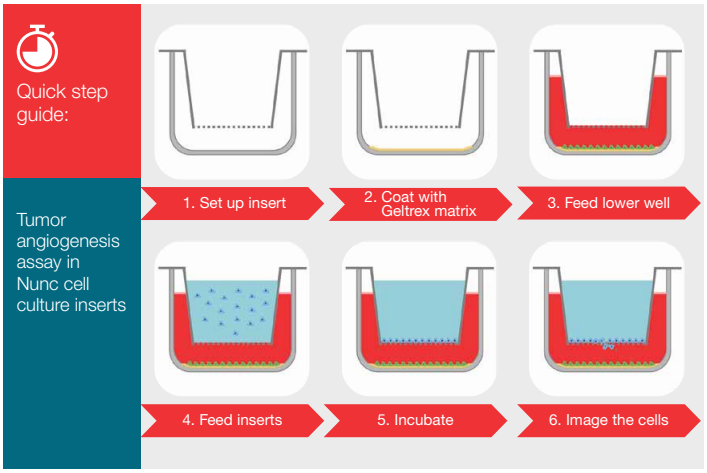

Figure 6. Quick step guide to perform a tumor angiogenesis assay in Nunc cell culture inserts.

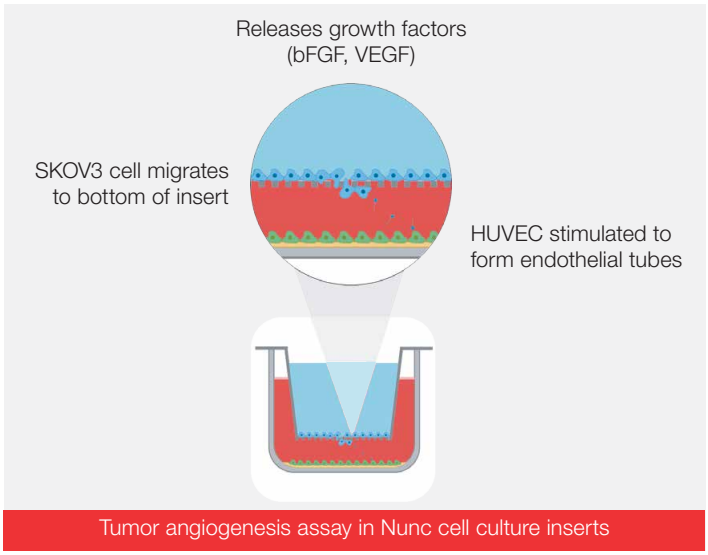

Figure 7. HUVEC and SKOV3 tumor angiogenesis assay in Nunc cell culture inserts.

Tumor angiogenesis assay steps

- Preparation of the culture insert system: The tumor angiogenesis assay was performed using Nunc cell culture inserts with 8 µm pores in a 24-well carrier plate system. The hanging height of the inserts was adjusted to the lowest position prior to feeding the wells.

- Geltrex matrix coating: Endothelial cells require extracellular matrix (ECM) coating on cell culture–treated surfaces to achieve successful tube formation. We thawed the Geltrex matrix at 4°C overnight prior to the day of the assay. See the note below for thawing steps. The lower wells of the multi-well plate were coated with Geltrex matrix at a concentration of 50 µL/cm^2 by adding 100 µL to each well in a 24-well multiplate and incubating for 1 hour at 37°C prior to the assay.

Note: Always thaw vials of Geltrex matrix at 4°C overnight prior to the day of the assay. Do not thaw the Geltrex matrix at room temperature, as it can polymerize faster at higher temperatures. Always keep Geltrex matrix ice-cold before coating the surfaces. Chill the multi-well plate and pipette tips for 5 minutes at 4°C prior to coating with Geltrex matrix. Ensure that the matrix is spread evenly over the surface after pipetting, as uneven coating can result in abnormal endothelial tube formation.

- Media preparation: We prepared three different media formulations for the tumor angiogenesis assay (Table 3). HUVECs in serum-free medium (Medium 200) were used as a negative control, and Medium 200 with bFGF and VEGF was included as a positive control for endothelial tube formation.

Table 3. Different media compositions required to perform tumor angiogenesis assay.

Cell type used	Medium (serum-free)	Supplements	Condition
HUVEC	Medium 200	–	Negative control
HUVEC	Medium 200	bFGF (60 ng/mL) + VEGF (25 ng/mL)	Positive control
SKOV3	DMEM	–	Test

- Trypsinization of HUVECs and SKOV3 cells: We removed the media from Nunc EasYFlask T25 flasks and washed the cells with 5 mL 1X PBS. We then added 2 mL of TrypLE Express Enzyme and incubated the flasks for 3–7 minutes until the cells were completely detached. The time needed to detach from the surface varied by cell type.

  – After complete detachment of the cells, the TrypLE enzyme was neutralized with 5 mL of the respective complete medium, and the cells were centrifuged at 200 x *g* for 5 minutes at room temperature.

