Video Article

Purification of Platelets from Mouse Blood

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Abstract

Platelets are purified from whole blood to study their functional properties, which should be free from red blood cells (RBC), white blood cells (WBC), and plasma proteins. We describe here purification of platelets from mouse blood using three-fold more iohexol gradient medium relative to blood sample volume and centrifugation in a swinging bucket rotor at 400 x *g* for 20 min at 20 °C. The recovery/yield of the purified platelets were 18.2-38.5%, and the purified platelets were in a resting state, which did not contain any significant number of RBC and WBC. The purified platelets treated with thrombin showed up to 93% activation, indicating their viability. We confirmed that the purified platelets are sufficiently pure using flow cytometric and microscopic evaluation. These platelets can be used for gene expression, activation, granule release, aggregation, and adhesion assays. This method can be used for purification of platelets from the blood of other species as well.

Video Link

The video component of this article can be found at https://www.jove.com/video/59803/

Introduction

Platelets are a component of blood that functions as an initiator of blood clotting in response to damage in the blood vessel. They gather at the site of injury to plug the vessel wall[1]. Platelets are anucleate fragments of cytoplasm derived from the megakaryocytes of the bone marrow under the influence of thrombopoietin and enter the circulation[2]. They are considered as metabolically active and capable of sensing extracellular environment by activating intracellular signaling cascades that result in platelet spreading, aggregation, and hemostatic plug formation[1,3]. Besides hemostasis/thrombosis and wound healing[4], platelets play an important role in host inflammatory responses, angiogenesis, and metastatis[3,5,6,7,8].

Platelets are purified from blood to study their biochemical and physiological properties, which should be free from other blood components. Since red blood cells (RBC) and white blood cells (WBC) contain significantly more RNA and proteins than platelets[9,10], the presence of even a small number of these cells can interfere with transcriptomic and proteomic analyses of RNA and proteins derived from platelets. We found that purified platelets activated with thrombin bind antibody such as anti-GPIIb/IIIa (JON/A) and anti-P-selectin more efficiently than whole blood platelets.

Since platelets are fragile, it is important to treat the samples as mildly as possible. If the platelets are activated, they release their granule contents and ultimately degrade. Therefore, to keep the platelets' functional properties intact, it is important to maintain platelet quiescence during isolation. Several protocols have described isolation of platelets from human, dog, rat, and non-human primate by various methods[1,10,11,12]. Some of the methods require multiple steps such as collection of platelet-rich plasma by centrifugation, filtration by separation column, negative selection of platelets with RBC- and WBC-specific antibody conjugated to magnetic beads, and so on, which are time-consuming and may degrade platelets and their contents.

Ford and his colleagues described platelet purification from human blood using iohexol medium[11]. This method uses a similar volume of blood sample and medium during purification. Since humans yield a higher volume of blood, it is relatively easy to purify the platelets.

Iohexol is a universal density gradient inert medium that is freely soluble in water and used in the fractionation of nucleic acids, proteins, polysaccharides, and nucleoproteins[13,14]. It has low osmolality and is non-toxic, thus making it an ideal medium for purification of intact living cells[11]. It is a non-particulate medium; therefore, the distribution of cells in a gradient can be determined using hemocytometer, flow cytometer, or spectrophotometer. It does not interfere with most of the enzymatic or chemical reaction of the cells or cellular fragments after dilution.

The mouse serves as an important animal model for many human diseases[15,16,17,18]. There are a few published articles that describe purification of mouse platelets[19,20]. However, the mouse yields a relatively smaller volume of blood, which makes it difficult to purify platelets. If the same small volume of gradient medium and blood samples is used, the platelet layer cannot be clearly separated from RBC-WBC layer after centrifugation. In this article, we have described a quick and simple method of mouse platelet purification with three-fold more iohexol gradient

medium relative to the blood sample volume and low speed centrifugation. We have also activated the purified platelets with thrombin and investigated their quality with flow cytometry and microscopy.

Protocol

Mouse blood collection should be conducted with appropriate institutional animal care and use committee approval.

NOTE: The platelet purification protocol is described in a flow diagram in **Figure 1**.

