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APPNOTE

Platelet Adhesion and Aggregation Assay

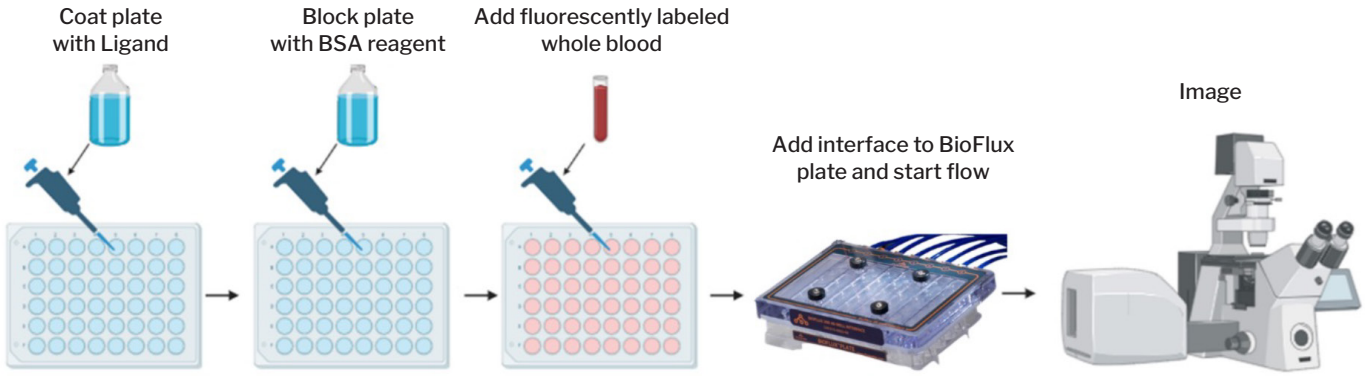
Microfluidic assays to enable multi-parametric investigations of platelet behavior under flow conditions.

In the cardiovascular system, shear stress generated by the blood flow in the circulation impacts platelet receptor expression and function. Therefore, understanding the complex biological relationships that contribute to hemostasis and thrombosis requires accurate physiological analysis of platelet function under shear flow. The BioFlux system is a microfluidic device and control instrument that is ideally suited for platelet adhesion and aggregation studies (Table 1). In contrast to parallel plate flow chambers, the BioFlux system does not need to be modified to accommodate pathological shear stress, has much smaller sample requirements, and has higher throughput capabilities. This application note describes platelet function assays performed using the BioFlux system. This note details the evaluation of 1) Platelet adhesion and aggregation on common extracellular matrix platelet ligands and 2) Platelet dose response to anti-platelet compounds. The assays presented here are easily adaptable to other substrates, drugs, compounds, donor blood, and host cell types.

Key Highlights:

1) Adherence to multiple substrates in the same experiment
2) High-throughput pharmacological screening
3) Easily replicate in vivo platelet/ vascular interactions

BioFlux platelet workflow



Questions this App Note Answers

1. How can the physiological relevance and throughput of platelet assays be increased?
2. What is the protocol for testing platelet adhesion and aggregation?
3. How can platelet biology be imaged in real-time?

Experimental design

Basic Experimental Setup

1) Coat channels with either collagen I, fibronectin (FCN), or von Willebrand factor (vWF) for 1 hour at room temperature (Figure 1).
2) Add 1X PBS (with Ca^{2+} and Mg^{2+}) with 0.5% BSA to block.
3) Flow at 5 dyn/cm^2 for 15 minutes.
4) Add whole blood, either treated with an anti-platelet compound or left untreated.
5) Attach the interface to the BioFlux plate and begin perfusion.

Note: 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.

Following setup, timelapse images were captured using a Zeiss inverted microscope. Montage software was used to analyze platelet adhesion, rolling, and aggregation from microscopy data. The high shear (0-200 dyn/cm^2) BioFlux plate was used to create conditions of very high shear in vitro. Briefly, a timelapse of 10 minutes at 125 dyn/cm^2 was recorded at 30-second intervals (Figure 2). Finally, Abciximab, a glycoprotein IIb/IIIa antagonist that is used to prevent platelet aggregation, was used as a test condition. A BioFlux 1000Z system was used to study the Abciximab dose response at 10 or 200 dyn/cm^2 (250-5000s^{-1}), using either fibrillar collagen (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 0-130 nM 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 using the BioFlux 1000Z.

Shear (dyn/cm^2)	Shear Rate (s^{-1} @ 4cP)	BioFlux (µL/5 min)	PPFC* (µL/5 min)
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	Not determined

*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.

