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Turbidimetry on human washed platelets: the effect of the Pannexin1 inhibitor Brilliant Blue FCF on collagen-induced aggregation.

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Corresponding Author:	Brenda Renata Kwak, Ph.D. Universite de Geneve Geneva, GE SWITZERLAND
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	Brenda.KwakChanson@unige.ch
Corresponding Author's Institution:	Universite de Geneve
Corresponding Author's Secondary Institution:	
First Author:	Filippo Molica, Ph.D.
First Author Secondary Information:	
Other Authors:	Filippo Molica, Ph.D.
	Severine Nolli
	Pierre Fontana, M.D. Ph. D.
Order of Authors Secondary Information:	
Abstract:	Turbidimetry is a laboratory technique that is applied to measure the aggregation of platelets suspended in either plasma (platelet-rich plasma, PRP) or in buffer (washed platelets), by the use of one or a combination of agonists. The use of washed platelets separated from their plasma environment and in the absence of anticoagulants allows for studying intrinsic platelet properties. Among the large panel of agonists, arachidonic acid (AA), adenosine di-phosphate (ADP), thrombin and collagen are the most frequently used. The aggregation response is quantified by measuring the relative optical density (OD) over time of platelet suspension under continuous stirring. Platelets in homogeneous suspension limit the passage of light after the addition of an agonist, platelet shape change occurs producing a small transitory increase in OD. Following this initial activation step, platelet clots form gradually, allowing the passage of light through the suspension as a result of decreased OD. The aggregation process is ultimately expressed as a percentage, compared to the OD of platelet-poor plasma or buffer. Rigorous calibration is thus essential at the beginning of each experiment. As a general rule: calibration to 0% is set by measuring the OD of a non-stimulated platelet suspension while measuring the OD of the suspension medium containing no platelets represents a value of 100%. Platelet aggregation is generally visualized as a real-time aggregation curve. Turbidimetry is one of the most commonly used laboratory techniques for the investigation of platelet function and is considered as the historical gold standard and used for the development of new pharmaceutical agents aimed at inhibiting platelet aggregation. Here, we describe detailed protocols for 1) preparation of human washed platelets and 2) turbidimetric analysis of collagen-induced aggregation of human washed platelets pretreated with the food dye Brilliant Blue FCF that was recently identified as an inhibitor of Pannexin1 (Panx1) channels.

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Turbidimetry on human washed platelets: the effect of the Pannexin1-inhibitor Brilliant Blue FCF on collagen-induced aggregation.

AUTHORS:
Filippo Molica, Séverine Nolli, Pierre Fontana, Brenda Renata Kwak

Filippo Molica, PhD
Department of Pathology and Immunology
University of Geneva
Geneva, Switzerland
filippo.molica@unige.ch

Séverine Nolli
Division of Angiology and Haemostasis & Geneva Platelet Group
Geneva University Hospitals
Geneva, Switzerland
severine.nolli@unige.ch

Pierre Fontana, MD PhD
Division of Angiology and Haemostasis & Geneva Platelet Group
Geneva University Hospitals
Geneva, Switzerland
pierre.fontana@hcuge.ch

Brenda Renata Kwak, PhD
Department of Pathology and Immunology
University of Geneva
Geneva, Switzerland
brenda.kwakchanson@unige.ch

CORRESPONDING AUTHOR:
Filippo Molica, PhD

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Platelet aggregation, washed platelets, collagen, turbidimetry, Pannexin1, Brilliant Blue FCF

SHORT ABSTRACT:
We describe a straightforward method for the isolation of washed platelets from human blood followed by agonist-induced platelet aggregation measurements by turbidimetry. As an example we apply this method for studying the aggregation response of human platelets to collagen after a pre-incubation with the Pannexin1 channel inhibitor Brilliant Blue FCF.

