FLUXION

APPLICATION NOTE

Platelet Adhesion and Aggregation Assays

Microfluidic assays enable multi-parametric investigation of platelet behavior under flow.

Relevant Research Areas: Vascular Biology, Thrombosis and Hemostasis, Cardiology, Oncology, Endocrinology

Introduction

Analysis of platelet function under flow is requisite to understanding the complex biological relationships contributing to hemostasis and thrombosis.[1] The function of platelet receptors and the eventual biological outcome are strongly influenced by fluid shear stress generated by the partially laminar flow of blood in the circulation.[2,3] Common *in vitro* methods used in research laboratories to study platelet biology under conditions of shear flow include light transmission aggregometers, cone and plate viscometers, perfusion chambers and more recently, microfluidic flow (perfusion chambers) cells.

Perfusion devices, such as parallel plate flow chambers (PPFC) and microfluidic devices, allow similar real-time insight into the dynamic process of platelet adhesion and aggregation behavior. However, with traditional PPFC, a large blood volume is required and the experimental throughput is especially low (1-2 conditions per hour). This precludes certain experiment types such as murine studies and studies from a single donor that must be performed very quickly after blood collection. The low throughput also prevents the use of a standard parallel-plate flow chamber for population studies.

The BioFlux system (Figure 1) is a microfluidic device and control instrument which is ideally suited for platelet adhesion and aggregation assays. Compared to conventional *in vitro* approaches to studying platelets, BioFlux offers higher throughput, enhanced real-time microscopy data, reduced sample volume requirements, and easier setup procedures.

BioFlux is quickly becoming the gold standard for timelapse platelet studies under defined shear stress. It provides real-time, high-resolution microscopy data in a low-volume microfluidic format.

- Very low sample volume required, enables murine, pediatric and single draw same donor studies over several parameters (Table 1)
- Rapid assembly enables maximum throughput, up to 144 conditions per hour
- Easy to use, no device assembly or preparation required
- Flexible format allows use of extracellular matrix, purified proteins, or cellular monolayers as binding substrates
- *In vitro* models can include human endothelial cells and human blood donors
- Up to 24 parallel conditions can be assessed with a single blood draw
- Covers a wide shear range to emulate physiological and pathological conditions
- Allows use of whole blood or purified platelets
- Enables tunable pulsatile flow

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

Shear dyn/cm2	Shear Rate s^{-1}@4cP	BioFlux µl/5min	PPFC* µl/5min
1	25	1.9	3,900
10	250	19.4	44,000
20	500	38.8	81,000
100	2500	141.4	480,000
200	5000	282.8	ND

* PPFC dimensions are 254 µm high and 1 cm wide.

Table 1. Average whole blood (human) consumption at 4cP for a typical 5 minute adhesion and aggregation assay in the BioFlux system compared to an average PPFC.

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Representative Applications

The BioFlux system can be used for many platelet biology assays, including:

- Investigation of kinetic platelet adhesion and aggregation behavioron different substrates over many parallel conditions [4]
- Exploration of the differences in thrombus formation from donors with different or mutant genotypes [5]
- Discovery and preclinical compound assessment for both anti-platelet and anti-thrombotic compounds
- Characterization of thrombosis pathways using knockout animals

This application note describes representative platelet function assays performed using the BioFlux system. Platelet adhesion and aggregation on common extracellular matrix platelet ligands was evaluated as well as dose response to anti-platelet compounds. The assays presented here are wholly adaptable to other substrates, compounds, donor blood, and host cell types.

