A Paper-Based Transwell Assay to Study Cancer-Cell Invasion in 3D

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Abstract

Cellular invasion is the gateway to metastasis, the leading cause of cancer-related deaths today. Therefore, in the fight against cancer, it is crucial to better understand the cues and conditions under which cellular invasion occurs. Such an understanding has the potential to lead to therapeutic treatments capable of halting a tumor in its progression towards a metastatic state. A popular current method of studying invasion is the modified Transwell assay, comprised of two chambers separated by a matrix-coated membrane through which cells can migrate in response to various chemokines. Although Transwell assays are readily available, customizable and provide easily interpretable data, they are expensive and offer little flexibility in assay setup. Here, we present a paper-based variation of the Transwell assay capable of generating similar datasets with equal reproducibility to the traditional setup at a fraction of the cost.

Introduction

Cellular movement plays an essential role in a variety of biological processes, including: organogenesis, tissue maintenance including angiogenesis and vascularization, and wound healing.[1-3] In the context of cancer, the invasion of tumorigenic cells into healthy tissue is a key step in metastasis.[4] To identify the regulators of these cellular responses, assays that can readily quantify movement are needed.

A popular assay for evaluating cellular migration—the movement of cells, independent of their extracellular environment—is the Transwell assay.[5] This assay consists of two chambers separated by a porous membrane. In a typical experiment probing the potential of a chemokine to enhance cellular movement, cells are placed on one side of the membrane and both chambers are filled with culture medium. The chemokine of interest is placed in the chamber opposite the cells. At the experiment's end, cells that migrated through the membrane are stained and enumerated. This assay has been adapted for invasion studies—cellular migration through an extracellular matrix (ECM), which must be remodeled prior to movement. In this modified Transwell assay the porous membrane is coated with a naturally derived or synthetic ECM.[6] Incorporating ECM serves two purposes: 1) the need for cells to remodel their extracellular environment is a key step prior to movement *in vivo* and not accounted for in traditional migration assays, and; 2) cells cultured in three-dimensional (3D) environments containing ECM yield more representative phenotypes than cells cultured as a monolayer.[7]

Despite the relevance of 3D culture formats, many labs rely on monolayer cultures and migration assays because their setup and analysis are straightforward and amenable to running many experiments in parallel. Transwell assays are commercially available, customizable, and provide data that is easily analyzed with a light microscope. Transwell inserts are available in various materials and with pore sizes suitable for cell culture (0.4 – 1.0 μm) or migration assays (3.0 – 8.0 μm). This platform can support individual experiments or be scaled to accommodate 6, 12, 24, or 96 experiments in parallel with well plate formats. Despite their simplicity, these devices are costly, with 96-well Transwell formats ranging from $100-300 per plate and single inserts ranging from $0.50-12.00 per insert. These prices increase considerably if the inserts are pre-coated with collagen (I, IV and fibrillar), fibronectin, or Matrigel®. Given the cost and

experimental options available, extensive experimental foresight is necessary to determine the optimal assay configuration.

Our laboratory has been developing paper-based invasion assays capable of quantifying cellular movement in defined extracellular gradients. Paper-based cultures are an emerging and highly modular platform to study cellular invasion, metabolism, and drug resistance in tissue-like structures.[8-10] Cells suspended in a hydrogel readily wick into the paper scaffolds, which provide a 3D environment of a defined and reproducible thickness. The cellulose fibers that compose the scaffolds provide structural integrity to cell-laden hydrogels, which are fragile and prone to cracking upon tactile manipulation. Much like the commercially available Transwell assays, the paper scaffolds can be easily patterned with any number, size, or shape of "zones" in which cells can be seeded and sequestered.[11]

Here, we adapt our previously published work to generate a paper-based Transwell assay. Paper scaffolds are advantageous for Transwell type applications because it eliminates the need for the inserts, introducing experimental flexibility (matrix type, cell type, culture size). The assay uses supplies commonly found in tissue culture laboratories, making it readily accessible and reducing the cost per assay significantly. To demonstrate the paper-based assays are comparable to traditional Transwell assays, capable of quantifying the invasion of cells in: 1) the presence of known chemokines; 2) in mono- and co-culture formats.