LONG ABSTRACT:
Turbidimetry is a laboratory technique that is applied to measure the aggregation of platelets suspended in either plasma (platelet-rich plasma, PRP) or in buffer (washed platelets), by the use of one or a combination of agonists. The use of washed platelets separated from their plasma environment and in the absence of anticoagulants allows for studying intrinsic platelet properties. Among the large panel of agonists, arachidonic acid (AA), adenosine di-phosphate (ADP), thrombin and collagen are the most frequently used. The aggregation response is quantified by measuring the relative optical density (OD) over time of platelet suspension under continuous stirring. Platelets in homogeneous suspension limit the passage of light after the addition of an agonist, platelet shape change occurs producing a small transitory increase in OD. Following this initial activation step, platelet clots form gradually, allowing the passage of light through the suspension as a result of decreased OD. The aggregation process is ultimately expressed as a percentage, compared to the OD of platelet-poor plasma or buffer. Rigorous calibration is thus essential at the beginning of each experiment. As a general rule: calibration to 0% is set by measuring the OD of a non-stimulated platelet suspension while measuring the OD of the suspension medium containing no platelets represents a value of 100%. Platelet aggregation is generally visualized as a real-time aggregation curve. Turbidimetry is one of the most commonly used laboratory techniques for the investigation of platelet function and is considered as the historical gold standard and used for the development of new pharmaceutical agents aimed at inhibiting platelet aggregation. Here, we describe detailed protocols for 1) preparation of human washed platelets and 2) turbidimetric analysis of collagen-induced aggregation of human washed platelets pretreated with the food dye Brilliant Blue FCF that was recently identified as an inhibitor of Pannexin1 (Panx1) channels.

INTRODUCTION:
Platelets are crucial components of blood and their main function—together with coagulation factors—is to stop bleeding after blood vessel injury. Platelets are small (2-3 µm) anuclear fragments derived from megakaryocytes of the bone marrow[1]. Platelets circulate in non-activated state, during which they appear as lens-shaped structures. Upon interruption of the endothelium, platelets gather to the site of blood vessel injury to plug the hole, a process called primary hemostasis. Initially, platelets attach to sub-endothelial molecules, such as collagen and von Willebrand factor, that are exposed as a result of the injury—adhesion step[2]. Then, they change shape and secrete chemical messengers—activation step. Finally, they connect to each other by bridging receptors—aggregation step. Primary hemostasis is followed by a secondary process involving activation of the coagulation cascade with fibrin deposition, which stabilizes the initial thrombus[2].

Acute ischemic events such as myocardial infarction[3] often result from thrombi that form because of physical disruption (rupture) of an atherosclerotic plaque. Current anti-platelet drugs are the cornerstone of the treatment of this widespread disease but their clinical benefit is limited by an increased risk for bleeding. The most prescribed drugs in cardiovascular patients, aspirin and anti-P2Y12 compounds, target the thromboxane A2 and the ADP pathways[4], respectively, which are the major pathways leading to platelet activation. However, innovative research towards new targets that would optimally balance antithrombotic effects and haemorragic risk is still necessary.

From the 1960s[5] to today, turbidimetric aggregometry has played a crucial role in research, enhancing our knowledge of platelet reactivity and in the monitoring of the potency of anti-thrombotic reagents in humans. Turbidimetry was initially applied to PRP extracted from blood samples. Indeed, blood collection performed in tubes containing citrate allows fast and large production of PRP without having any effect on platelet integrity and function. However, the short-term stability (about 3 h) of PRP and the remaining plasmatic enzymes, such as thrombin, and the low calcium concentration associated with potentially artefactual aggregation profiles are of major inconvenience for the use of PRP. An important step forward has been the development of a method for platelet isolation with additional centrifugation and washing steps[6]. In short, PRP is isolated from whole blood collected on acid-citrate-dextrose (ACD) and platelets are isolated after serial centrifugation steps before being resuspended in an iso-osmotic phosphate buffer (Tyrode's buffer) containing glucose, human serum albumin and divalent cations (Ca^{2+} and Mg^{2+}). To avoid changes in platelet reactivity, the pH of Tyrode's buffer is carefully kept at 7.35-7.4. Moreover, undesired activation of platelets is prevented by adding prostacyclin (PGI_2) before some centrifugation steps. Finally, addition of apyrase prevents washed platelets from becoming resistant against the action of ATP/ADP. The resulting platelet suspension lacks coagulant factors and the stability of platelets is increased by at least two-fold as compared to PRP solutions. In addition, the fact that platelets are inactive but intact warrants the reproducibility of turbidimetric measurements and provides the ability to study the action of agonists or antagonists of platelet aggregation in an optimal way.

