FLUXION

APPLICATION NOTE

# Angiogenesis

## Novel microfluidic assay for cancer cell invasion with real-time imaging

### Introduction

Understanding the molecular and cellular mechanisms of cancer progression from primary malignancy to metastatic disease is critical to development of successful treatments. Cell invasion from the primary tumor and tumor-induced angiogenesis are just two of many phenomena contributing to the pathology of metastatic disease. Both biological processes involve migration and transmigration of cells in response to chemoattractants.

Conventional In vitro analysis of these phenomena typically involves deposition of a basement membrane derived matrix (i.e. Matrigel) in a static well plate, often times using a porous cell culture insert. These inserts can be difficult to process and often preclude capturing data in real-time. Here, we demonstrate a microfluidic method to follow angiogenesis and cell invasion in real time. The method enables acquisition of high content data by microscopy using microfluidic flow cells called BioFlux Plates. These devices are optimized for imaging and leverage the parallel flow capabilities of the BioFlux system to create a unique cell invasion environment (Figure 1).

### Application Overview

The microfluidic channels of the BioFlux 24-well plate were filled with matrix in one half of the channel while the other half was maintained in the fluid phase. Using this technique, we tested endothelial cell response to VEGF, bFGF and fumagilin. It was shown that VEGF and bFGF impregnated in the matrix promoted angiogenesis while fumagilin abrogated the effect. We also investigated invasion of serum starved HT1080 and MCF-7 cells into FBS containing matrix and found that, as predicted, only HT1080 cells successfully invaded the matrix. The method is amenable to screening multiple cell types, environmental conditions and compounds in parallel. High resolution microscopy data is produced which can be quantified to determine cell behavior and compound efficacy.

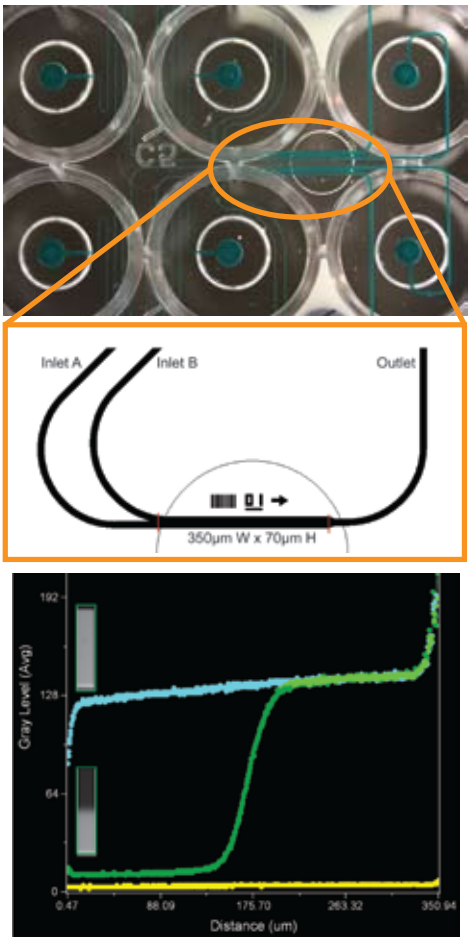



**Figure 1: Creation of two microenvironments within a microfluidic channel is achieved using a flow channel with two parallel flow inputs.**
A network of microfluidic channels are integrated into an SBS-standard well plate (top).
Each experimental zone has two fluidic inputs. When flowed under laminar fluid dynamic conditions, fluids originating from inlet A and B travel in non-mixing streams from beginning to the end of the channel (middle).
Laminar flow conditions were demonstrated by flowing fluorescent dye from one inlet and non-fluorescent buffer from the other while varying the pneumatic pressures on each well. Pressurizing the buffer well resulted in a channel with no fluorescence (yellow trace), while pressurizing the dye inlet resulted in a channel with all fluorescence (blue trace). Equal pressures applied to both inlets resulted in equal-sized zones of fluorescent dye and non-fluorescent buffer (green trace).