Materials and Methods

Reagents. All reagents were used as received, unless otherwise noted. Dulbecco Modified Eagle Medium (DMEM), Dulbecco phosphate-buffered saline (PBS), HEPES, RPMI 1640 medium, and penicillin-streptomycin were purchased from ThermoFisher Scientific. Fetal Bovine Serum (FBS) was purchased from VWR.

Cell Lines and Culture Maintenance. MDA-MB-231 (M231) and MCF7 cell lines were purchased from ATCC. Reduction mammoplasty fibroblast (RMF) and HGF-overexpressing reduction mammary fibroblast (HGF-RMF) lines were provided Dr. Melissa Troester and Dr. Bonnie Sloane and were previously characterized.[12-14] The M231 and MCF7 lines were engineered to constitutively express mCherry fluorescent protein with LPP-MCHR-Lv105-025 lentiviral particles. Both fibroblast cell lines were engineered to constitutively express enhanced

green fluorescent protein with eGFPLPP-EGFP-Lv105-025 lentiviral particles. All cell lines were transduced with lentiviral particles obtained from GeneCopoeia according to the manufacturer's protocol. Single colony clones were selected and maintained as adherent cultures at 37 °C and 5% CO_2 until needed. M231-mCherry cells were cultured in RPMI 1640 medium supplemented with FBS (10% v/v) and penicillin-streptomycin (1% v/v). The MCF7-mCherry, RMF-eGFP and RMF-HGF-eGFP cells were cultured in DMEM supplemented with FBS (10% v/v), penicillin-streptomycin (1% v/v), HEPES (25 mM). Culture medium was exchanged every 48 h, and cells were passed upon reaching 70-80% confluency.

Paper Scaffold Preparation. Wax-patterned paper-based scaffolds were prepared as described previously.[10] Whatman 105 lens paper was patterned with black, non-fluorescent wax from a Xerox ColorQube®8870 printer. Each scaffold was then baked at 150 °C for 15 min to allow the wax to melt and penetrate entirely through the paper scaffold. Prior to use, each scaffold was sterilized in a laminar flow hood overnight under ultraviolet (UV). The design used in this work is a single-zone scaffold, which fits directly into the well of a 96-well plate. Each zone is 3.3 mm in diameter.

Paper-Based Invasion Assays. Prior to usage, the M231-mCherry and MCF7-mCherry were placed in serum-free medium for 4h. To prepare cell suspensions for seeding, the cells were detached from the culture flask, pelleted, suspended in rat tail collagen I (1.2 mg/mL) at 150,000 cells/µL, and placed on ice to avoid gelation. Cell-containing zones were seeded with 0.5 µL of cell-laden collagen. Additional zones were seeded with collagen containing no cells. These collagen-only scaffolds served as controls and allowed us to account for background fluorescence arising from the scaffolds.

After seeding, the single-zone scaffolds were placed in a clear-bottomed 96-well plate. Each well contained 300 µL of culture medium. In the monoculture format, the medium contained either 0, 0.5, 1, 5% v/v of FBS. In the co-culture format, 32,000 fibroblasts suspended in 300 µL DMEM with 1% FBS were added to each well. The fibroblasts were given 4h to attach to the surface of the plate before paper scaffolds were placed in the wells. The plate was then incubated at 37 °C and 5% CO_2 for 48 h, after which time the paper zones were removed from the medium and imaged.

Image Acquisition and Analysis. The number of cells at the bottom of the 96-well plates and in the paper scaffolds were quantified from fluorescence images obtained with a Typhoon 9400 scanner (GE Life Sciences). The mCherry-expressing cells were imaged with a 526 nm laser and a 610 ± 30 nm emission filter. All imagers were obtained at a 100 µm resolution and analyzed with ImageJ software. Reported values were obtained by subtracting the background fluorescence values both in the paper zones and in the wells. Papers containing only seeded collagen served as the blank for the signal in the paper zones, and wells filled only with 300 µL of medium served as the blank for the signal in the wells.

Proliferation Assay. Cell suspensions (200 µL, 2500 cells/mL) of M231-mCherry cells suspended in RPMI medium containing varying concentrations of FBS (0, 0.5, 1, 5% v/v) were pipetted into a 96-well plate. The cells were incubated at 37 °C and 5% CO_2, and the culture medium (100 µL) was exchanged every day. Cell numbers were assessed every 24 h after initial seeding with the Cell-Titer Glo (Promega) luminescence viability assay, according to the manufacturer's protocol. Luminescence values were measured on a SpectraMax M5 plate reader (Molecular Devices).

