CELL Microsystems®

APPNOTE

High-Throughput Cell Adhesion

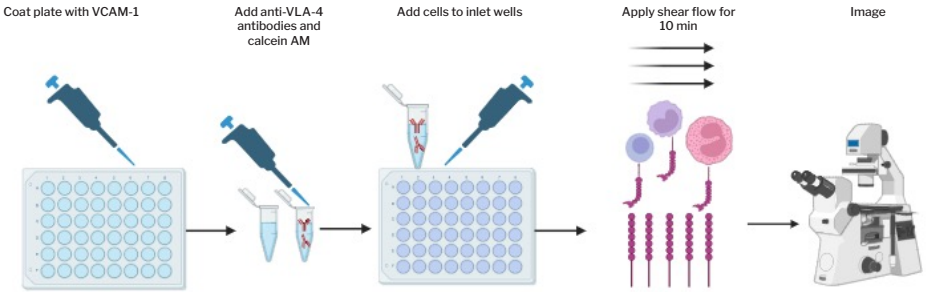
Screening of integrin antagonists under physiological shear flow conditions

Introduction

Cell adhesion governs numerous biological phenomena in health and disease. Adhesion molecules are expressed on the surfaces of numerous cells, including leukocytes and endothelial cells. At a site of infection or injury, inflammatory cytokines increase adhesion molecule expression, promoting leukocyte rolling and adhesion. The result of these interactions is the transmigration of leukocytes to the underlying tissue and amplification of the inflammatory response. Cell adhesion is impacted by shear flow, with areas of high shear flow having fewer adhesion molecules compared to areas with low shear flow. This can contribute to certain areas of tissue becoming more susceptible to cancer metastasis and progression of other diseases. The molecular interactions governing adhesion between white blood cells, cancer cells, and the cells of the endothelium are the focus of targeted therapeutic drug development. However, traditional in vitro assays of static cellular adhesion are non-physiological and animal models are time-consuming and expensive, contributing to long and expensive drug discovery and development timelines.

The BioFlux Shear Flow System provides a straightforward method to accelerate data production by simulating in vivo environmental conditions, including shear flow and temperature. In this application note we present a high-throughput, physiological assay that can be used as a screening method of antibodies and compounds that impact cell adhesion.

BioFlux Cell Adhesion Workflow



Key Highlights:

1. Reduce cell adhesion assay time from 6-18 hours to 3 hours
2. Mitigate the effects of inconsistent washing of traditional adhesion assays
3. Increase the speed and accuracy of dose-response testing

Questions this App Note Answers

1. Can the number and morphological characteristics of cells attached to adhesion molecules be visualized?
2. How can the dose-response of multiple compounds be tested in the same experiment?
3. Does physiological flow alter the effects of anti-adhesion compounds?

Methods

Cell Preparation

Jurkat cells (TIB-152; ATCC) were cultured in RPMI-1640 (UCSF Cell culture facility) supplemented with fetal bovine serum (10% v/v; Hyclone), penicillin-streptomycin (1X v/v; USCF CCF), HEPES (10 mM; USCF CCF), and glutamine (2 mM; UCSF CCF).

Adhesion Inhibition Assay

Three BioFlux 48-well (24-channel; Figure 1) plates were coated with VCAM-1 (10 µg/mL; R&D Systems) in Hank's balanced salt solution (USCF CCF) under flow at 1 dyn/cm^2 for 10 minutes, followed by 50 minutes of settling and attachment time (0.1 dyn/cm^2 to prevent backflow). Jurkat cells were centrifuged at 200 x g for 5 minutes; the supernatant was discarded and cells were resuspended in PBS, bovine serum albumin (BSA) (1% w/v), and calcein AM (4 µM) (Invitrogen). Cells were counted and aliquoted into 24 microcentrifuge tubes at a concentration of 1.2 x 10^6 cells/mL. Antibodies (BBA37 [R&D Systems], MAB1955 [Millipore], and MAB16983 [Millipore]) were added to the cells (Table 1) and incubated for 1 hour with gentle rocking at room temperature. On a separate plate, a set of cells were stained with EPR1355Y (Epitomics), an antibody against the intracellular epitope, VLA-4. VCAM-1 coated channels were washed and blocked for 10 minutes with 1% BSA in PBS at 1 dyn/cm^2. BSA in PBS was then removed from the BioFlux plate. Cells and PBS were added to the inlet and outlet wells respectively. Cells were perfused from the inlet wells for 10 minutes at 0.8 dyn/cm^2 and channels were washed for 10 minutes at 0.8 dyn/cm^2 from the outlet wells. Micrographs were captured using a QICam (QImaging, Surrey, B.C.) and BioFlux Montage software on a Nikon TS100 microscope. Cell counting and morphometry analyses were performed using BioFlux Montage software; data were plotted using GraphPad Prism 5 (Dotmatics).

