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Calcium test: preparations, imaging

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Abstract

This protocol describes a method for recording the calcium response of single platelets in surface-immobilized platelets with a calcium-sensitive dye in a plane-parallel flow chamber using TIRF microscopy.

This includes: preparation of two types of adhesive coatings in flow chambers, collection and preparation of whole blood for the experiment, design and execution of the experiment.

Note: silanization of coverslips and assembly of flow chambers are described in a separate protocol.

Materials

1. Human thrombin (Hematologic Technologies, Essex Junction, VT, USA);
2. Non-fibrillar human collagen type I (IMTEK, Moscow, Russia);
3. CalBryte-590 AM (AAT Bioquest, Sunnyvale, CA, USA);
4. VM64 antibodies against PECAM-1/CD31 (RRID:AB_782149) was a kind gift from Prof. A. V. Mazurov [3].

All other reagents were from Sigma-Aldrich (San Diego, CA, USA) unless otherwise indicated.

5. ADP
6. Pluronic F-127

Before start

A typical experiment consists of two runs with different flow chambers: one with a collagen coating and one with anti-CD31 coating. The steps for each chamber are identical.

Experiments were performed with whole blood of healthy donors and patients. Whole blood was collected into 1.6 ml hirudin-containing tubes (S-Monovette®, SARSTEDT AG & Co. KG, Nümbrecht, Germany). Samples were stored in a water bath at 37°C until the start of the experiment.

This protocol involves two buffer solutions:

1. Tyrode's buffer (as in [1])
137 mM NaCl, 2.7 mM KCl, 12 mM NaHCO3, 0.36 mM NaH2PO4, 1 mM MgCl2, 2mM CaCl2, 5 mM HEPES (pH 7.5), 0.36% BSA, 1 g/l D-glucose, pH 7.35

2. BRB-80 buffer (as in [2])
80 mM PIPES/KOH, pH 6.9, 1 mM EGTA, 1 mM MgCl2

Preparation of collagen coating

46m 30s

1 Collagen type I coating simulates physiological conditions near the site of blood vessel damage. 15m

Prepare the following solutions and items:

1. 100 µl of 1 mg/ml human collagen type I diluted in 0.01M acetic acid (A)
2. 100 µl of 5% BSA solution in Tyrode's buffer (B)
3. 100 µl of Tyrode's buffer (C)
4. 1 ml syringe
5. Empty flow chamber

2 Connect the syringe to the output tube of the flow chamber and place the input tube into the tube with solution A. 30s

3 Drive in the solution by pulling the syringe plug and wait for 15 minutes. Always leave a small amount of solution in the tube to prevent air from entering the flow chamber. 15m

4 Replace solution A tube with solution B tube. Drive in the solution and wait for 15 minutes. 15m

5 Replace solution B tube with solution C tube. Drive the solution in. Now the collagen-coated flow chamber is ready for use. 1m

Preparation of antibody coating

39m 30s

6 Anti-CD31 (VM64) coating simulates conditions for low-activated platelets in the periphery of the thrombus. 15m

Prepare the following solutions and items:

1. 3 × 100 µl BRB-80 buffer (W1, W2, W3)
2. 100 µl of 40 µg/ml of anti-CD31 (VM64) dissolved in BRB-80 (A)
3. 100 µl of 1% Pluronic F-127 dissolved in BRB-80 (P)
4. 100 µl of Tyrode's buffer (T)
5. 1 ml syringe
6. Empty flow chamber

7 Connect the syringe to the output tube of the flow chamber and place the input tube into the tube with 100 µl of solution W1. Drive in the W1 solution. 30s

8 Replace solution W1 tube with solution A tube. Drive in the solution and wait for 15 minutes. 15m

9 Replace solution A tube with solution W2 tube. Drive in the solution. 30s

10 Replace solution W2 tube with solution P tube. Drive the solution in and wait for 7 minutes. 7m

11 Replace solution P tube with solution W3 tube. Drive in the solution. 30s

12 Replace the solution W3 tube with the solution T tube. Drive in the solution. Now the VM64-coated flow chamber is ready for use. 1m

Platelet fluorescent labeling 30m

13 Load 300 µl of whole hirudinated blood with 2 µM of CalBryte-590 AM and 0.1 u/ml of apyrase, incubate in a water bath set at 37°C for 30 minutes. Use immediately. 30m

Materials and equipment for imaging

14
1. Whole blood loaded with CalBryte-590 (as per previous step)
2. Flow chambers loaded with VM64 and collagen type I
3. Syringe pump SPLab1, Shenchen
4. 1 ml syringe with a needle that fits tightly into a silicone tube with an inner diameter of 0.5 mm;
5. A fluorescence microscope equipped with a video camera (*)
6. Three 200 µl portions of Tyrode's buffer
7. 200 µl of 10 µM ADP solution in Tyrode's buffer
8. 200 µl of 10 µM ADP and 5 nM thrombin solution in Tyrode's buffer.