  – The supernatant was discarded, and the cells were resuspended in 1 mL of serum-free medium. The SKOV3 cells were resuspended in serum-free DMEM, and the HUVECs were resuspended in serum-free Medium 200. Finally, the cells were counted using the Countess 3 FL Automated Cell Counter.

Labeling the cells with CellTracker dyes: Endothelial tube formation can be visualized in real time by staining cells with fluorescent dye at the time of seeding. Multiple dyes can be used to differentiate between different cell types. We used Invitrogen™ CellTracker™ Green dye to stain the HUVECs and CellTracker™ Red dye to stain the SKOV3 cells prior to seeding in the plate.

- We prepared working solutions of the CellTracker dyes in serum-free medium, then added the dyes to the cell suspensions to achieve a final concentration of 10 µM.
- We then incubated the cell suspensions for 20–30 minutes and centrifuged the cells at 200 x *g* for 5 minutes at room temperature. The supernatant was discarded, and 1 mL of fresh serum-free medium was added to the cells. The cells were stained with CellTracker dyes and visualized in realtime via fluorescence microscopy. **Note:** Protect the fluorescent dyes and the stained cells from direct exposure of light to limit quenching.
- After staining the HUVECs and SKOV3 cells with the dyes, we adjusted the seeding density of the cells. We used a seeding density of 200,000 cells/cm^2 for the SKOV3 cells and 35,000 cells/cm^2 for the HUVECs in a 24-well plate system.
- We prepared a single tube containing SKOV3 cells in medium without any supplements. The HUVECs were prepared in two different tubes, one with growth medium containing bFGF and VEGF and the other with growth medium only. Medium with supplements served as the positive control for the experiment and unsupplemented medium served as the negative control (Table 3).
- Upon bringing the Geltrex matrix–coated multi-well plate to the biosafety cabinet, we removed the inserts from the carrier plate. We seeded 1 mL of the HUVEC suspension (75,000 cells/well) to all the lower wells of the plate (positive control, negative control, and test). The plate was then incubated at 37°C for 30 minutes to allow cells to settle at the bottom of the plate.
- We seeded 0.5 mL of SKOV3 cell suspension (100,000 cells/insert) to the test inserts. For the positive and negative control well inserts, we added the same media that we fed in the lower wells and ensured that the SKOV3 cells were not seeded to the control wells. We closed the lid and incubated the plate at 37°C in 5% CO_2 for 24 hours.

Visualizing endothelial tube formation

Upon activation, HUVECs on a Geltrex matrix–coated surface form endothelial tubes that can be visualized using either a bright-field or fluorescence microscope. When cells are labeled with fluorescent dyes, it is possible to observe tube formation in real time. We labeled the HUVECs with CellTracker Green dye, which enabled us to examine the SKOV3-induced tube formation over time. SKOV3 cells labeled with CellTracker Red dye were easy to distinguish from HUVECs stained green.