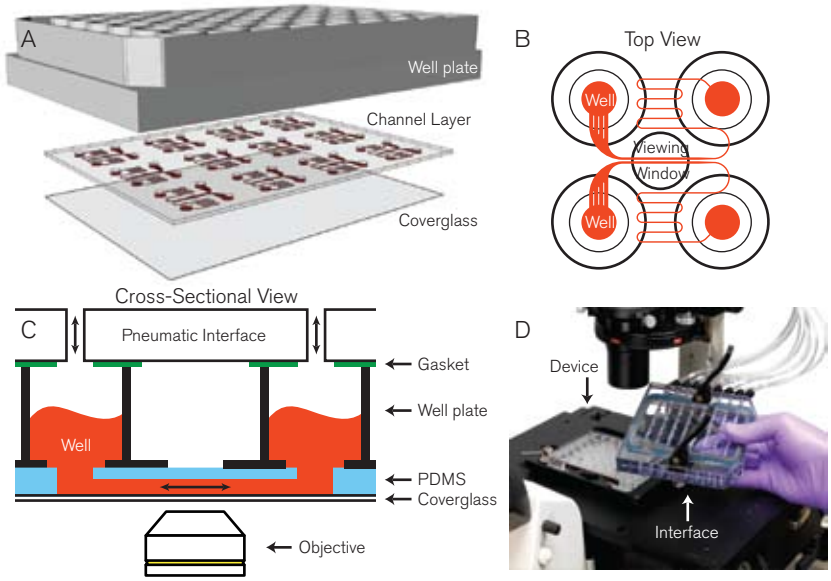


Figure 2: Introduction to the BioFlux system. (A) The BioFlux plate consists of a well plate upper coupled to a polymer-based layer which forms the structure of the channels. The polymer layer is bonded to a #1.5 (180µm) glass cover slip which serves as the bottom of the channel and imaging surface. (B) Schematic of BioFlux channels. Two independent channels are shown. This pattern is repeated to form up to 24 identical channels in one device. (C) An airtight interface is attached to the BioFlux plate to allow tight pneumatic flow control with the BioFlux controller (Figure 1) (D) Data are captured using an inverted microscope (BioFlux 1000 system is shown).

Principles of Operation and Experimental Setup

The BioFlux well-plate microfluidic device (Figure 2) can be used for controlled perfusion studies using the following workflow:

1. Channels are coated with a ligand of interest, for example purified, collagen I.
2. Channels are blocked with BSA containing buffer.
3. Whole blood labeled either non-specifically with a dye such as Calcein AM or specifically with a fluorescently conjugated anti-platelet antibody is added to the prepared channels. Blood treated with anti-platelet compounds can also be used in the channels (Figure 3)
4. The interface is placed on the BioFlux plate and perfusion is triggered using the BioFlux controller software.
5. Timelapse microscopy data are captured. Adhesion and aggregation size, intensity, morphology, and the like are analyzed across multiple fields of view per condition.

In the case of platelet adhesion to a cellular monolayer, cells can be grown *in situ* and the blood is added under perfusion directly to the cells.

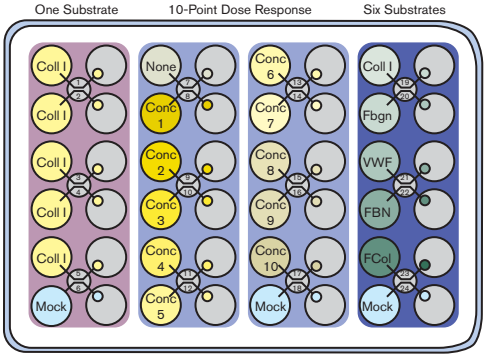


Figure 3: Possible experimental layouts are shown (from left to right) for evaluation of replicate samples on one substrate, a 10-point dose response, and testing of different substrates.

Data Processing for Platelet adhesion and platelet aggregation with BioFlux Montage

BioFlux Montage has a number of automated routines for the analysis of platelet function. For data captured using widefield fluorescence illumination, typical analysis begins with quantitation of platelet percent coverage of the substrate and/or measurement of fluorescent intensity. Both modes of analysis as well as many others are enabled by Montage (Table 2, Figure 4).

Image Name	Time (min)	Average Intensity	Total Intensity	Area Coverage (%)
nd001_t00001	0	153	1343147	0.8
nd001_t00002	0.5	154	1289274	0.7
nd001_t00003	1.0	153.2	1681546	1.0
nd001_00004	1.5	155.1	3339409	1.9
nd001_00005	2.0	156.8	7124616	4.0
nd001_00006	2.5	160.3	11778134	6.5
nd001_00007	3.0	164.1	25088123	13.5
nd001_00008	3.5	170.3	35801640	18.5
nd001_00009	4.0	174.8	44941254	22.7
nd001_00010	4.5	178.4	51519341	25.4
nd001_00011	5.0	180.8	61121133	29.8
nd001_00012	5.5	184.3	86428366	41.3
nd001_00013	6.0	190.6	106005402	49.0
nd001_0014	6.5	198.8	121459769	53.8
nd001_0015	7.0	205.5	134104749	57.5

Table 2. Sample data output from an automated analysis of platelet adhesion using BioFlux Montage. For each image in a time series, data are shown for both overall fluorescence intensity and intensity in the context of surface coverage.