Transwell Invasion Assay. To prepare modified Transwell assays, the inserts of a Corning HTS Transwell 96-well plate were coated with 50 µL of rat tail collagen I (1.2 mg/mL) and allowed to dry overnight in a laminar flow hood. The inserts contained a polycarbonate membrane with an 8 µm-diameter pore size. A series of cell suspensions were generated in serum-free RPMI medium and 50 µL aliquots were pipetted onto the collagen-coated inserts at a final cell density of 75,000, 50,000, and 25,000 cells/insert respectively. Prior to placing the cells on the collagen-coated insert, M231-mCherry cells were incubated in serum-free RPMI medium (4 h).

The inserts were placed in a 96-well plate, and each insert was in direct contact with 150 µL of RPMI with 0, 0.5, 1, or 5% v/v of FBS. The plate was incubated for 48 h at 37 °C and 5% CO_2. To quantify the number of cells that invaded through the porous membrane, we analyzed both the well plate and the bottom of the inserts. Fluorescence images of the well plate were obtained, as described above, to quantify the number of cells that had fallen off the membrane during incubation. The inserts were fixed with ice cold 100% methanol for 10 min, dried, and then stained with a crystal violet solution for 10 min. The inserts were washed with deionized

water to remove excess staining solution and allowed to completely dry overnight. A light microscope was used to enumerate the number of stained cells.

Results & Discussion

Single zone, paper-based Transwell assays are compatible with traditional 96 well plates. Single-zone paper-based scaffolds, comprised of one region supporting cell culture surrounded by a border of wax, fit directly into a well of a commercial 96-well plate. Isolating each cell culture allowed us to easily expose cells to various conditions while using commercially available plates, making the technique accessible to anyone with access to a wax printer. In a typical assay format, fluorescently labeled cells are suspended in an extracellular matrix (ECM) and then seeded into the paper scaffolds. Cell-containing scaffolds are placed in a 96-well plate and incubated in culture medium containing a particular exogenous agent. A drawback of this setup is the cells are exposed to a single culture condition throughout the incubation period, opposed to the dissipating gradients formed in the traditional Transwell assays.

To quantify the number of invasive cells present in a population, or in the case of potential chemokine that promote cells to adopt an invasive phenotype, we quantify the number of cells that escape the scaffold and fall to the bottom of the well plate. Thus, we can simply separate the highly invasive and the non-motile cells by removing the paper scaffold from the well at the end of the experiment. To quantify the number of cells in both the plate and paper scaffolds we image each with a fluorescence flatbed scanner. Cells that constitutively express fluorescent proteins such as mCherry can be determined with calibration curves that relate fluorescence intensity and cell number. We have shown that there is a linear relationship between fluorescence intensity and cell number in the paper scaffolds.[15] These cells can also be quantified with viability assays such as CellTiter-Glo.

Paper-based and traditional Transwell assays show similar invasion trends. To determine if the paper-based assays provided similar trends to those obtained in traditional Transwell assays, we compared the invasion of MDA-MB-231 across setups when exposed to different FBS concentrations.

In both setups the cells were placed in contact with collagen I, with cells being placed atop a collagen coated membrane in the traditional Transwell assay, and cells being suspended in

collage prior to seeding in the paper scaffolds. We selected collagen I because: 1) it is readily purified, allowing us to probe responses to chemokines in a relatively growth factor-free environment; 2) elevated levels have been associated with breast increased tumor intravasation and metastasis; and 3) it is a common membrane coating in Transwell assays.[16] Other ECM mimics like Matrigel® are compatible with our assay and are used in earlier paper-based studies.[9] We exposed the cells to increasing concentrations of FBS (Figure 1c), which is a commonly used chemokine and known to promote cellular movement.[17]

We found that in both formats, increasing concentrations of FBS resulted in a higher number of cells invading through the collagen. In the paper scaffolds, the number of cells in the paper scaffolds decreases while the number of cells at the bottom well plate increased with increasing FBS concentration (Figure 1d). In the traditional Transwell assay, the number of cells that crossed the porous membrane increased with increasing FBS concentration (Figure 1b). Additionally, fluorescence intensity measured at the bottom of the wells of the traditional Tranwell assay appears to increase with increasing FBS concentration (Figure 3). When comparing 0% to 5%, we see a 1.12-fold increase in cells in a Traditional assay and a 1.31-fold increase in cells in the bottom well for our paper-based setup. These results suggest that our setup is comparable to traditional Transwell assays.