1. Collection of blood

1. Add 25 µL of 3.2% sodium citrate (pH 7.2) as an anti-coagulant and 0.4 mM of Gly-Pro-Arg-Pro (GPRP) in a polypropylene tube.
2. Collect approximately 200 µL of blood from the C57BL/6 mouse using retro-orbital bleeding.
   NOTE: Blood can be collected from any type of mouse strain regardless of age or gender.
   1. Use 2 mL of isoflurane in any type of chamber to anesthetize the mouse.
      NOTE: Depth of anesthesia is checked by signs of increased respiration and decreased movement. The mouse should not respond to movement of the anesthesia chamber, or to being handled. If the mouse is responsive to movement and handling, it requires more time to keep in the anesthesia chamber.
   2. 1.2.2 Open either right or left eyelid of the mouse and insert a 7.5 cm (0.12 cm diameter) capillary tube into the vascular plexus of the eye.
   3. Twist the capillary tube one half turn while applying pressure to the capillary tube to break the vessels and initiate blood flow. Hold the animal upside down over the collection vial to be filled by capillary action.
   4. Once the appropriate volume of blood is collected, remove the capillary tube and close the eye by applying mild pressure for 30 s on the eyelid with gauze. Once bleeding is stopped, return the mouse to its cage and monitor for bleeding.
   5. Check eyes for trauma 1-2 days after retro-orbital bleeding. Remove any mouse from the study showing signs of significant trauma to the eye as a result of the procedure and euthanize.
      NOTE: The mouse should not undergo any kind of treatment which can inhibit platelets for at least two weeks before blood collection. The blood sample must be used for platelet purification within one hour of bleeding the mouse.

2. Platelet purification

NOTE: Platelet purification should be carried out at room temperature to prevent their degradation. Make sure that temperature of the reagents and instruments are between 18 to 22 °C. To prevent platelet degradation, fast pipetting and vigorous shaking should be avoided during the procedure.

1. Add 600 µL of iohexol gradient medium (12% iohexol powder in 0.85% sodium chloride, 5 mM Tricine, pH 7.2)[11] into an 1.5 mL tube.
2. Add 200 µL of blood sample slowly down the side of the tube on top of the gradient medium with a wide bore pipette tip so that blood and iohexol medium do not mix (**Figure 2A**).
3. Centrifuge the sample containing tube at 400 x *g* for 20 min at 20 °C in a swinging bucket rotor with slow acceleration and deceleration.
4. Collect most of the platelet-rich layer and a small fraction of platelet-poor layer (total 300 to 400 µL) using a wide bore pipette tip without disturbing the RBC and WBC layers (**Figure 2B**). Transfer the platelet sample to a new tube.
   NOTE: If the platelet count is less in the blood or a smaller volume of blood is used, the platelet-rich layer and platelet-poor layer can be seen as a single layer.
5. Add 1 mL of PBS to the platelet sample, mix by inverting, and centrifuge at 800 x *g* for 10 min at 20 °C in a swinging bucket rotor. Discard the solution completely and add 200 µL of PBS to resuspend the platelets with mild pipetting.
   NOTE: This step is optional, as it removes the gradient medium and plasma proteins and increases the sensitivity of platelets to agonists such as thrombin. Since different centrifuges have different programs, the slow acceleration/deceleration can be determined by reading the user manual of the centrifuge.
6. Transfer 30 µL of this sample into a new tube to count platelets. The remaining platelets can be used for biochemical and physiological assays such as platelet activation, aggregation, granule release, etc.
7. For RNA or protein extraction, centrifuge the platelet sample at 800 x *g* for 10 min to pellet the platelets. Discard supernatant completely without disturbing the pellet. Add the desired volume of RNA or protein lysis buffer to lyse the platelets.

3. Counting the platelets

1. Count the whole blood cells without dilution and purified platelets (diluted 2 to 5-fold with PBS) using an automated cell analyzer. Use 30 to 50 µL samples for counting.
2. Calculate the total number of platelets by multiplying by the volume of the sample.
3. Calculate the percentage of yield/recovery of purified platelets.
4. Count the RBC and WBC in the purified platelet sample (diluted 50 to 100-fold with PBS) with a hemocytometer and microscope.

4. Platelet activation

1. In a tube, add 1 to 2 million purified platelets in up to 100 µL of staining buffer (PBS with 2% fetal bovine serum, FBS).