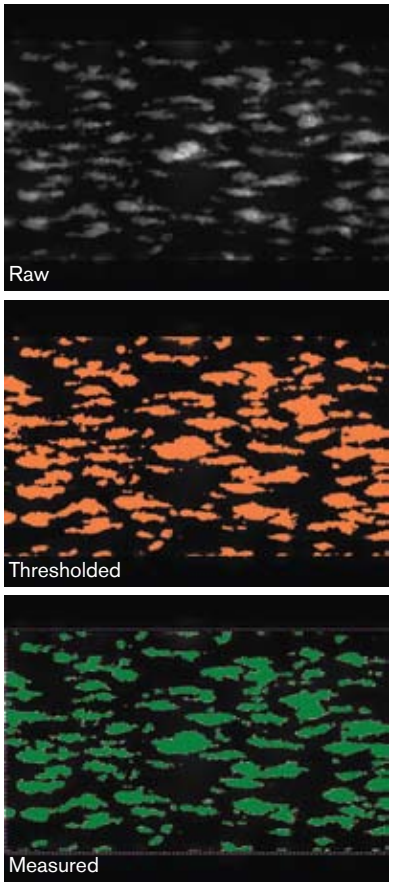


Figure 4: Platelet aggregation on Collagen. Shown (top to bottom) are the raw image, the image thresholded for analysis and the measurement performed by Montage.

Results

1. Platelet adhesion and aggregation at physiological flow on different substrates.

Platelet adhesion, rolling and aggregation were evaluated on three different, physiologically relevant matrices using the low shear device: collagen I, fibronectin (FCN), and vWF, [3] at either 10 or 20 dyn/cm^2. On vWF, we observed massive and uniform platelet attachment and rolling at both shear values. Rolling velocity was tabulated for 10 dyn/cm^2 at 2.95 µm/s. The attachment was reversible; cessation of shear resulted in platelets floating free of the ligand. No aggregation was observed at either shear stress with vWF (Figure 5A). The interaction of platelets with FCN is thought to be important at shear rates above 20dyn/cm^2. Thus as expected, platelets (whole blood) perfused over the FCN substrate formed unstable, partially reversible small aggregates at both shear values with an average aggregate size of 95 µm^2 (Figure 5B). Consistent with the literature, we found the most potent mediator of platelet aggregation was collagen I. [6, 7] Exposure to collagen I mediated rapid and large stable platelet aggregates within 2 to 3 minutes. The average size of aggregates under control conditions was 2000 µm^2 (Figure 5C).

