FLUXION

APPLICATION NOTE

Wound Healing

Novel method to perform quantitative wounding assays using BioFlux

Introduction

Scratch wound assays are commonly performed to assess cellular migration and cell proliferation, often in response to therapeutic drug application. Wounding assays are also used to further understand the molecular phenomena related to wound healing, which includes cell signaling, immune and healing response to bacterial and fungal infections, and tissue remodeling. Most wounding assays are performed using a mechanical means to generate a break in a confluent monolayer of cells. Often this involves drawing a pipet tip or a pin over the monolayer. This physically damages the monolayer as desired, but also damages the cells, causing contents to leak and potentially complicating the interpretation of data. The other drawback of this method is that wound size and shape is highly variable contributing to difficultly in data analysis and inability to directly compare experimental conditions.

In this study we used the BioFlux 200 System (Figure 1) to wound cells enzymatically with trypsin, which eliminates cell damage at the wound edge. The microfluidic channels in the BioFlux plate are identical within <5%, conferring the ability to generate wounds by fluid flow that are highly regular in shape and similar in size. We studied both migration and proliferation following wounding by microscopy using a promoter of cell migration, epidermal growth factor, and a motility and cell cycle inhibitor, cytochlasin D.

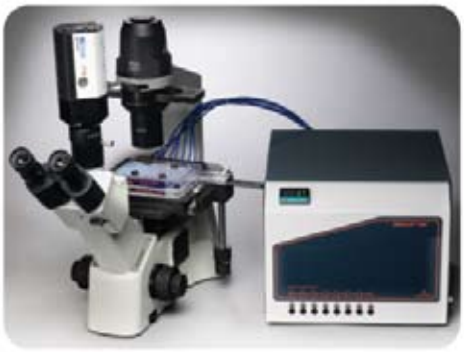


Figure 1: The BioFlux System for live cell assays under controlled shear flow.

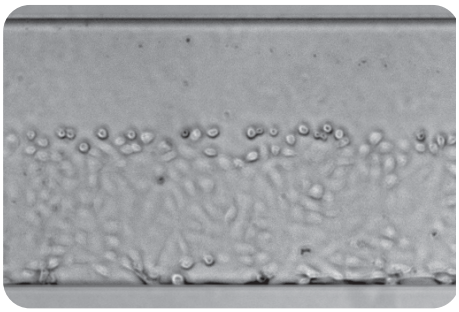


Figure 2: A BioFlux Plate channel viewed on an inverted microscope. Epithelial cells were seeded into the channel and allowed to attach. This was followed by trypsinization on the top part of the channel to remove cells. This acheives a wounding effect and provides a platform for following the remaining cells over time.