Using this method, we have shown in a recent study that inhibiting the formation of Panx1 channels by a genetic approach (knock-out mice) or decreasing Panx1 channel activity by pharmacological approaches reduced collagen-induced platelet aggregation[7]. Panx1 forms ATP-release channels, which are ubiquitously expressed in many cell types including human platelets[7,8]. In fact, we demonstrated by turbidimetry on human washed platelets that a 7 min preincubation with a panel of more-or-less specific chemical blockers (probenecid, mefloquine and [10]Panx1 peptides) prior to the addition of various agonists, inhibited specifically collagen-induced platelet aggregation while platelet responses to AA and ADP were not affected. We demonstrated that ATP release through Panx1 channels specifically interferes in the GPVI signaling pathway leading to collagen-induced aggregation. Interestingly, multiple FDA-approved compounds with applications in other diseases (probenecid, mefloquine) affect the activity of Panx1 channels in platelets. On one hand, this opens new therapeutic perspectives to selectively modify platelet reactivity. On the other hand, one should consider potential secondary effects of these compounds. In this context, the safe food dye Brilliant Blue FCF used in multiple candies and energy drinks has been described as a selective inhibitor of Panx1[9]. We describe here a protocol for the isolation of human washed platelets and turbidimetric measurements of platelet aggregation adapted to investigate the effect of the Brilliant Blue FCF dye as an antagonist of platelet aggregation.

PROTOCOL:

Five unrelated healthy volunteers were recruited for blood sampling for platelet isolation and aggregation tests. Written informed consent was obtained and the protocol was approved by the Central Ethics Committee of the University Hospitals of Geneva. All volunteers certified to be healthy and to have not taken any platelet-interfering drugs during at least the 10 days preceding the experiments.

1. Buffer preparation for human blood collection and washed platelet isolation

1.1) Prepare a 100 mL aqueous solution of acid-citrate-dextrose (ACD) by dissolving 1.4 g citric acid monohydrate ($C_6H_8O_7$*H_2O, 66.6 mM), 2.5 g trisodium citrate dihydrate ($Na_3C_6H_5O_7$*2 H_2O, 85 mM) and 2 g of anhydrous D(+)-glucose. The pH of the solution is about 4.5.

1.2) Prepare stock solutions for Tyrode's buffer as follows

1.2.1) Prepare stock solution 1 by dissolving 80 g NaCl, 2 g KCl, 10 g $NaHCO_3$ and 0.58 g NaH_2PO_4*H_2O in 500 mL of distilled H_2O. The respective final concentrations are 2.73 M, 53.6 mM, 238 mM and 8.4 mM. Keep the solution at 4 °C.

1.2.2) Prepare stock solution 2 by dissolving 10.15 g $MgCl_2$*6 H_2O (100 mM) in 500 mL distilled H_2O. Keep solution at 4 °C.

1.2.3) Prepare stock solution 3 by dissolving 10.95 g (100 mM) $CaCl_2$*6 H_2O in 500 mL distilled H_2O. Keep solution at 4 °C.

1.3) Prepare Tyrode's buffer by diluting 2.5 mL of stock solution 1 in a final volume of 50 mL with distilled H_2O. This corresponds to final concentrations of 136.5 mM NaCl, 2.68 mM KCl, 11.9 mM $NaHCO_3$ and 0.42 mM NaH_2PO_4*H_2O. Adjust the pH to 7.35 and sterilize by filtering with 0.22-µm filters.

1.4) Prepare Tyrode's albumin 0.35% buffer (TA buffer[7]) by diluting 5 mL of stock solution 1, 1 mL of stock solution 2, 2 mL of stock solution 3, 0.5 mL 1M HEPES, 1.8 mL of 200 g/L human serum albumin and 0.1 g of anhydrous D(+)-glucose in a final volume of 100 mL distilled H_2O.

1.4.1) Adjust the pH to 7.35 with 1N HCl and set the osmolarity to 295 mOsm/L by adding distilled H_2O (10% of total volume). Final concentrations in this solution are: 124 mM NaCl, 2.44 mM KCl, 10.82 mM $NaHCO_3$, 0.38 mM NaH_2PO_4*H_2O, 0.91 mM $MgCl_2$*6 H_2O, 1.82 mM $CaCl_2$*6 H_2O. Keep TA buffer at 37 °C during the whole experiment.

2. Blood collection

2.1) Collect 45-50 mL of venous blood, from the antecubital vein using a 19-gauge needle and no tourniquet, into 50 mL tubes containing ACD anticoagulant (1 volume ACD for 6 volumes of blood). Discard the first 1-2 mL of blood to avoid the presence of thrombin and tissue factor.

2.1.1) After collection, mix the blood with the ACD by gently inverting the tube. Incubate the sample for 10 min at 37 °C.