BioFlux plate

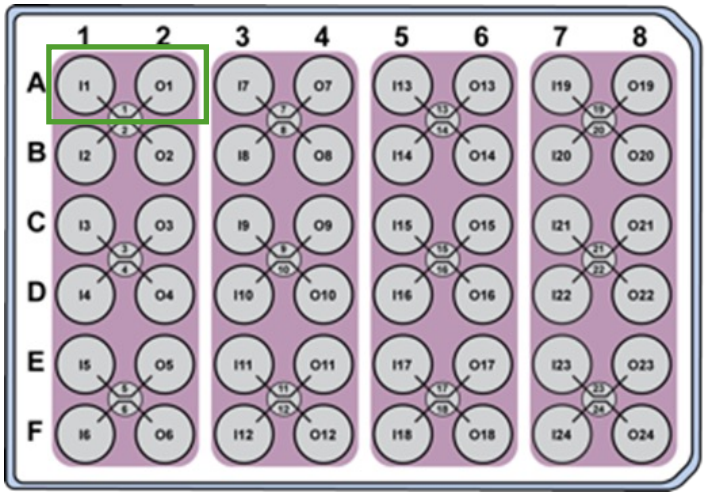


Figure 1. 48-well BioFlux plate. The green square highlights an inlet (A1) and outlet (A2) well with a viewing channel connecting the two wells. Each pair of inlet and outlet wells represent independent assays.

Table 1. Antibodies used for inhibition studies

Channel	Drug	Concentration	Details
1	BBA37	0	Anti-human integrin alpha4/VLA-4/CD49d monoclonal raised against whole cell lysates, murine IgG1, protein G purified
2		100 fM	
3		100 pM	
4		300 pM	
5		1 nM	
6		3 nM	
7		10 nM	
8		30 nM	
9	MAB1955	0	Anti-alpha4beta1, IgG3 clone P4C2 murine ascites
10		1/106	
11		1/105	
12		1/50,000	
13		1/15,000	
14		1/10,000	
15		1/5,000	
16		1/2,000	
17	MAB16983	0	Anti-human integrin alpha4 monoclonal raised against T-cells murine, IgG1, clone P1H4 protein A purified
18		100 fM	
19		100 pM	
20		1 nM	
21		10 nM	
22		100 nM	
23		200 nM	
24		N/A	

Results

We investigated the inhibition of cell adhesion to VCAM-1 and determined IC_{50} values for 3 monoclonal antibodies against VLA-4.

BBA37

BBA37 was tested at a range of concentrations from 100 fM to 30 nM. Inhibition increased sharply between the pM and nM range with an IC_{50} of approximately 600 pM. Adhesion was completely inhibited at 1 nM (Figure 2A), which is 30 times less antibody than the manufacturer's recommendation for U937 cells in a static assay (R&D Systems).

MAB1955

MAB1955 treated cells exhibited a reduction in adhesion between 1/50000 and 1/15000 dilutions with an IC_{50} at 1/32000. Complete inhibition was achieved at a concentration of 1/15000, 15 times lower than the recommendation (Millipore) (Figure 2B).

MAB16983

MAB16983 treated cells never reached complete inhibition of adhesion. Maximum inhibition was a reduction of 30% of the control, plateauing at >100 nM with an IC_{50} of 10 (Figure 2C). We did not test above 200 nM as the volume of antibody required was prohibitory. Interestingly, homotypic aggregation of cells was observed at a concentration of 1 nM (data were excluded from the dose-response curve) but not at any other concentration. The average size cell cluster area at 1 nM was 66% larger at 280 pixels^2 compared to 79 pixles^2 for single cells (Figure 3). The overall distribution sizes and degree of circularity were wide when compared to the adhesion of monodisperse cells (Figure 4).