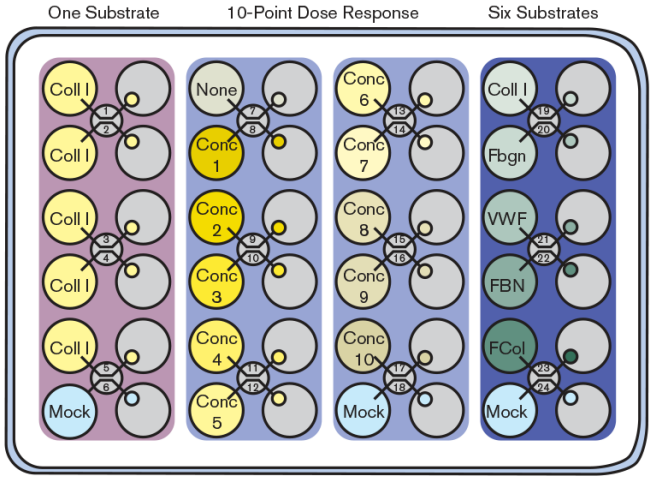


Figure 1. Possible experimental layouts are shown (from left to right) for evaluation of replicate samples on one substrate, a 10-point dose-response, and testing of different substrates.

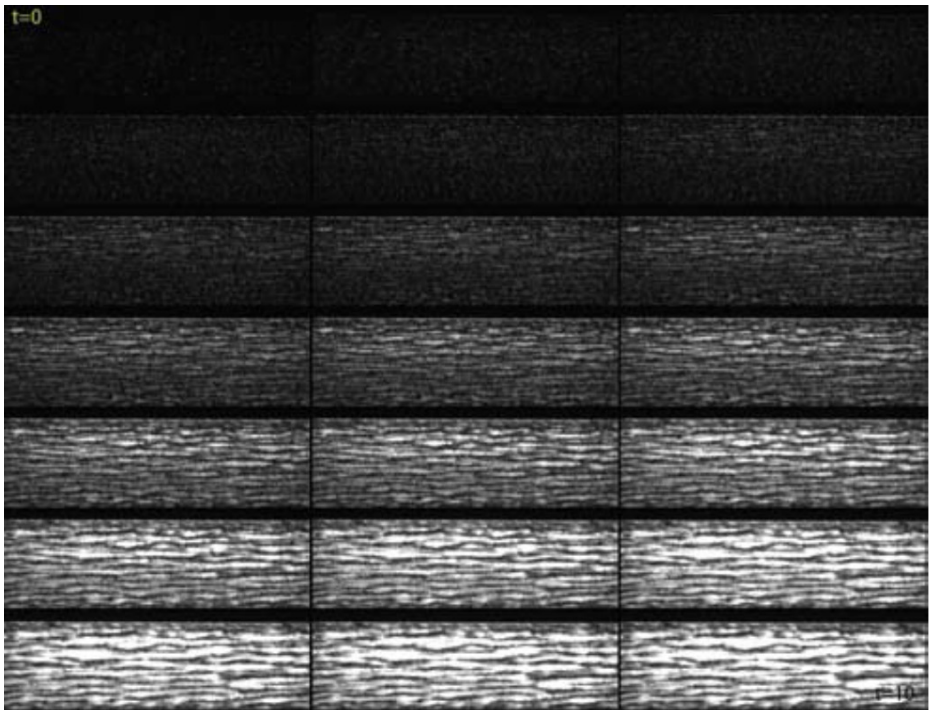
Results

The BioFlux Montage software has multiple 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 3).

Time (min)	Average Intensity	Total Intensity	Area Coverage (%)
0	153	1343147	0.8
0.5	154	1289274	0.7
1.0	153.2	1681546	1.0
1.5	155.1	3339409	1.9
2.0	156.8	7124616	4.0
2.5	160.3	11778134	6.5
3.0	164.1	25088123	13.5
3.5	170.3	35801640	18.5
4.0	174.8	44941254	22.7
4.5	178.4	51519341	25.4
5.0	180.8	61121133	29.8
5.5	184.3	86428366	41.3
6.0	190.6	106005402	49.0
6.5	198.8	121459769	53.8
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 in the context of surface coverage.