To confirm that these invasion trends were a result of cellular movement and not a result of cellular proliferation, which increases with increasing concentrations of serum, we measured the proliferation of M231-mcherry cells in monolayer cultures at each FBS concentration over a 5-day period (Figure 2). We found that cells cultured in medium containing 0.5% or less of FBS did not demonstrate any significant growth over a 5 day period. Significant growth occurred in 1% and 5% FBS only after 72 h of incubation. When cultured in 5% FBS, the doubling time of M231-mcherry is 54 h. We note that at higher FBS concentrations (5%), cells are exposed to culture conditions that promote proliferation. Given that our assay was within 48h, we believe that the increase in cells in the bottom well is a result of cellular movement but note that shorter time intervals or lower concentrations of FBS would prevent any complications from cellular proliferation.

Paper-based and traditional Transwell assays have similar reproducibility. Using the invasion results from both assays for increasing concentrations of FBS, we compared the reproducibility

of our paper-based to the traditional Transwell assay. To compare the reproducibility between the two culture platforms, we compared the experimental conditions with the least and most invasion (0 and 5% FBS, respectively). For 0% FBS, the relative standard deviation (RSD) of cells at the bottom of the well plate was 13.4% for the paper-based assay and 18.3% for the traditional Transwell assay. For 5% FBS, the paper-based format had an RSD 10.4% and the traditional Transwell assay 11.7%. The similarity between these values suggests that our paper-based assay is as consistent as its traditional counterpart.

Paper-based Transwell assays support direct and indirect co-culture formats. We demonstrated that the Transwell assay on paper is similar to traditional assays in that we can look at the addition of exogenous chemicals and paracrine signaling. Co-cultures of cells are necessary to evaluate cell-cell signaling and its role during chemotaxis.[18] In some cases, one cell line will excrete factors that signal for the invasion of neighboring cells.[19] To illustrate this capability, we co-cultured MCF7-mcherry cells with one of two mammary fibroblast cell lines. Fibroblasts are known to release factors such as hepatocyte growth factor (HGF) the transforming growth factor beta (TGFβ), both of which promote EMT and increase cellular movement.[14] In one setup we used an RMF line and in another we utilized an RMF line that was engineered to overexpress HGF.

In these experiments, we placed paper scaffolds containing MCF7-mCherry cells (75,000) suspended in collagen into wells containing: 1) conditioned medium obtained from RMF-eGFP cells; 2) conditioned medium obtained from RMF-HGF-eGFP cells; 3) 32,000 RMF-eGFP cells, which formed a monolayer at the bottom of the well, or; 4) 32,000 RMF-HGF-eGFP cells, which formed a monolayer at the bottom of the well. Figure 1 is a schematic of the co-culture setup used in this work.

Compared to wells without any exogenous factors, we found that RMF conditioned media or RMF in the well plate did not elicit a more invasive response. However, when cultured with conditioned media from ($p < 0.05$) or in the presence of RMF-HGF-eGFP ($p < 0.0001$), MCF7-mCherry cells were more invasive than without any co-cultured line. This posits that MCF7-mCherry invasion is sensitive to concentrations of HGF.

Conclusion

Cellular movement is crucial in a number of biological processes, ranging from tumor progression to the maintenance and repair of healthy tissue. To elucidate the chemical signals that promote cellular movement, or in the case of invasive cancers the drugs that inhibit this movement, *in vitro* assays are invaluable. Transwell assays are a popular method of studying cellular migration due to its: 1) customizability of culture size, membrane pore size and membrane material; 2) ease of assay setup and data interpretation; and 3) ability to be modified to study invasion using ECM-mimic membrane coatings. However, their considerable cost and inflexibility in assay variation once plates are purchased represent significant assay limitations.

Here we have shown that a paper-based variation of the modified Transwell assay can generate datasets similar to traditional Transwell assays with a high degree of reproducibility. These paper scaffolds are made using materials typically found in tissue culture laboratories, thus significantly reducing cost. The ability to manufacture these scaffolds in-house also allows for a large degree of flexibility in assay design. It has been shown in this work that the paper variation of a Transwell assay can be used to study the response of cells to varying concentrations of exogeneous chemokines. Furthermore, it has proven amenable to co-culture setups to further probe the effects of paracrine signaling between tumor cells and tumor-associated fibroblasts on the progression of metastatic behavior in cancerous cells.