2. For multiple number of samples, make a master mix of antibodies with 1 µL (0.5 mg/mL) of the following: PE-Cy7 rat anti-mouse TER 119, PE rat anti-mouse CD45, FITC rat anti-mouse CD41, and APC rat anti-mouse/human CD62P (P-selectin) to detect RBC, WBC, platelets, and activated platelets, respectively. Add the master mix to the tubes for staining the cells. Do not add thrombin.
3. In another tube, add 1 to 2 million of purified platelets in up to 100 µL of staining buffer, and 0.4 mM GPRP peptide. Add thrombin to activate the platelets and stain the cells following step 4.2.
   NOTE: GPRP should be added to the sample before adding thrombin to prevent aggregate or clot formation through platelet activation. Thrombin concentration should be optimized to obtain good activation of platelets. After platelet activation, event counts are decreased during acquisition of samples by flow cytometry.
4. As a positive control, add 1 µL of whole blood in up to 100 µL staining buffer in a tube and 0.4 mM GPRP peptide. Add thrombin to activate platelets. As a negative control, add 1 µL of whole blood in up to 100 µL of staining buffer. Do not add thrombin in negative control sample. Stain the cells following step 4.2.
5. For flow cytometry isotype control, label 5 tubes with unstained, PE-Cy7, PE, FITC, and APC. Add 100 µL of staining buffer, 1 µL of blood, and 1 µL of the respective antibody to the labeled tubes to stain the cells.
6. Incubate samples to stain for 30 min at room temperature in the dark.
7. Wash the samples by adding 1 mL of staining buffer to each tube. Centrifuge at 800 x *g* for 5 min at room temperature. Discard the staining buffer carefully without dislodging the pellet.
8. Add 300 µL of staining buffer to each tube, mix mildly, and transfer to a FACS tube. Store the stained samples in the dark until analyzed with a flow cytometer.

5. Flow cytometry analyses of platelets

1. Create a new experiment and generate log forward scatter vs log side scatter dot plots and assign gates for Ter119 PE-Cy7 (RBC), CD45 PE (WBC), CD41 FITC (platelets), and CD62P APC (activated platelets) populations according to the gating strategy chosen for the experiment in the FACS DIVA software.
   1. Open the population hierarchy by choosing **Show Population Hierarchy** under the **Populations** tab. Edit the population hierarchy by checking the gating strategy and properly renaming the gates.
   2. Open statistics view by choosing **Create Statistics View**. Edit statistics view according to user preferences by right clicking the statistics view and selecting **Edit Statistics View**.
   3. Choose a color for each gate that enhances the visual aspect of the layout by double clicking on the box corresponding to the population.
2. In the **Cytometer** window, click on the **Parameters** tab and adjust the voltages for parameters to visualize the population of interest on the plots.
3. Adjust voltages for each of the parameters so that the negative population can be distinguished from the positive population. Before recording the samples for the compensation control, check that the single-stained positive controls are on scale (ensure that the positive peaks are visible and not too far to the right).
   NOTE: If the positive peaks are off scale, the voltages should be adjusted to see the positive populations.
4. Record the single-color stained cells for compensation controls (unstained, PE-Cy7, PE, FITC, and APC).
5. If the positive control gates are not set up properly, adjust them by changing voltages.
6. Go to **Experiment > Compensation Setup > Calculate Compensation**. Choose **Apply Only** in the resulting window.
7. Switch to the global worksheet by clicking on the toggle button in the top, left of the worksheet window.
8. Load the positive control sample and acquire enough cells to adjust the compensation and the gates.
9. Load the sample again and check that each plot shows the expected cell population based on the fluorochrome stained antibody. Acquire and record 100,000 events for whole blood samples and 10,000 events for purified platelets. Load the samples one by one until acquisition is completed.
10. Analyze the flow cytometry data with the software with appropriate plots and gates (**Figure 3**)

Representative Results

The summary of platelet purification is described in a flow diagram (**Figure 1**). Steps include collection of blood from mouse using retro-orbital bleeding in the presence of an anticoagulant, addition of blood sample onto the iohexol gradient medium, centrifugation in a swinging bucket rotor at 400 x *g* for 20 min at 20 °C. The quality of the purified platelets was evaluated with microscopy and flow cytometry after staining with antibody to detect any contaminating cells and activated platelets.

