Introduction

The BioFlux system (Figure 1) offers a physiologically-relevant platform for running vascular biology assays such as platelet adhesion and thrombosis. These assays often require the use of whole blood or its components to maximize biological relevance. Fluxion’s Well Plate Microfluidic™ technology makes it easy to run whole blood assays because all of the fluidic reagents are neatly contained in the well plate rather than being distributed through a messy network of tubing (Figure 2). The microfluidic format uses substantially less blood (up to 100 times less) than conventional flow cells. This enables more experimental conditions using the same human donor or mouse model.

This protocol is for running platelet adhesion and aggregation assays on various extracellular matrix proteins at physiological shear flow rates.

Caution: Any time that whole blood is used as a reagent, there are basic safety steps that should always be followed. Please refer to your institutional lab safety manuals and training to take the necessary precautions. Also refer to Technical Note 1042, “Whole Blood Assays” for additional procedures.

Required Materials

- Fresh Whole blood, sodium citrate anticoagulant
- PBS plus calcium/magnesium
- 0.5% BSA in PBS
- Collagen I, human fibronectin (Invitrogen), vWF (HTI, HCVWF-0190)
- Calcein AM [ex492/em513nm] (Invitrogen, 4mM stock in DMSO)
- BioFlux plates - 48-well 0-20dyne/cm² plate (can be adapted for 24-well configurations)

Physiological Shear Flow

In normal human physiology, shear flow ranges from 2-10 dyne/cm² (50-350 s⁻¹) in the veins and large arteries. The BioFlux 0-20dyne/cm² plates are suitable for experiments in this physiological range. Shear flow is most often reported as shear stress $\tau$ (units: dyne/cm²) or shear rate $\gamma$ (units: s⁻¹). These measurements are related by the equation: $\tau = \mu \cdot \gamma$ in which $\mu$ is the viscosity of the blood.

<table>
<thead>
<tr>
<th>Vessel</th>
<th>Diameter (cm)</th>
<th>Wall Shear Rate (s⁻¹)</th>
<th>Wall Shear Stress (dyn/cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ascending aorta</td>
<td>2.3 - 4.5</td>
<td>50 - 300</td>
<td>2 - 10</td>
</tr>
<tr>
<td>Femoral artery</td>
<td>0.5</td>
<td>350</td>
<td>10</td>
</tr>
<tr>
<td>Large veins</td>
<td>0.5 - 10</td>
<td>200</td>
<td>7</td>
</tr>
<tr>
<td>Inferior vena cava</td>
<td>2.0</td>
<td>50</td>
<td>2</td>
</tr>
</tbody>
</table>

Source: Platelets, 2002, de Groot et al.

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

Figure 2: Whole blood and reagents can be added to BioFlux plates just like standard well plates. Underneath the bottom of the well plate is a series of microfluidic channels where the experiments take place (see below).

Figure 3: Representative image of a whole blood assay run in two BioFlux plate channels (top and bottom). Whole blood was labeled with calcein AM and run across a vWF coating at 125 dyne/cm² shear stress (3125 1/s).
Platelet Adhesion and Aggregation
Assay protocol for physiological shear ranges

Protocol Steps

1. Use the appropriate Biosafety level for your fluid of interest.

2. Whole blood or washed platelets can be labeled with Calcein AM at a final concentration of 4µM for 1 hour at room temperature prior to the experiment. This step can be done simultaneously with any treatment to be applied to the whole blood – such as applying a blocking antibody.

3. While the blood is incubating with the Calcein AM, you may prime and coat the channels to be used in the experiment. Recommended concentrations for common coatings are as follows: collagen I at 200µg/ml, vWF at 100µg/ml, and fibronectin at 50µg/ml. Concentrations may need to be modified to suit individual application requirements. Follow the manufacturer’s directions for dilution of collagen and fibronectin.

   Coat the channels from the outlet wells for 1 hour at room temperature. Include buffer alone for a ‘no coating’ control.

   - Add 100 µl diluted coating to the outlet well and perfuse at 2 dyn/cm² until you see a bead of liquid in the inlet well. Continue perfusion until inlet inner punch is filled with coating (Figure 4).

   - Stop flow. Incubate for 1 hour at room temp.

4. Perfuse the channels with PBS at 5dyn/cm² from the outlet to wash off excess coating and to neutralize the diluent. Block the coating with 0.5% BSA in PBS for 10 minutes by perfusion at 5 dyn/cm².

5. Add 1 ml prepared blood to the outlet well of the channel. Working quickly, place plate on the microscope.

   Warning!!! Before beginning this experiment, please be aware that when operating at high shear, fluid in the wells will be depleted rapidly. It is best to set an audible alarm/timer for 10 minutes after you begin flow to ensure that you do not run the channel dry.

6. Begin perfusion from outlet side of the channel. If thrombus formation is desired, it is sufficient to perfuse at 10dyn/cm². Thrombi will begin forming on collagen I immediately. Coordinate image capture accordingly using fluorescence microscopy in the FITC range (see microscopy notes in following section). It is best to begin timelapse capture on a region of interest immediately as you begin flow and continue capturing until the desired outcome is achieved.

7. Alternatively, you can set an end time for the experiment and capture data after flow is halted or slowed – collagen I induced thrombi are stable under these conditions. You may wash with PBS after thrombus formation if brightfield imaging is desired.
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Image Acquisition and Sample Results

Image acquisition should be performed using a suitable inverted fluorescence microscope or BioFlux 1000 workstation. Care should be taken to ensure that the correct filters are being used for Calcein AM (excitation:492nm, emission 513nm) or other fluorophores being used. The sample should be shielded from ambient light as best as possible using a darkroom or an opaque cover on the sample.

The number of images acquired in each experimental channel can be determined based on the desired level of statistical sampling and natural variation of the assay. A typical sampling plan for this application would be to use a 4X objective and sample 3 fields of view in each channel. Use of the 4X objective can reduce background fluorescence of non-adherent platelets. An automated stage, such as the one included with the BioFlux 1000 system, can facilitate the automatic acquisition of these fields of view when many channels are being run in parallel.

Figure 5 shows representative results of platelet assays using the BioFlux system. Data should be used for reference only. Actual results may vary based on blood source (i.e. donor variability, hematocrit, etc.) and experimental conditions.

Image Analysis

Images can be analyzed in BioFlux Montage software or other similar image analysis program. Typical analysis endpoints will use the microscopy images to characterize the extent of platelet adhesion and aggregation under varying conditions such as inhibitor compounds, shear stress and coatings. When using BioFlux Montage, the automated modules for “Percent Adhesion” and “Fluorescence Adhesion” and “Intensity” are often used in platelet assays and can quickly generate data for large image sets.

As a general primer and for other software packages, a representative image analysis protocol is provided here:

1. Open the image(s) to be analyzed.

2. Threshold the image to isolate the fluorescently-labeled platelets (light objects) aggregated on the ligand (Figure 6). This step utilizes the difference in pixel contrast between the labeled cells and background. Thus, it is important to maximize the signal (i.e. selecting the right fluorophore, aligning the fluorescent lamp, using the proper objectives) and minimize the background (i.e. blocking out ambient light, avoiding autofluorescent media).

3. Calculate thresholded area in the software for each image in the data set. Express each area as a percentage of the control group. For example, a compound screening assay might report percent adhesion area normalized to a no-antibody control.

4. Data is generated in tabular format and can be plotted in Microsoft Excel, GraphPad Prism, or other suitable software package.