Moving forward, we hope to interrogate the juxtacrine signaling relationship between fibroblasts and epithelial breast cancer cells utilizing a direct coculture assay in paper scaffolds, as opposed to the indirect coculture previously described in this work. Additionally, we intend to probe the differences in gene expression, cell viability and drug resistance between cells migrating to the bottom of wells and those remaining in the paper. This will help to elucidate how phenotype and behavior differ between invasive and non-motile cells from a single population.

Acknowledgements

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Figures

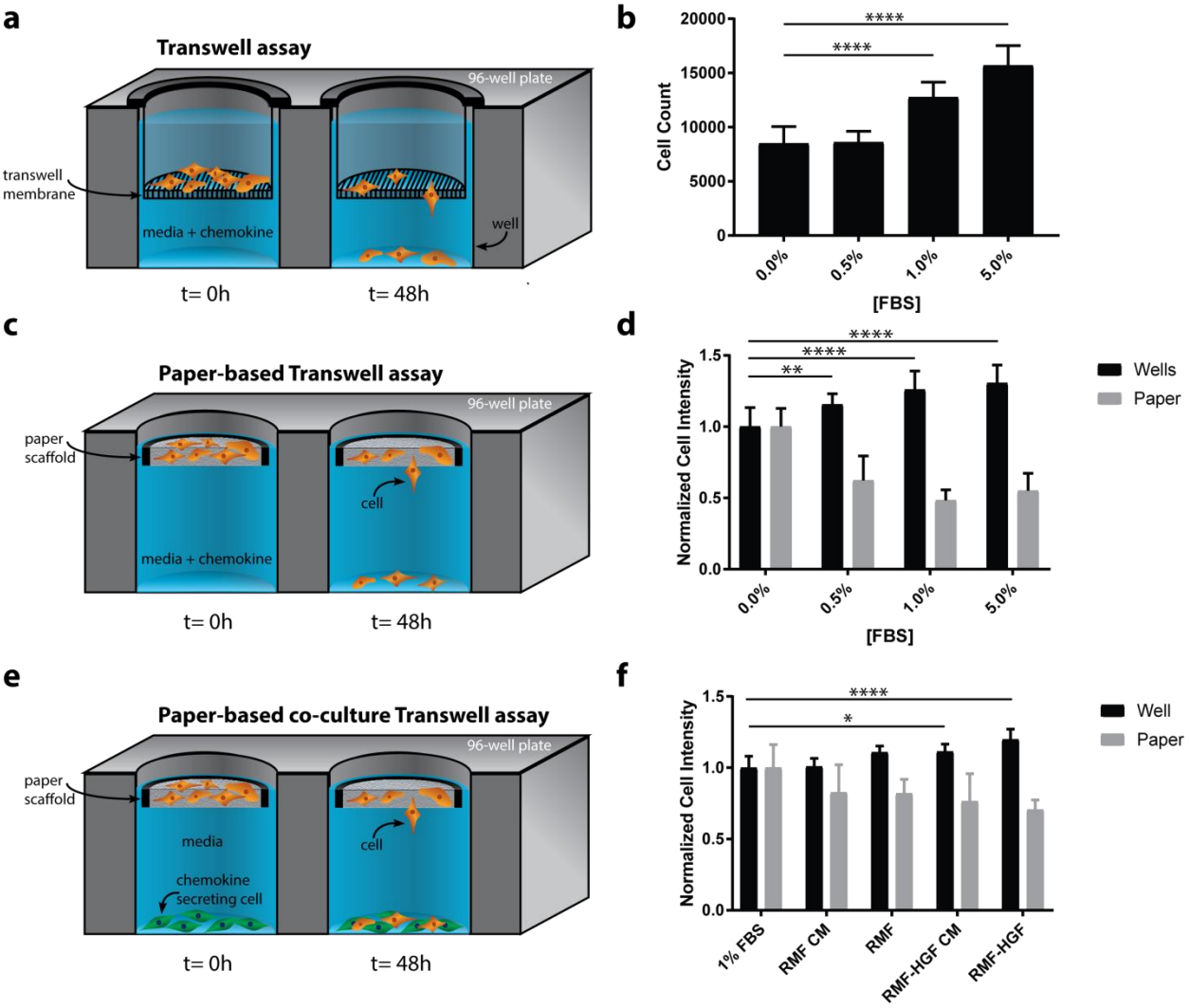

Figure 1. Paper-based Transwell assay. (a) and (b) depict a traditional Transwell assay and invasion data obtained from varying FBS concentrations. All [FBS] are significant from one another with $p < 0.0001$ except for 0% vs 0.5%. (c) and (d) depict the paper-based comparison of