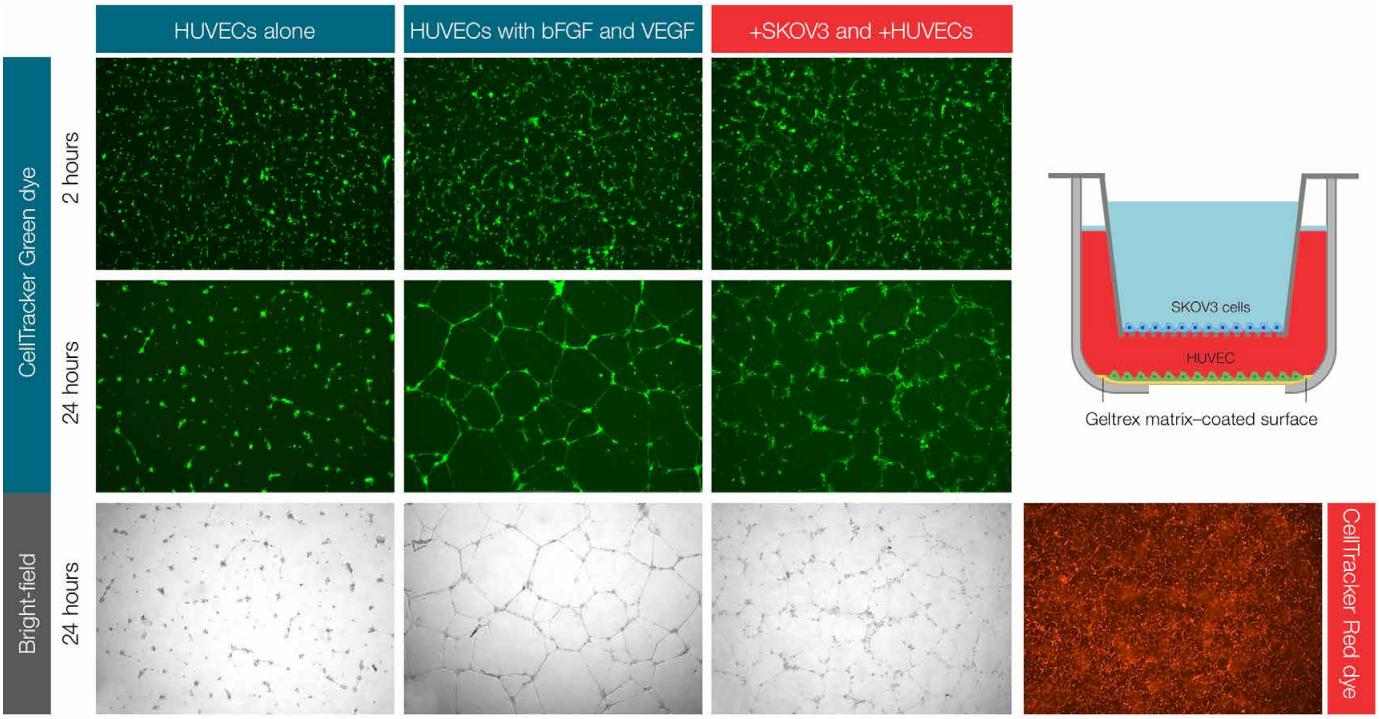

Figure 8. Tumor angiogenesis model shown in Nunc cell culture inserts in a 24-well carrier plate system. HUVECs (75,000 cells/well) were seeded on the Geltrex matrix–coated lower wells of the plate and SKOV3 cells (100,000 cells/well) were seeded on the inserts. The cells were incubated for 24 hours and analyzed for endothelial tube formation in the lower wells. The HUVECs were labeled with CellTracker Green dye, and the SKOV3 cells were labeled with CellTracker Red dye. The cells were imaged with the EVOS M7000 Imaging System at 4X objective. **(A)** Negative control: HUVECs seeded on the lower well without any supplements formed no endothelial tubes. **(B)** Positive control: HUVECs seeded on the lower well along with supplements bFGF (60 ng/mL) and VEGF (25 ng/mL) formed endothelial tubes. **(C)** HUVECs seeded on the lower wells and SKOV3 cells seeded on the inserts without any supplements formed notable endothelial tubes, confirming tumor angiogenesis. **(D)** Evidence showing SKOV3 cells migrated to the bottom of the cell culture inserts stained with CellTracker Red dye.

Results

Ovarian cancer cells have been shown to secrete VEGF in the cancer microenvironment and have been reported to promote tumor angiogenesis in endothelial cells [7, 8, 10]. In the Nunc cell culture insert system, SKOV3 cells seeded at the inserts actively migrated through the porous membrane and induced endothelial tube formation by HUVECs seeded in the lower wells. Control wells without SKOV3 cells failed to generate endothelial tubes (Figure 8).

Conclusions

Nunc cell culture inserts and the Nunc carrier plate system serve as an excellent platform to conduct cell-barrier assays and can be used to develop various co-culture models. Nunc cell culture inserts provided an efficient system for establishing an *in vitro* angiogenesis model to study cell migration and co-culturing of cancer cells to demonstrate tumor angiogenesis. Versatile pore size options with adjustable hanging heights offer convenience and flexibility over other cell culture vessels for such studies. Nunc cell culture inserts are not strictly limited to angiogenesis. They can be extended to other applications like transport studies, cell invasion assays, and tissue engineering. More application notes for Nunc cell culture inserts about establishing a human skin model, co-culturing tumor-associated macrophages, and other applications can be found **here**.

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Ordering information

Materials	Pore size	Cat. No.
Nunc cell culture inserts and carrier plate systems		
24-well carrier plate with cell culture inserts	0.4 micron	141002
24-well carrier plate with cell culture inserts	3.0 micron	141004
24-well carrier plate with cell culture inserts	8.0 micron	141006
24-well carrier plate (no inserts)	—	141008
12-well carrier plate with cell culture inserts	0.4 micron	141078
12-well carrier plate with cell culture inserts	3.0 micron	141080
12-well carrier plate with cell culture inserts	8.0 micron	141082
12-well carrier plate (no inserts)	—	141086
6-well carrier plate with cell culture inserts	0.4 micron	140640
6-well carrier plate with cell culture inserts	3.0 micron	140642
6-well carrier plate with cell culture inserts	8.0 micron	140644
Cells, media, and supplements		
Human Umbilical Vein Endothelial Cells (HUVEC)	—	C0035C
Human Large Vessel Endothelial Cell Basal Medium (Medium 200)	—	M200500
Low Serum Growth Supplement (LSGS)	—	S00310
Human bFGF Recombinant Protein	—	PHG0261
Human VEGF Recombinant Protein	—	PHC9391
Imaging and reagents		
CellTracker Green CMFDA Dye	—	C7025
CellTracker Red CMTPX Dye	—	C34552
EVOS M7000 Imaging System	—	AMF7000

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