### Materials and Methods

**Please refer to the companion protocol for recommended experimental techniques.**

A 24-well Bioflux device was chilled at -20°C for 10 minutes prior to preparation. Channels were primed with CO2-independent media (Invitrogen, Carlsbad,CA) supplemented with 1% FBS from the outlet well. After priming, the Bioflux device was placed on a flat wet ice pack. Undiluted Matrigel (BD Biosciences, Franklin Lakes, NJ) either alone or supplemented with VEGF(10ng/ml), bFGF (1ng/ml) or fumagilin (200 or 400 ng/ml) was added to the "B" inlet wells and media was added to the "A" inlet wells. Flow from both inlet wells was initiated with the device on the ice pack for a few seconds. Flow was continued with the device off the ice pack for 3 minutes; at the 3 minute mark, the device was placed on the glass-bottomed Bioflux heating plate preheated to 38°C. Flow was continued for 30 additional seconds. The perfusion was stopped with the plate on the heater and incubation at 38°C continued for 20 minutes. An additional incubation at room temperature was included to solidify the gel for 20 minutes (Figure 2).

## Materials and Methods (cont.)

Following gel formation, the A-well was used to coat the remainder of the channel with 1/40 v/v diluted Matrigel in HBSS. The diluted Matrigel was perfused into the fluid space inside the channel. The device was incubated at 37°C for 30 minutes.

Cells were introduced into the plate from the A wells. Either P2 HUVEC, HT1080 colon carcinoma cells or MCF7 cells were added to the A wells at 1 x106 cells per ml. Cells were perfused into the main viewing window until an 30% density was reached. Flow was stopped. Cells were incubated for 1 hour without flow. After cells had attached, perfusion at 0.4 dyn/cm2 was commenced with 1% FCS supplemented CO2-independent media. For HT1080 and MCF7 cells, flow was continued for 15hours under continuous timelapse. For endothelial cells, flow was continued for 36 hours.

## Results

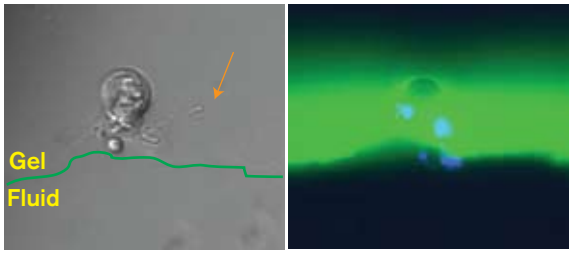
***Endothelial cell matrix invasion is abrogated by fumagilin***

HUVECs were grown under serum starvation conditions under 1 dyn/cm2 shear flow for 36 hours. Individual cells were observed invading the gel matrix under conditions of Matrigel alone, VEGF, bFGF. Sprouting and process formation, which is an early step of angiogenesis, was noted under these conditions as well. The addition of fumagilin, a angiogenesis inhibitor, abrogated the invasion and the cells multiplied outside of the gel (Figure 3).

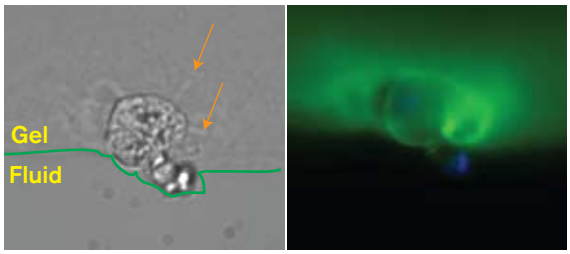
***Invasive carcinoma cells migrated into the matrix while non-invasive cells remained inactive.***

Non-invasive cells (MCF7) and invasive cells (HT1080) were used to test cancer cell invasion into the Matrigel matrix. The cells were cultured overnight in the prepared BioFlux channels and imaged in time-lapse. The HT1080 cells began to migrate after 4 hours of perfusion into the matrix and continued invasion throughout the entire experiment. In contrast, the MCF7 cells were completely non-invasive, as expected (Figure 4).

**Control Matrix**




**bFGF**




**Fumagilin (400ng/ml)**

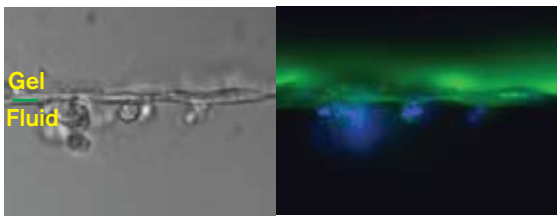



**Figure 3. Invasion of endothelial cells into the Matrigel matrix.** After 36 hours wheat germ agluttinen (green) was used to stain cells and gel for 15 min post-flow. Nuclei are stained with Hoescht 33342 (blue).