Materials and Methods

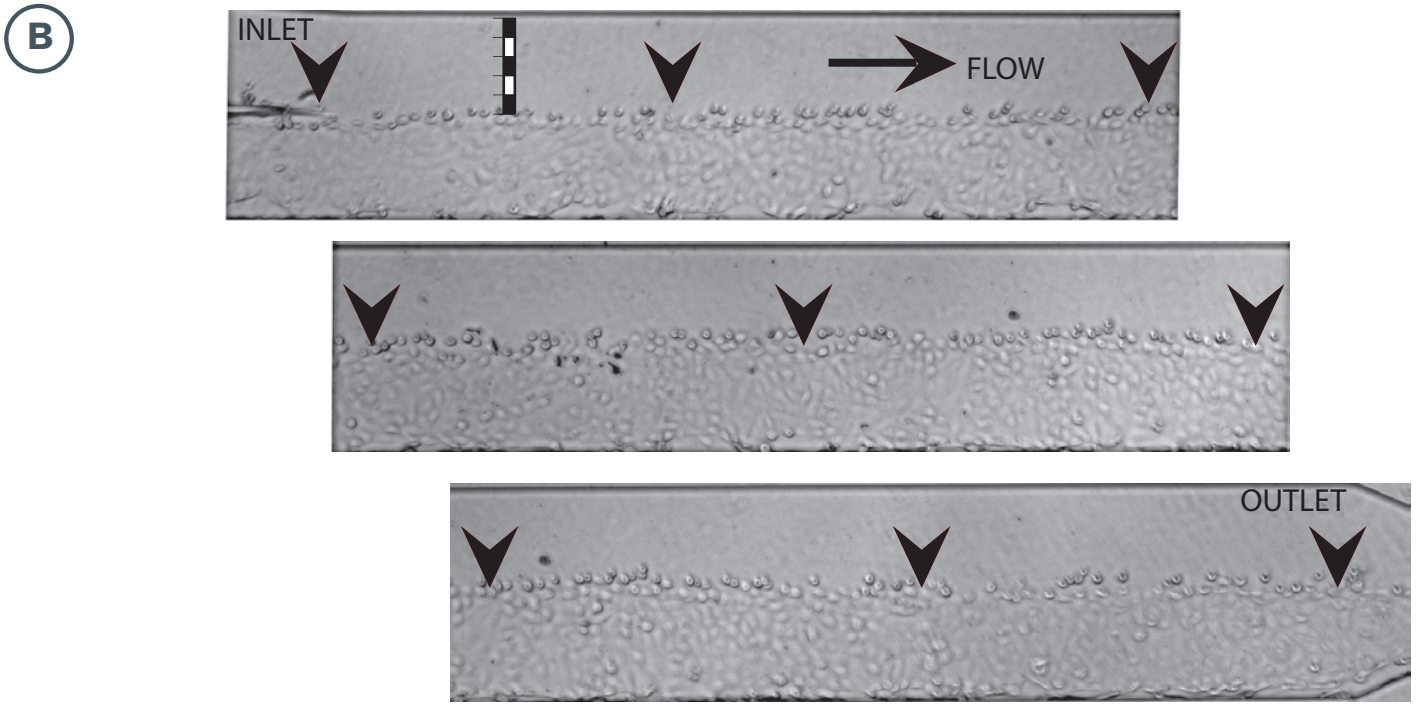
Channels of 24-well BioFlux Plates (Figure 2) were coated with Matrigel (BD Biosciences, Franklin Lakes, NJ) at a non-gelling concentration (1/50 v/v in Hank's buffered salt solution), followed by an incubation at 37˚C for 1 hour. Rat lung epithelial cells were seeded at 10^7 cells/mL in the channels. Cells were allowed to attach to the channel surface for 1 hour. Williams complete media with 10% fetal calf serum was added to the inlet wells of the plate to feed the cells by gravity flow overnight. Wounding experiments were performed the next day.

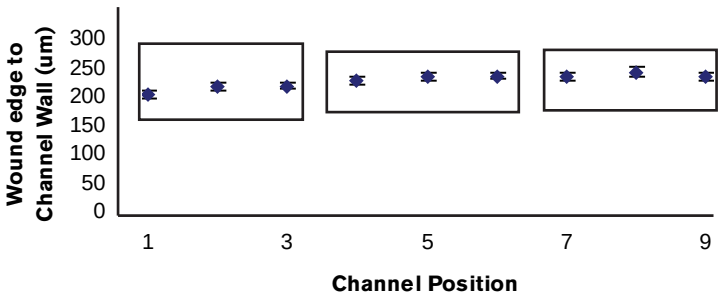
The pneumatic control lines of the instrument were rearranged to allowed inlet A and inlet B to flow simultaneously and to be controlled independently (Figure 3A). Inlet wells B were washed out with PBS without divalent cations and Trypsin EDTA (0.25%) was added. Inlet A wells were filled with William's complete plus serum. Flow was initiated from both inlets concurrently at 1 dyn/cm^2 for 10 minutes followed by a short pulse of high shear (10 dyn/cm^2) to remove rounded cells. Perfusion from Inlet B was stopped. Perfusion from inlet A was continued for 10 minutes to neutralize residual trypsin. Media and trypsin were removed from wells and replaced with William's complete plus or minus serum. At the same time in separate channels, Cytochalasin D (CytoD) or epidermal growth factor (EGF) were added at the following concentrations to either serum plus or serum minus media: 1ug/ mL and 5 ug/mL or 10ng/mL and 100ng/mL for respective compounds. Plates were placed in the incubator and data were collected at 2, 4, 5.5, 20 and 24 hours post-wounding.

Figure 3. Creating wounded monolayers in the BioFlux system

(A) Flow patterns in channel during wound formation.

(B) Measurements of wound size in microns were made throughout the length of the viewing window at nine positions in total. The same regions were measured for each channel (black arrowheads) from the leading edge of the wound to the outer wall of the channel (black and white bar). The size of the wound increased slightly as the distance from the inlet increased, regions 7-9. Error bars indicate standard error of the mean for 9 measurements.





Results

Wound Size

Following trypsinization, wound sizes were measured from the leading edge spanning the cell-free area to the channel wall. The average wound size across the channel from the inlet to the outlet as determined by measurements at 9 points along the length of the viewing window was 224.5 µM ± 5.4%. The wound size increased towards the outlet by 32 µM which could represent some diffusion and inactivation of trypsin as the length increased. The average wound size for the first 3 positions in the channel was 210 µM± 3.8% (Figure 4B). This area was used for the remainder of the migration and proliferation studies as it represents the largest gap to close, ~160 µM.