The iohexol gradient medium and the blood sample formed two separate layers in the tube if the blood was slowly added onto the medium (**Figure 2A**). However, if there is delay in this step, most of the blood sample could diffuse into the medium and it might be difficult to purify the platelets. The wide-bore pipette tips ensured no physical stress to the platelets which is important for maintaining integrity of the platelets and preventing their degradation. During centrifugation, slow acceleration helped prevent inadvertent mixing of the blood sample with iohexol medium and slow deceleration helped maintain the gradient after centrifugation. The top (straw color) layer contained the plasma and did not contain any types of intact blood cells, the second layer (whitish) from the top contained majority of the platelets which was collected using a wide-bore pipette tip, the third layer (transparent) was platelet poor which was partly collected carefully to avoid aspiration of bottom layer (red) RBC and WBC (**Figure 2B**). The platelet-rich layer and platelet poor layer could not be seen separated if the platelet counts were low in the blood sample. The RBC and WBC layer could not be seen as separate layers since blood volume was small. However, WBC forms a white layer, called buffy coat between platelet poor layer and RBC layer if a larger volume of blood is used for purification[10,11]. It is important to carry out the whole procedure at 18 to 22 °C. Both the higher temperature and refrigeration of the samples yielded lower platelet counts due to degradation.

We found 18.2 to 38.5% (27.1 ± 6.5%, n=12) recovery/yield of the purified platelets. To investigate their viability, purified platelet samples were activated with thrombin. Flow cytometric analysis of platelets samples were done after staining with the markers for platelets, activated platelets, RBC, and WBC. The whole blood showed distinct populations of RBC, WBC, and platelets on a logarithmic forward scatter vs side scatter dot

plot (**Figure 3A**), whereas the purified platelets showed a distinct population with negligible numbers of RBC and WBC (**Figure 3B**). The whole blood showed RBC and WBC after staining them with anti-mouse Ter119 and anti-mouse CD45 antibody (**Figure 3C**) whereas the purified platelets showed negligible number of RBC and WBC (**Figure 3D**). We evaluated the purified platelet sample with a microscope and did not see any significant number of intact RBC and WBC. Therefore, the RBC and WBC events which were shown in **Figure 3D** might be the fragments of RBC and WBC containing Ter119 and CD45, respectively. Purified platelets which were not treated with thrombin showed almost no activation indicating their resting state (**Figure 3E**), whereas purified platelets treated with thrombin showed 71% activation (up to 93% activation was observed in some samples) (**Figure 3F**) indicating their viability. We also performed microscopic evaluation of purified platelet samples not treated with thrombin which showed no platelet aggregation (**Figure 4A**), however, purified platelets formed aggregates after being treated with thrombin (**Figure 4B**), which further indicate their viability. Based on flow cytometric and microscopic studies, we confirmed that our purified platelets were good quality.

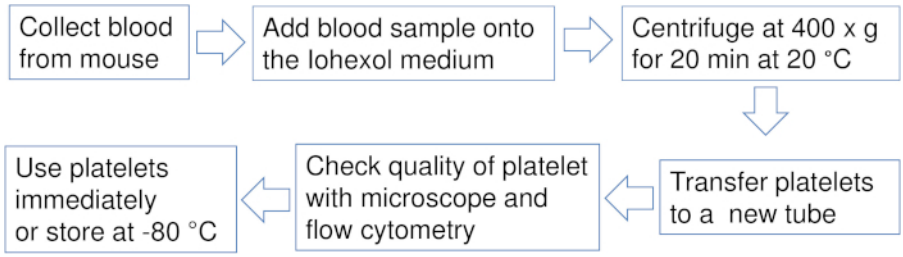

Figure 1. Schematic diagram for mouse platelet purification. Blood sample from mouse is collected in the presence of an anticoagulant. The blood sample is slowly added onto the Iohexol gradient medium and centrifuged at 400 x *g* for 20 min at 20 °C. The platelet layer is transferred to a new tube. The quality of purified platelets is checked with microscopy and flow cytometry. The platelets can be used immediately or stored at −80 °C. Please click here to view a larger version of this figure.