A



B

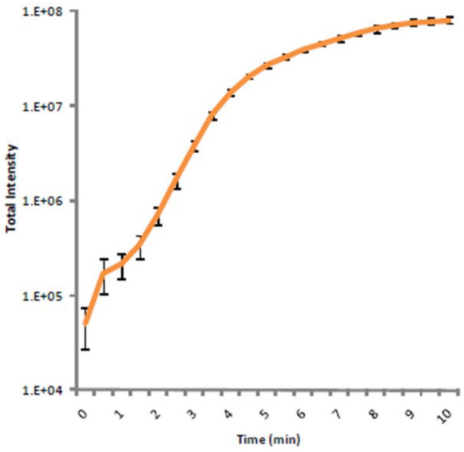


Figure 2. Highly reproducible thrombus formation. Platelet aggregation on vWF at high shear. (A) 30-second interval timelapse of 10 minutes at 125 dyn/cm^2. The width of the channel is 250 µm and flow is from left to right. Graph shown (B) is the intensity (arbitrary units) over time, 6 replicate channels.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

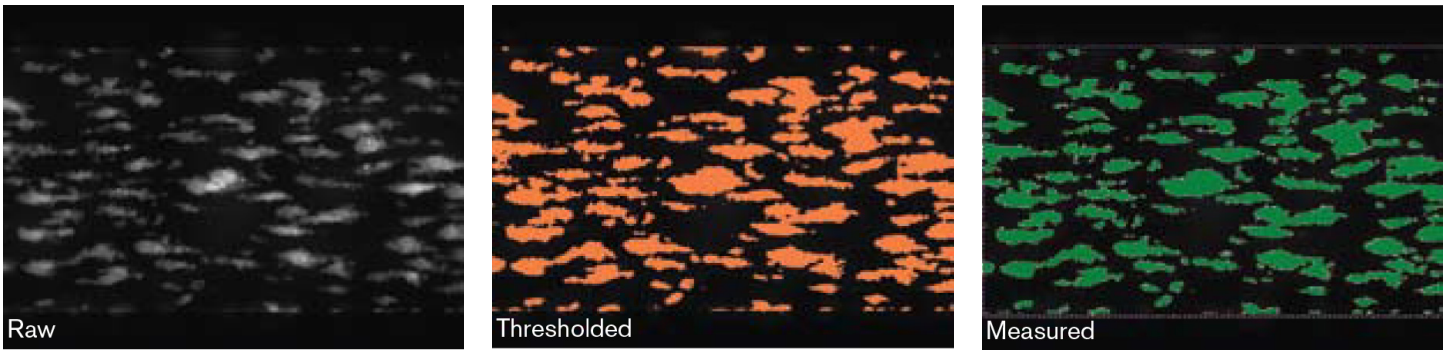


Figure 3. Platelet aggregation on collagen. (Left) Raw image, (Middle) the image thresholded for analysis, and (Right) the measurement performed by Montage.

Platelet adhesion and aggregation at physiological flow on different substrates

Platelet adhesion, rolling, and aggregation were evaluated on three physiologically relevant matrices using a low shear plate: vWF, FCN, or collagen I at either 10 or 20 dyn/cm^2. On vWF, we observed massive and uniform platelet attachment and rolling at both shear values. Average rolling velocity at 10 dyn/cm^2 was measured 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 4A). Consistent with the literature, we found that collagen I was the most potent mediator of platelet aggregation. Exposure to collagen I mediated large stable platelet aggregates within 2 to 3 minutes. As expected, platelets 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 4B). The average size of aggregates under control conditions was 2000 µm^2 (Figure 4C).

High shear platelet aggregation on Von Willebrand factor

vWF is present in both whole blood and on the surface of endothelial cells. vWF becomes an important ligand for platelet adhesion at very high shear and can initiate thrombus formation under pathological conditions. The high shear (0-200 dyn/cm^2) BioFlux plate was used to induce thrombus formation and time versus intensity for 6 replicate channels was calculated (Figure 2B).

Examination of Abciximab dose-response on collagen I and vWF

Platelet binding to glycoprotein VI and integrin a2b1 initiates platelet aggregation by fibrinogen. On fibrillar collagen I, the IC_{50} value for Abciximab was 7.53 nM with maximum inhibition at 95% of the control (Figure 5A). On collagen I (bovine, sheared), the IC_{50} was 7.37 nM with a surface coverage maximized at 90% of the control (Figure 5B). Abciximab-mediated inhibition of platelet adhesion on vWF matrix had a measured IC_{50} of lower than 7 nM. Complete inhibition was achieved with vWF treatment alone. For all treatments, maximum inhibition was achieved at 30 nM (Figure 5B).