Cell Migration

Cells were allowed to recover in media without serum to parse out migration from proliferation. Measurements were made from the leading edge of the wound to the channel wall. This tracks the increase in cellular migration as movement of the leading edge towards the free wall. The peak distance in migration was observed at 5.5 hours post wounding. The highest concentration of EGF led to the furthest migration under starvation conditions at 47 µM. Predictably, both the media alone control and the lower concentration of EGF led to migration as well (Figure 4A). The CytoD treated cells rounded as early as 2 hours post-wounding and the monolayer began to contract. The anomaly in the migration distance for the 5 ug/mL CytoD treated monolayer occurred as clumps of rounded cells massed in the channel and was erroneously measured as an increase in the distance to the wall. The cellular morphology observed for EGF and media alone treated cells was typical fibroblast to cobblestone types, whereas the CytoD treated cells never achieved this morphology. By 20 hours post-wounding, however, all cells began to round up and cells were lost from the monolayer, most likely due to the media conditions which are unfavorable for cell viability.

Wound Closure

To study cell proliferation leading to wound closure, all treatments were delivered in complete media containing fetal calf serum which is permissive for growth. The cells in the media alone control were tightly packed together reminiscent of the non-wounded control. This sample closed the wound without spreading out, and by 20 hours the gap was completely closed (Figure 4B). Cells treated with EGF behaved differently from the control. EGF treatment caused cells to migrate away from the wall and spread out (Figure 5) before closing the wound by proliferating towards the walls. Cells with the highest EGF concentration remained disordered and never closed the gap. Cells exposed to the lowest concentration however came close to complete wound closure within 24 hours.

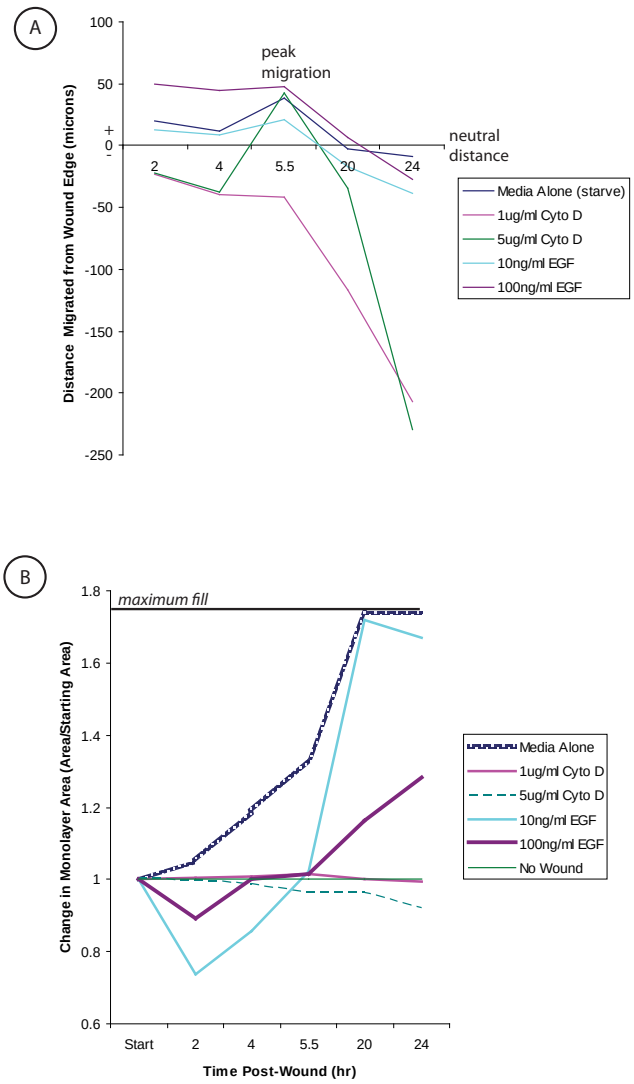


Figure 4: Cell migration and proliferation after wounding.
Measurements were made at 2,4,5.5,20 and 24 hours post-wounding.

(A) Cell migration was measured as the distance from the monolayer wall to the leading edge of the wound in three positions for each channel, only the first field of view was used (Figure 3B) as the wound was the greatest size in this region. Distance migrated was calculated by subtracting the leading edge distance from the starting position of the leading edge in microns. Positive values express growth; negative values indicate contraction of or loss of cells from the monolayer.