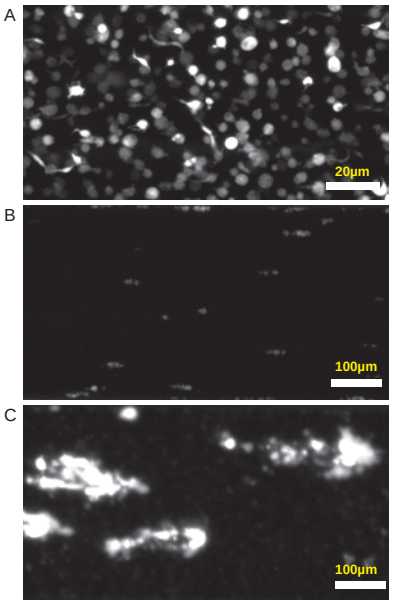
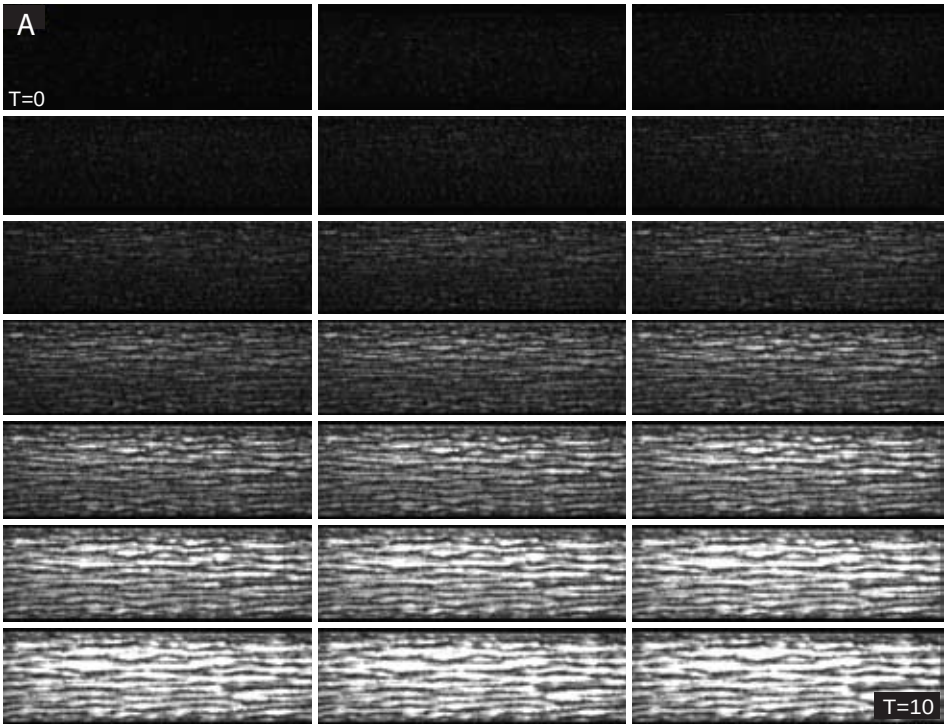


Figure 5: Platelet adhesion and aggregation on different substrates (A) vWF (B) Fibronectin and (C) sheared Collagen I.

Results, continued

2. High shear platelet aggregation on Von Willebrand factor (VWF).

VWF is present both in whole blood and on the surface of endothelial cells in the vasculature. vWF becomes an important ligand for platelet adhesion at very high shear and can initiate thrombus formation under pathological conditions. The 0-200dyn/cm^2 BioFlux plate is used to create conditions of very high shear *in vitro* (Figure 6). Channel geometry for the high shear device is shown in Figure 2B.



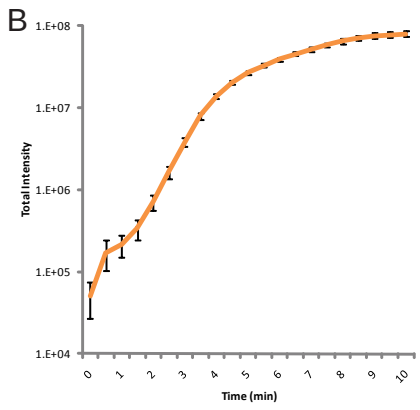


Figure 6: Highly reproducible thrombus formation. Platelet aggregation on vWF at high shear (A) Timelapse of 10 minutes at 125 dyn/cm^2, at 30 sec intervals. The width of the channel is 250 µm, and flow is from left to right. Shown (B) is the intensity (arbitrary units) over for the timelapse, 6 replicate channels. Error bars designate standard deviation for 18 data points, 3 per channel.