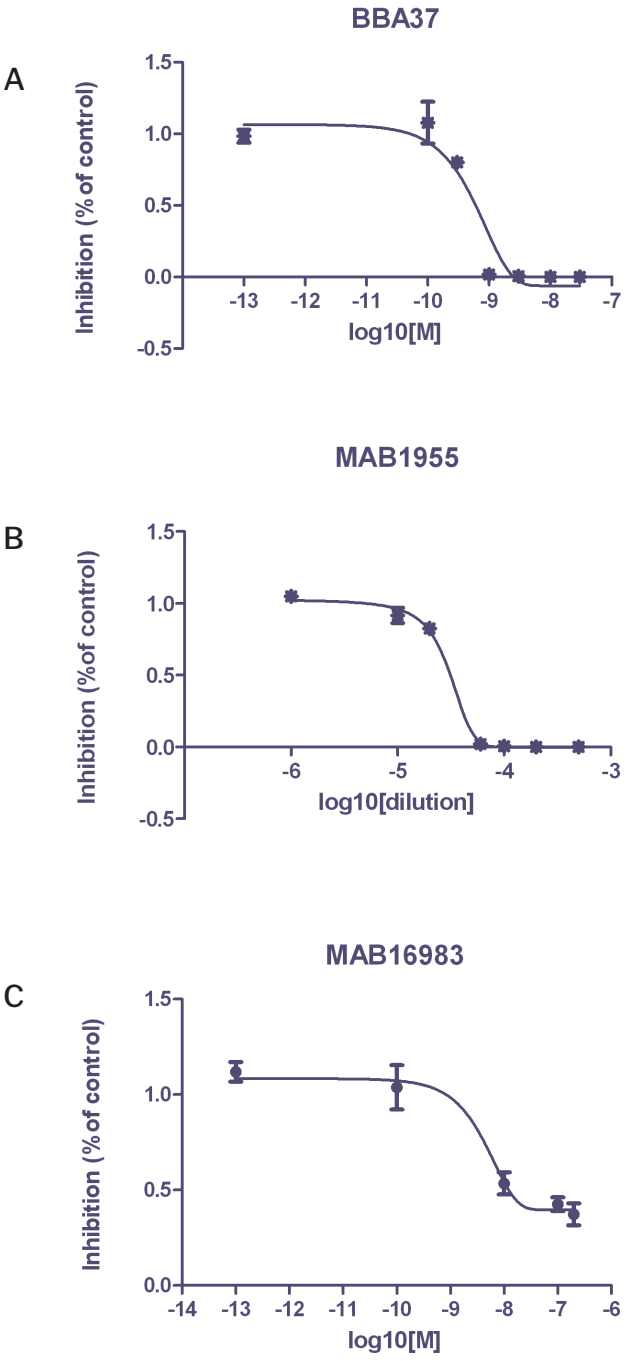


Figure 2. Reduction in cell adhesion to VCAM-1 following treatment with anti-VLA-4 antibodies. Percent inhibition of adhesion when treated with BBA37 (A), MAB1955 (B), and MAB16983 (C). Cell counts were determined using BioFlux Montage software integrated morphometry analysis. Inhibition level was expressed as number of cells adhering after treatment over number of cells attached in control samples. Error bars represent SEM for three experiments.