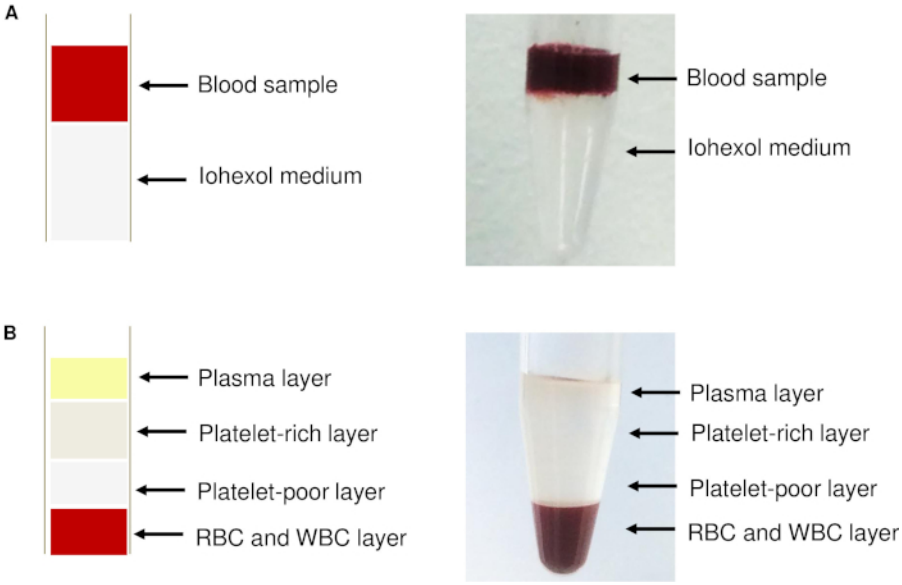

Figure 2. Mouse blood before and after centrifugation with Iohexol gradient medium. (**A**) Iohexol and blood form two separate layers before centrifugation if the blood sample is added carefully. Left panel shows schematic diagram and right panel shows the actual image. (**B**) Four separate layers are seen after centrifugation of whole blood with gradient medium. Left panel shows schematic diagram of the layers and right panel shows the actual image. Please click here to view a larger version of this figure.