## Summary

- Two inlet channels were used to create a side-by-side hydrogel (stationary) / fluid (under flow) environment.
- Under these conditions, cells were introduced and grown in the fluid side of the channel.
- Compounds introduced in the gel or the fluid created gradients in the opposite phase.
- Endothelial cells grown under flow responded by invading and beginning to sprout into the gel matrix. Fumagilin abrogated this effect.
- HT1080 cells were able to migrate and grow within the gel matrix while MCF-7 cells were not.

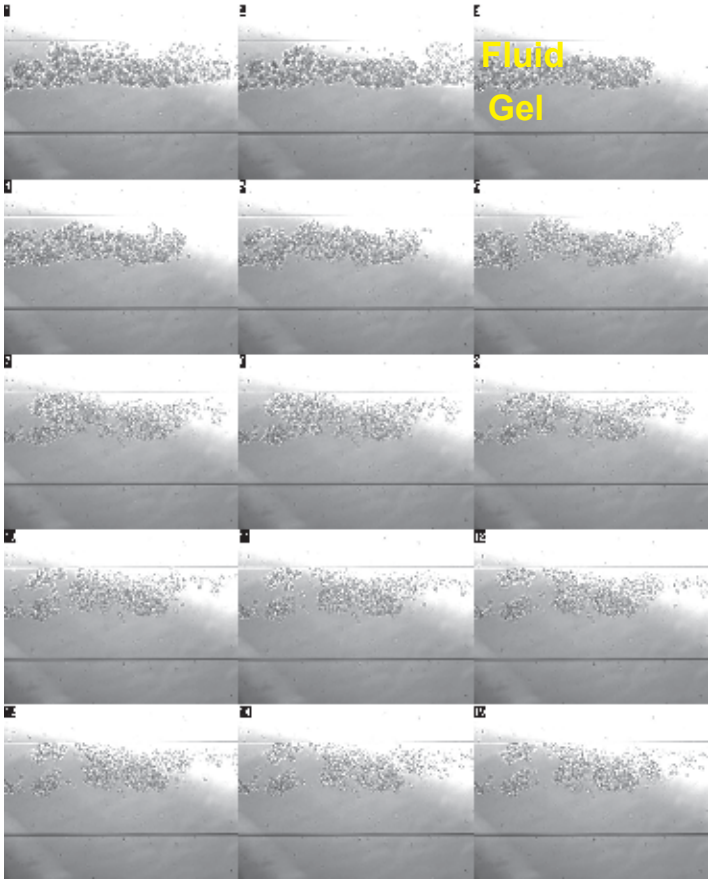
## HT1080 Colon Carcinoma Cells

Time 0


Time 15h

## MCF7 Breast Cancer Cells

Time 0




Time 15h

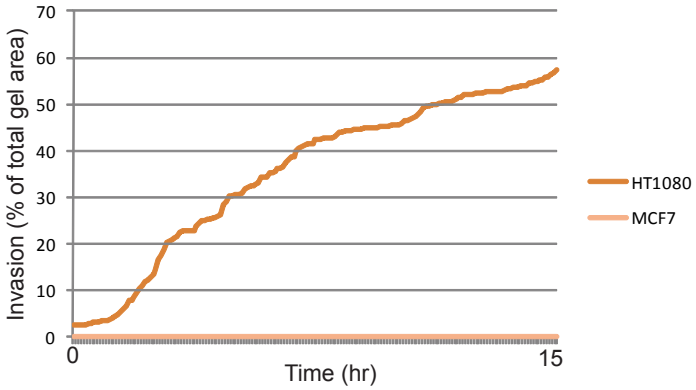



**Figure 4. Cancer cell invasion into the Matrigel matrix.** Timelapse over 15 hours of either invasive (HT1080) or non-invasive cancer cells (MCF7) into Matrigel (top). Cell invasiveness expressed as pixel intensity over total gelled area of the channel as a function of time (left).