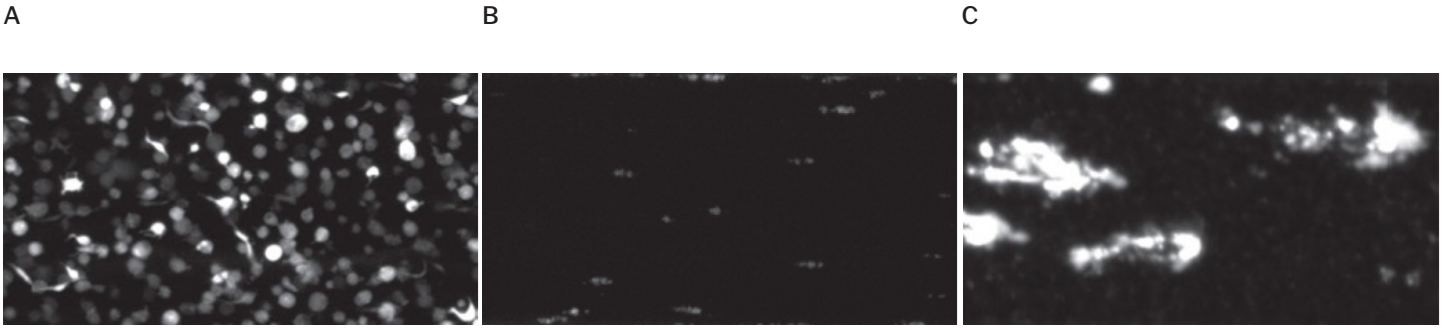


Figure 4. Platelet adhesion and aggregation on various substrates. (A) vWF, (B) fibronectin, (C) sheared collagen I.

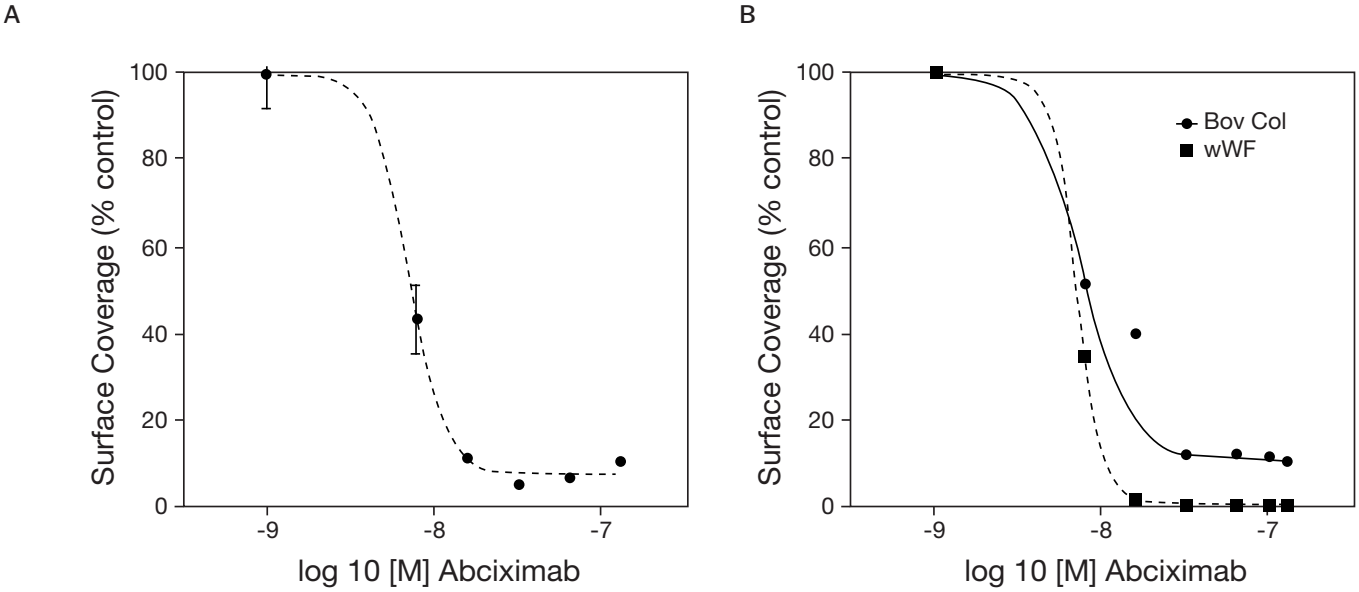


Figure 5. Abciximab dose response using BioFlux. Whole human blood was perfused over coated chambers for 5 minutes at 10 dyn/cm^2 (collagen I) or 200 dyn/cm^2 (vWF). Micrographs for 3 fields of view per channel were captured for each condition. (A) Each Abciximab concentration was assessed in duplicate 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 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 low shear (0-20 dyn/cm^2) plate at 10 dyn/cm^2 (■) IC_{50} dose response for Abciximab on vWF using a high shear (0-200 dyn/cm^2) plate at 200 dyn/cm^2.

Discussion

Using the BioFlux cellular analysis system, we have demonstrated the in vitro assessment of platelet adhesion, rolling, and aggregation under *in vivo* like shear flow conditions. In addition, we have demonstrated how the plate-based BioFlux system can be leveraged for high-throughput pharmaceutical screening.

The BioFlux system was able to accurately obtain aggregate size for various substrates at multiple flow rates, including pathological shear. 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.

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.

For more information on the presented data or BioFlux Technology, visit cellmicrosystems.com

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