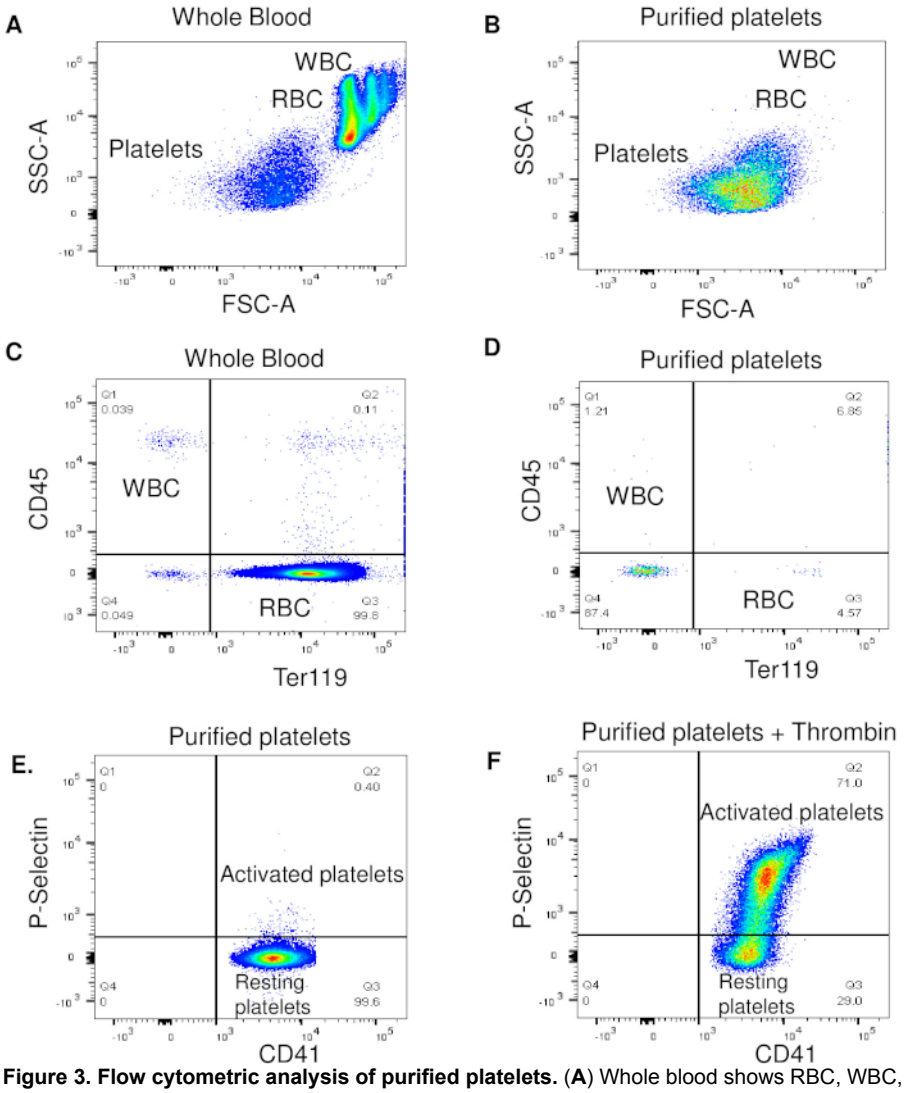

Figure 3. Flow cytometric analysis of purified platelets. (**A**) Whole blood shows RBC, WBC, and platelet populations. (**B**) Purified platelets show minor numbers of RBC and WBC events. (**C**) Whole blood shows RBC and WBC after staining with anti-mouse Ter119 and anti-mouse CD45. (**D**) Purified platelets show negligible number of RBC and WBC events after staining with anti-mouse Ter119 and anti-mouse CD45. (**E**) Purified platelets stained with anti-mouse CD41 and anti-mouse/human P-selectin show almost no platelet activation. (**F**) Purified platelets treated with thrombin and stained with anti-mouse CD41 and anti-mouse/human P-selectin show activation of platelets. Please click here to view a larger version of this figure.

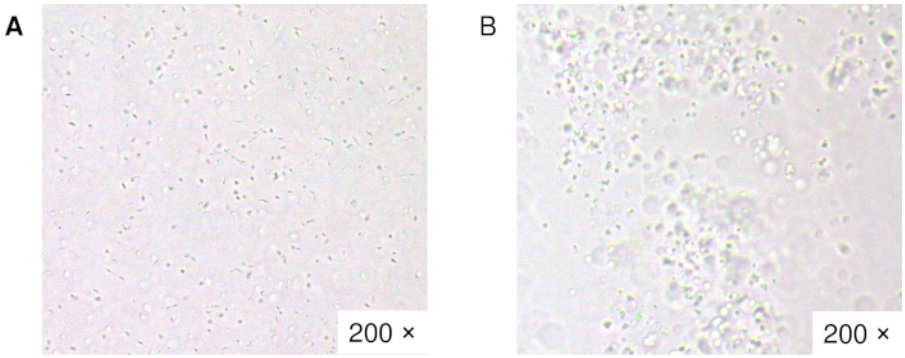

Figure 4. Microscopic evaluation of purified platelets. (**A**) Purified platelets do not show any aggregation. (**B**) Purified platelets treated with thrombin show aggregation. Both figures are 200x magnification, ocular lens 10x and objective lens 20x. Please click here to view a larger version of this figure.

Discussion

Commonly, platelets are isolated by low-speed centrifugation which yields platelet-rich plasma that contains a significant number of blood cells, cellular debris, and plasma proteins which can interfere with the biochemical and physiological assays and needs further purification[21]. Therefore, it is important to use a rapid and simple method which can yield pure platelets without major contaminants. The protocol presented here describes the purification of platelets from mouse blood using a gradient medium with low speed centrifugation. The parameters used in this protocol maximize the yield and minimize the degradation of platelets. The gradient medium and plasma proteins can be removed by including a washing step after platelet collection which can increase the sensitivity of platelets to agonist or antibody. Most importantly, the purified platelets are in resting state, but they can be activated in the presence of an agonist, such as thrombin. We have found that thrombin activity varies from one source to another. Therefore, thrombin concentration should be optimized empirically to obtain good activation of platelets.

Due to a small volume of mouse blood, it is inconvenient to purify platelets because platelets can be mixed with RBC and WBC during aspiration. We have resolved this issue by optimizing the ratio of blood sample and gradient medium. We have found that three-fold more gradient medium relative to the blood volume can clearly separate the platelet layer from the serum and RBC-WBC layers which facilitates easy collection of pure platelets.

Platelet purification should be carried out at room temperature between 18 to 22 °C to prevent their degradation. It has been reported that platelets degrade if stored in the refrigerator or temperatures above 27 °C[11]. Fast pipetting and vigorous shaking should be avoided to prevent platelet degradation during purification. Since different centrifuges have different programs, the acceleration/deceleration should be determined empirically to maintain the gradient.

If several mouse blood samples are used in the purification, the bleeding should be done in less than one hour and purification should be completed within next one hour. If blood samples are left for more than 2 hours, platelets cannot be purified due to their degradation. If more platelets are needed for any study, the mouse blood can be collected by terminal procedures such as cardiac puncture or inferior vena cava bleeding, which yield 800 to 1000 µL blood, and platelet purification should be carried out in multiple tubes and combined after purification.

In conclusion, we have described a simple and rapid protocol for platelet purification from a small volume of mouse blood. This method can also be used for platelet purification from other species as well. The purified platelets can be used for gene expression, activation and granule release, aggregation, and adhesion assays upon stimulation with various agonists.

Disclosures

The authors declare no conflict of interest.

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