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**Assay Protocol**

# Micropatterning Matrigel in the BioFlux Microfluidic channels for Invasion or Angiogenesis Assays

## Required Materials

- Matrigel (BD Biosciences, Standard Matrigel cat # 356234 or Reduced Growth Factor Matrigel #356230)
- $CO_2$-independent Media (Invitrogen, cat #18045088) supplemented as needed with Glutamax, 1%v/v FBS (and Pen/Strep if desired) or other appropriate, prepared media.
- BioFlux 24-well plate (Fluxion, cat #910-0009)
- Heater plate (Fluxion, cat #900-0047)
- Access to a -20°C freezer and wet ice in a bucket or a slightly defrosted, gel ice pack

**Special notes:**

1. *Matrigel is very temperature sensitive*. It will only remain in a liquid form when below 4°C. It is critical to thaw Matrigel as directed (overnight at 4°), otherwise it might gel or crystallize in an inconsistent manner and the protocol will not work as desired.
2. A BioFlux glass heating plate P/N 900-0047 must be used – preheated to 38°C for the gelling step
3. Balance well volumes on all sides of the channel.
4. When placing the BioFlux plate in the freezer and on ice be extremely careful not to push the glass down hard onto any hard surface.
5. Re-attach tubing to the interface per the table below and perform all flow steps using the BioFlux 48-well Tubing Harness (900-0013) and 48-well plate software interface.

*Connect tubing from BioFlux Controller (rear panel) to interface as follows: This allows you to control each row of inlet wells as needed.*

| Number on Controller/Software Template | Number on 24-well interface | Controls wells on plate | Channels Impacted |
|---|---|---|---|
| **1** | **1** | **Left A** | **Channels 1-4** |
| **5** | **2** | **Left B** | **Channels 1-4** |
| **2** | **3** | **Left O** | **Channels 1-4** |
| **3** | **4** | **Right A** | **Channels 5-8** |
| **7** | **5** | **Right B** | **Channels 5-8** |
| **4** | **6** | **Right O** | **Channels 5-8** |

**Assay Protocol**

1. Chill the BioFlux 24-well plate at -20°C for 10 minute prior to starting the experiment.
2. If Matrigel is to be prepared with compounds process samples on ice into pre-chilled eppendorf tubes and pipet tips (set in a box of ice or dry ice). Take care to prepare stock solutions of compound at a high enough concentration such that the Matrigel is not significantly diluted (plan on adding less than 10µl of compound to 500 µl of Matrigel).
3. Remove from freezer. Prime device from the outlet well with 100 µl of chilled media plus 1% FBS. Plate is to be placed on a room temperature surface to avoid freezing the media in the channels.
Only prime until there is ≤5µl of fluid in the inlet wells. To accomplish this flow at 5 dyn/cm$^2$ for approximately 30 seconds, while watching the inner reservoir of the inlet wells for the tiny bead of fluid. Return plate to ice or ice pack once priming is completed.
4. Add 100 µl of chilled media to all inlet 'A' wells
5. Add 100 µl of prepared Matrigel mixtures to all inlet 'B' wells.
6. Begin flow immediately for three minutes on ice at 1.3 dyn/cm$^2$ for inlet 'A' and inlet 'B' at 1.8 dyn/cm$^2$. Note the shear force applied to inlet 'B' can be increased or decreases appropriately to provide the desired coverage of the channel.
7. Continue flowing for exactly 3 minutes off the ice and once complete transfer the plate onto the glass heater and flow for an additional 3 minutes and stop.
8. Leave the BioFlux plate on heater for 20 minutes without disturbing it.
9. After the 20 minute incubation, remove the plate from the heater and place at room temperature on the bench top for an additional 10 min.
*Once gel has formed, avoid perfusion from outlet well and avoid fast perfusion above 2dyn/cm$^2$.Gel and fluid compartments of the channel should be observable using phase contrast as a slight line along the middle of the channel.*
10. Before adding cells, coat with an experiment specific adhesion molecule in the fluid side of the channel. Example, 1/40 v/v diluted Matrigel, perfused from inlet A as directed (not from outlet)
NOTE: ALL SUBSEQUENT PERFUSION STEPS SHOULD BE PERFORMED ONLY FROM INLET A.

*Following this protocol, one should expect the BioFlux 24-well plates to have gel coverage in about 25-30% of the channel. If desired, experiment with the force ratio between the inlet streams to customize the percentage of gel coverage.*