(*) we used Nikon Eclipse Ti-E with CFI Apochromat TIRF 100XC Oil objective, Nikon LU-N4 laser excitation source, Nikon TI-TIRF-E TIRF module, Andor DU-897 digital EMCCD camera. To record the position and shape of platelets, the DIC mode was used, and to record the fluorescent signal, low-angle TIRF mode with an angle of 48 degrees was

used. The power of the 561 nm laser was 5% of the maximum. Frames were collected at 12.25 FPS with 80 ms exposure and no delay.

Flow chamber imaging

1h 5m

15 Place the first flow chamber on the microscope table and adjust the microscope settings. 7m

16 Put one tube of a flow chamber into the tube containing blood loaded with a fluorscent due and attach the other to the syringe pump. Use an extension tube when necessary. Set the pump at 33 µl/min rate. 3m

In most cases, such a rate is enough to wash away unbound cells in several minutes.

17 Wash in nearly 150 µl of whole blood loaded with fluorophore. Observe how platelets are retained and adhere to the coating within the field of view. Wait until approximately 10-15 single cells adhere. 5m

Note: If cells do not adhere well, reducing the speed to 7-15 µl/min may help.

18 Wash in 150 µl of Tyrode's buffer, then replace the tube and rinse the chamber with fresh 150 µl of buffer to remove the free-flowing platelets. If the chamber is not sufficiently clear of unattached cells, wash in another 150µl. Set the pump at 33 µl/min rate until the end of the experiment. 5m

Note: the tube needs to be replaced because of blood remains in the first one.

19 Select a field of view with 10-30 platelets that do not form thrombi or overlap with each other. Take a picture of this field in DIC mode, replace the tube with buffer. Start a recording a 5-minute fluorescence video simultaneously with starting the pump. The buffer must be pumped through the chamber during the entire recording period. 5m

This step allows to measure the level of platelet activation without adding an activator, the base activation level.

20 Change the field of view and take a picture in DIC mode. Replace the tube for 200 µl of 10 µM ADP solution in Tyrode's buffer, record fluorescent signal for 5 minutes while pump is working. The activator usually arrives in the middle of the chamber in 30-50 seconds after the pump is turned on. 5m

21 Change the field of view and take a picture in DIC mode. Replace the tube for 200 µl of 10 µM ADP and 5 nM thrombin solution in Tyrode's buffer, record fluorescent signal for 5 minutes while pump is working. 5m

22 Repeat all steps for the second flow chamber. 30m

Protocol references

1) Ignatova, A. A., Suntsova, E. V., Pshonkin, A. V., Martyanov, A. A., Ponomarenko, E. A., Polokhov, D. M., ... & Panteleev, M. A. (2021). Platelet function and bleeding at different phases of childhood immune thrombocytopenia. Scientific Reports, 11(1), 9401. doi: 10.1038/s41598-021-88900-6

2) Gudimchuk, N., Tarasovetc, E. V., Mustyatsa, V., Drobyshev, A. L., Vitre, B., Cleveland, D. W., ... & Grishchuk, E. L. (2018). Probing mitotic CENP-E kinesin with the tethered cargo motion assay and laser tweezers. Biophysical journal, 114(11), 2640-2652. doi: 10.1016/j.bpj.2018.04.017

3) Mazurov AV, Vinogradov DV, Kabaeva NV, Antonova GN, Romanov YA, Vlasik TN, Antonov AS, Smirnov VN (1991) A Monoclonal Antibody, VM64, Reacts with a 130 kDa Glycoprotein Common to Platelets and Endothelial Cells: Heterogeneity in Antibody Binding to Human Aortic Endothelial Cells. Thromb Haemost 66:494–499. doi:10.1055/S-0038-1646445.