the same assay. All [FBS] show significant cell movement compared to 0% ($p < 0.01$ for 0.5%, $p < 0.0001$ for 1% and 5% for signal in wells). (e) and (f) depict the indirect coculture setup. Only wells containing RMF-HGF-eGFP cells ($p < 0.0001$) or their conditioned media ($p < 0.05$) elicited significant cell movement.

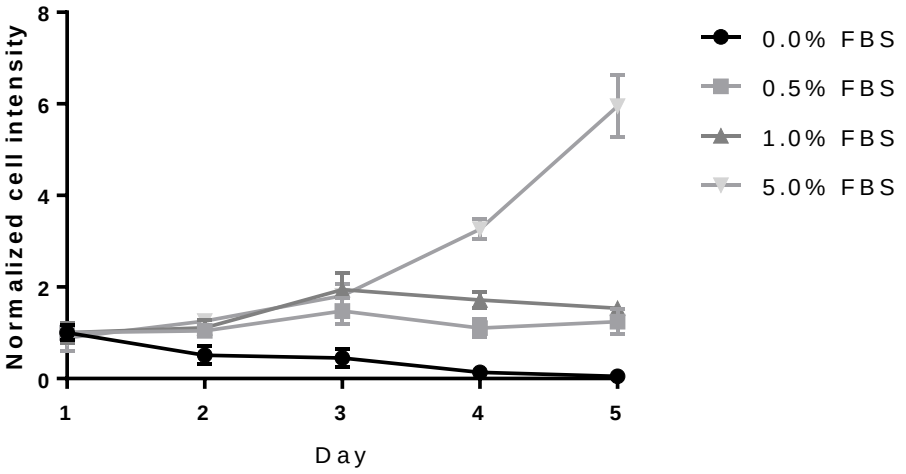

Figure 2. Proliferation in 2D monolayer of M231-mcherry cells after 5 days. Cell proliferation is significant in 1% and 5% FBS after Day 3. The doubling time in 5% FBS is 54h. Day 1 = 24h.

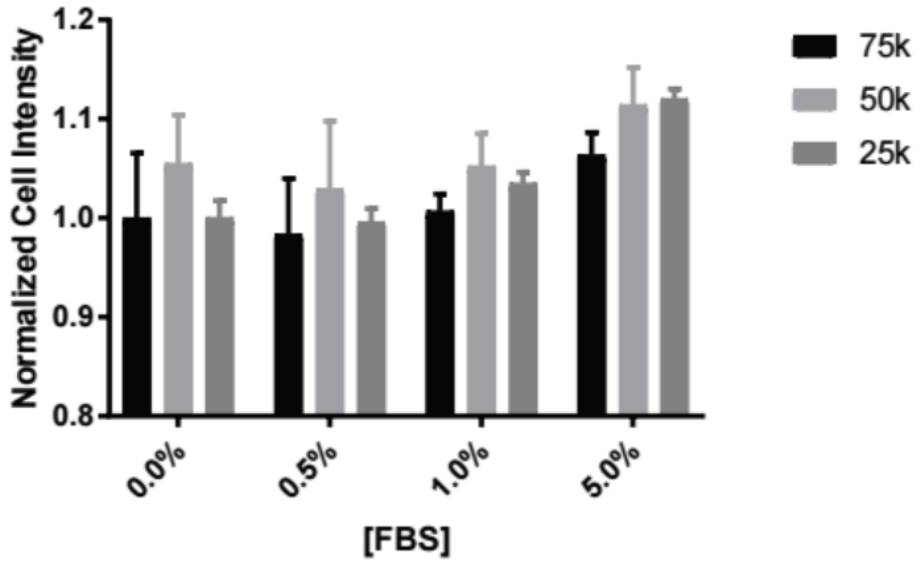

Figure 3. Fluorescence data from the bottom of the traditional Transwell assay at densities of 75,000, 50,000 and 25,000 cells/insert.

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