(B) Cell proliferation was measured as the total area covered by cells in microns squared. The change in area shown is expressed as the area measured for each time point divided by the original area. Complete confluence of the monolayer is indicated by the bar at the top of the chart. Values below 1 indicate loss of monolayer are compared to starting size.

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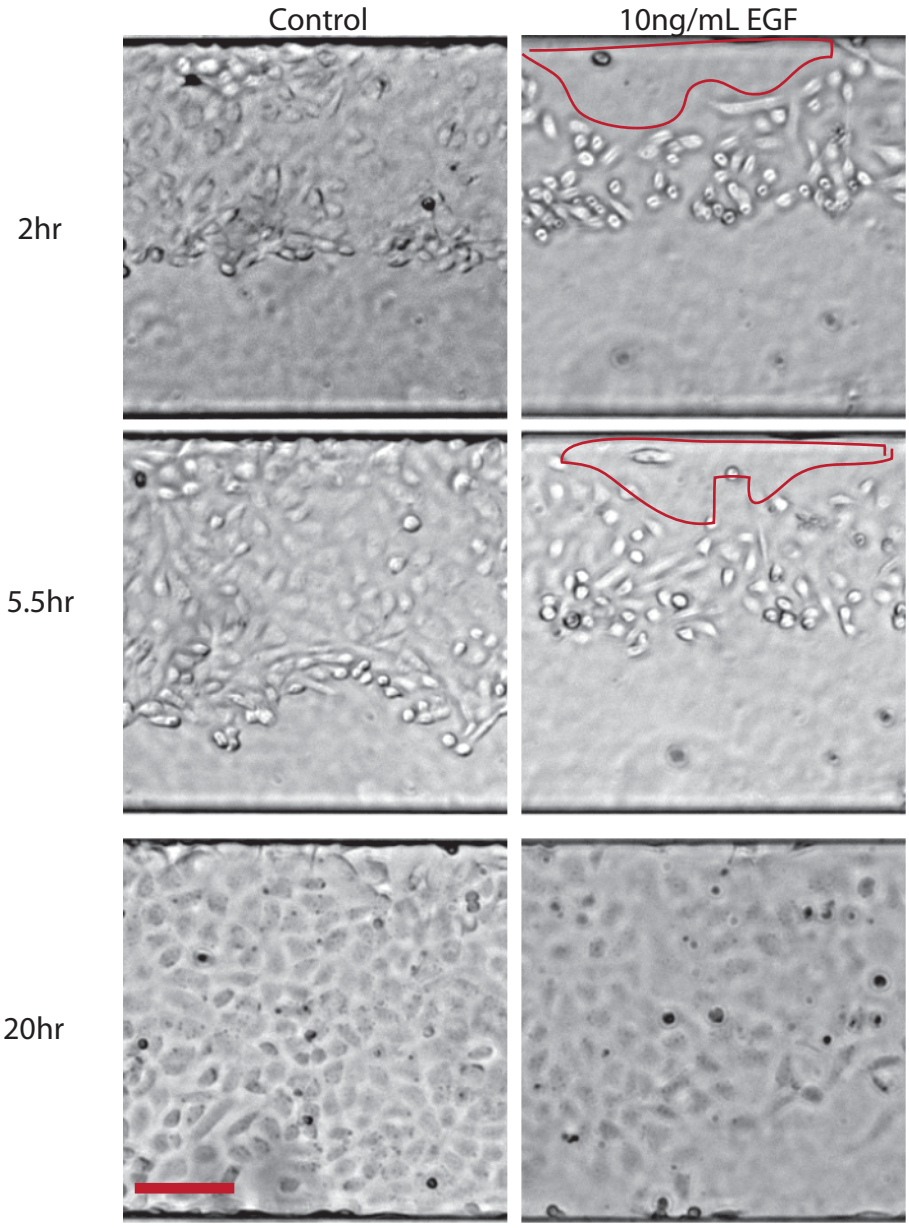

Figure 5: Cell morphology during proliferation. Cells treated with EGF displayed increased spreading and disordered morphology prior to proliferation under permissive conditions in contrast to the control monolayers that maintained a tightly packed cobblestone appearance and proliferated in an orderly manner from leading edge to far channel wall. Red outlines highlight cells moving away from non-wounded channel wall. Scale bar is 100 µM.

Summary

Using the BioFlux 200 system, we generated tightly controlled wounds with rat lung epithelial cells with a reproducibility of 5.4%. %. Because the wounds were generated by enzymatic means and fluid flow, the cells at the leading edge of the wound remained intact; thus cytosolic leakage was not a factor in the interpretation of the data. We demonstrated that cell migration can be followed using media starvation conditions with different compounds. We also showed that cell proliferation can be assessed in the same manner.

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