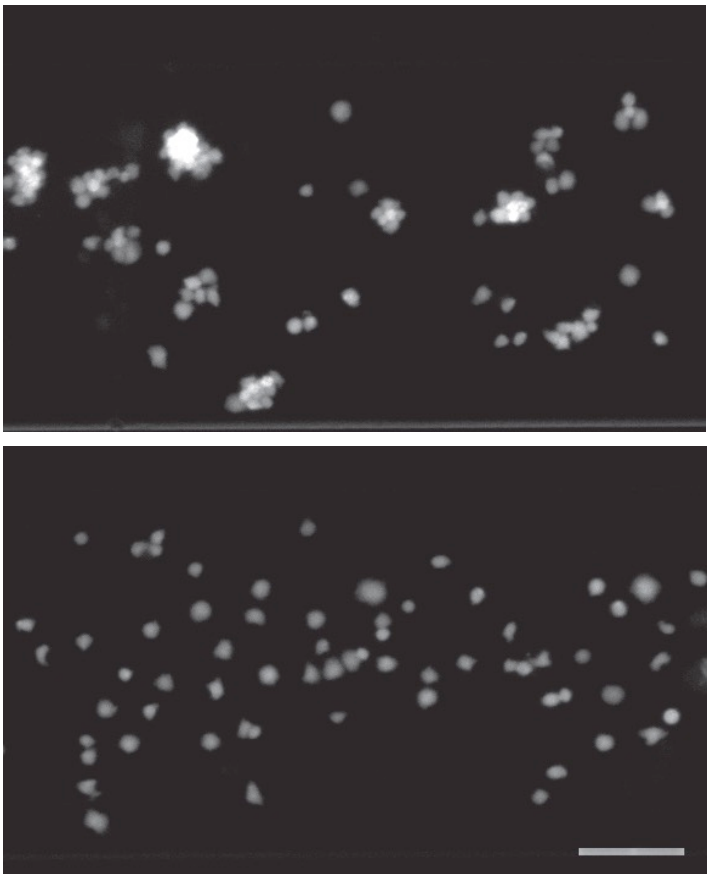


Figure 3. Homotypic aggregation. Jurkat cells stained with calcein AM treated with 1 nM MAB16983 (top) or an antibody against an intracellular epitope of VLA-4 (EPR1355y) (bottom) are shown. 4X objective, scale bar is 75 microns.

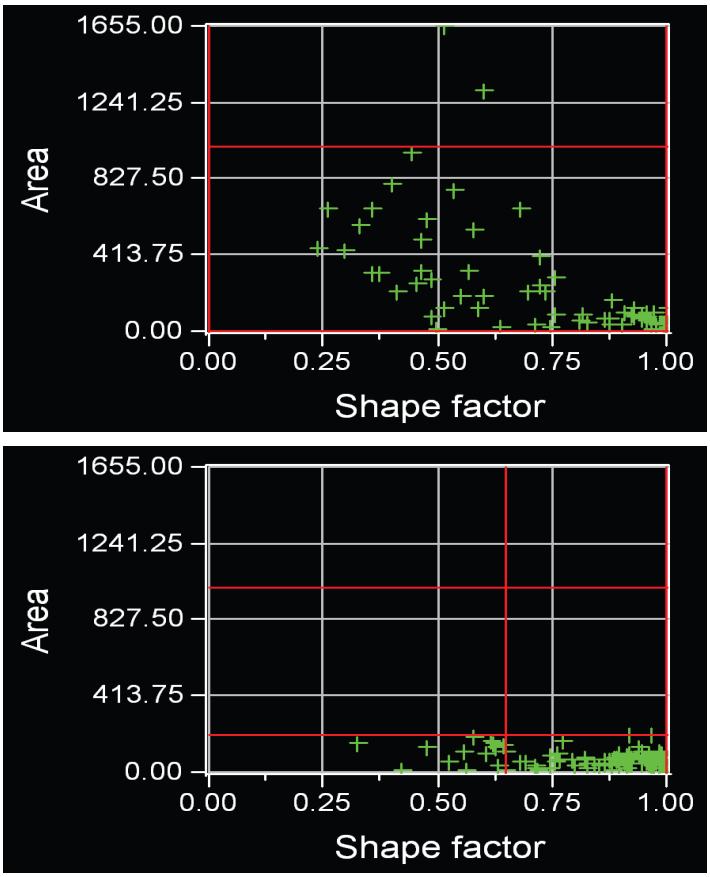


Figure 4. Cell morphology metrics. Cell morphology metrics plotted for 1 nM MAB16983 treated cells (top) and EPR1355Y (bottom) are shown. A value of 1 for shape factor denotes a perfect circle and units area are pixels^2.

Discussion

Inhibition of VLA-4 in patients is thought to be a viable avenue of immune suppression in diseases where inflammation has become pathological. Using 3 monoclonal antibodies against VLA-4, we demonstrated a dose-dependent reduction in cell adhesion, and in 2 of the 3 antibodies, complete blockade of adhesion of Jurkat cells to endothelial cells under shear flow. The MAB16983 antibody exhibited a large reduction in adhesion; however, inhibition plateaued, and complete inhibition of adhesion was never achieved. This antibody also promoted aggregation at one concentration, which would be an undesirable trait as a therapeutic. We hypothesize this to be the result of epitope-dependent VLA-4 crosslinking facilitated by the antibody. It is likely that this observation would not have been detected in a microplate assay scored on a plate reader.

As all of the liquid handling steps were done using microfluidic flow within the BioFlux plate, in comparison to traditional static adhesion assays, the BioFlux adhesion assay was more streamlined and physiologically relevant. This was demonstrated by a **2-6 fold reduction in time** to complete the assay in comparison to a traditional microtiter plate-based adhesion screen. Additionally, the inhibitory antibody concentrations of BBA37 and MAB1955 were significantly reduced compared to the data provided by the manufacturers. This suggests that, in comparison to static testing, the properties of adhesion inhibition are altered under physiological conditions and may be more relevant to what occurs in vivo.

For more information on BioFlux Technology,
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801 Capitola Drive, Suite 10
Durham, NC 27713
info@cellmicrosystems.com