Results, continued

3. Many data points from small amounts of blood: Examination of Abciximab dose response on collagen I and VWF.

Abciximab treatment was used as the test condition for the BioFlux devices. Platelet binding to glycoprotein VI and integrin a2b1 initiates intracellular signals which activate the major platelet integrin αIIbβ3 (GPIIb/IIIa) to aggregate platelets by fibrinogen. [8] The drug Abciximab blocks fibrinogen binding to activated αIIbβ3 preventing platelet aggregation. BioFlux devices were used to study the Abciximab dose response at 10 or 200 dyn/cm^2 (250 or 5000s-1), using either fibrillar collagen I (10 dyn/cm^2), collagen I (bovine, sheared) (10 dyn/cm^2), or vWF (200 dyn/cm^2) as adhesion matrices. Dose responses to Abciximab for 3 donors were examined simultaneously from 0-130nM of Abciximab on fibrillar collagen I, followed by a single donor for collagen I (bovine, sheared) and vWF. **Data were collected for 40 independent experimental conditions within 1 hour using the BioFlux 1000 Imaging System.** On fibrillar collagen I, the IC_{50} value for Abciximab was 7.53nM with maximum inhibition at 95% of the control (Figure 7A). On collagen I (bovine, sheared), the IC50 was 7.37nM with a surface coverage maximized at 90% of the control (Figure 7A). Abciximab-mediated inhibition for platelet adhesion on vWF matrix had a measured IC50 of lower than 7nM; complete inhibition (100%) was achieved with vWF (Figure 7B). For all treatments the maximum level inhibition was reached at 30nM (Figure 7). The fundamental difference in inhibition levels between collagen and vWF can be attributed to platelet adhesion to collagen I at a basal level which is not blocked by Abciximab. In contrast, initial platelet adhesion to VWF is blocked by Abciximab. Therefore, inhibition of platelet surface coverage of the chamber surface is likely to be more pronounced with vWF. [3]

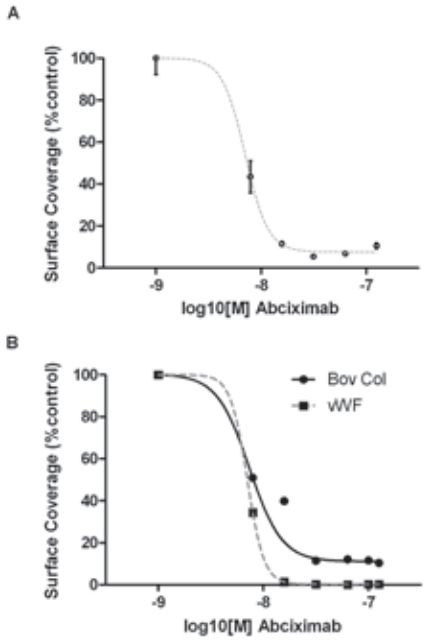


Figure 7: Abciximab dose response using BioFlux. Whole human blood was perfused over coated chambers for 5 minutes at 10dyn/cm^2 (collagen I) or 200dyn/cm^2 (vWF). Micrographs for 3 fields of view per channel were captured for each condition. (A) Each Abciximab concentration was assessed in duplicate for fibrillar collagen for 3 donors (1 draw each). Percent thrombus formation was expressed as platelet surface coverage for treatment over control. Error bars denote standard deviation across the three donors. (B) Inhibition of thrombus formation data generated using BioFlux devices at low and high shear on different coatings using the same donor (1 draw) (●) IC_{50} dose response for Abciximab on collagen I using 0-20dyn/cm^2 microfluidic device at 10 dyn/cm^2 (■) IC_{50} dose response for Abciximab on vWF using a 0-200dyn/cm^2 microfluidic device at 200 dyn/cm^2.

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Summary

- Perfusion chamber parallelization and low reagent use easily enable previously difficult platelet studies.
- Platform flexibility provides both physiological and pathological conditions with one system.
- Kinetic data acquisition delivers the whole picture of platelet behavior.
- Integrated data routines (BioFlux Montage) facilitate analysis of kinetic platelet behavior data.
- Other Relevant applications include:
  - Platelet adhesion to endothelial cells
  - Platelet: leukocyte interactions
  - Cell adhesion (Figure 9)
  - Vascular biology
  - Vascular permeability
  - Endothelial cells under shear

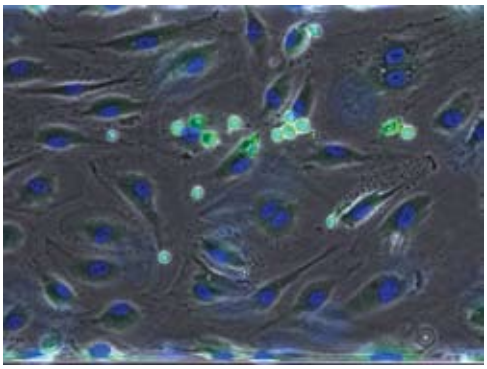

Figure 9: A representative microscopic field following a mononuclear cell adhesion assay on primary human aortic endothelial cells.

References

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