3. Preparation of human washed platelets

3.1) Pre-heat the centrifuge to 37 °C. All centrifugation steps below are performed at this temperature.

3.2) Dispatch the collected blood into 15 mL tubes (5 mL per tube) and centrifuge at 250 x g for 13 min to obtain PRP.

Note: This centrifugation step results in the production of three layers in the sample: 1) The upper layer, composed of plasma, platelets, and a small fraction of white blood cells. 2) The intermediate layer, a portion rich in white blood cells. 3) The bottom layer, which is essentially composed of red blood cells.

3.3) Collect the PRP by pipetting the upper layer carefully into a new 15 mL tube to maximally prevent contamination with red and white blood cells, and incubate for 10 min at 37 °C.

3.4) Centrifuge the PRP at 2200 x g for 12 min (for 5 mL PRP).

Note: This centrifugation step should be performed with low brake or without brake.

3.5) Remove the supernatant (platelet-poor plasma), and carefully resuspend the pellet with 10 mL of TA buffer containing 2 µL/mL of heparin (5000 U/mL) and 2.5 µL/mL of 25 µM PGI_2 using a plastic Pasteur pipet. Incubate for 10 min at 37 °C.

3.6) Add 2.5 µL/mL of 25 µM PGI_2 and centrifuge for 8 min at 1900 x g (with low brake or without brake).

3.7) Remove the supernatant and resuspend the pellet with 5 mL TA buffer containing 2.5 µL/mL of 25 µM PGI_2 using a plastic Pasteur pipet. Incubate for 10 min at 37°C.

3.8) During the incubation period, pipet 150 µL of the platelet suspension into a 1.5-mL tube and count platelets, using an automatized cell counter (that detects the size of blood cells by measuring the changes in direct-current resistance).

3.9) After the 10 min incubation, add 2.5 µL/mL of 25 µM PGI_2 to the platelet suspension and immediately centrifuge at 1900 x g for 8 min.

3.10) Remove the supernatant and resuspend the pellet to a concentration of 250,000 platelets/µL with an adequate volume of TA buffer (*i.e.* if the cell count is 500,000 per µL, resuspend in 10 mL TA buffer) containing 32 µL/mL of apyrase at 0.01 U/mL (final concentration

0.32 U/mL).

Note: High concentration of apyrase is used to avoid the desensitization of P2X1 receptors induced by spontaneous secretion of ATP[10,11] in absence of agonists. This is important because collagen-induced responses are induced by fast paracrine/autocrine activation of P2X1 by ATP released from activated platelets. If the platelet signaling pathway does not critically require preservation of P2X1 function, use 0.02 U/mL apyrase. Several studies (reviewed in Mahaut-Smith *et al*.[10]) demonstrated that 0.02 U/mL apyrase avoids ADP receptor P2Y1 desensitization with negligible P2X1 responses.

3.11) Incubate the cell suspension for at least 30 min at 37 °C before performing the aggregometric measurements. The preparation is stable for 5 to 8 h.

4. Aggregometry

4.1) Prepare fibrinogen (56 mg/mL) in Tyrode's buffer.

4.2) Pipet 260 µL of platelet suspension into glass cuvettes (Figure 1A; left cuvette) containing 10 µL of fibrinogen (56 mg/mL) and a magnetic stirring rod, then incubate the suspension for 2-3 min at 37 °C in incubation wells present in the aggregometer (Figure 1B and 1C).

4.3) Pre-incubate with the Panx1 inhibitor Brilliant Blue FCF by adding 10 µL of a 2.8 mM or 28 mM stock solution (final concentration 100 µM and 1 mM, respectively) for 7 min at 37 °C.

4.4) Calibrate the aggregometer to an assumptive 100% aggregation value by measuring the OD of a cuvette containing 10 µL fibrinogen (56 mg/mL), 10 µL Brilliant Blue FCF (2.8 mM or 28 mM) and TA buffer without platelets.

4.4.1) Place the cuvette in an aggregation well under automatic stirring and press the corresponding button on the keyboard of the computer linked to the aggregometer (*i.e.* press F1 if aggregation well 1 is used).

Note: This experiment described below includes Brilliant Blue FCF. The compound used for calibration has to be adjusted to the experimental condition.

4.5) Calibrate the aggregometer to an assumptive 0% aggregation value by using the same platelet sample that will be used for the experiment under automatic stirring.

4.5.1) Place the cuvette in the aggregation well and press the corresponding button on the keyboard of the computer linked to the aggregometer. Wait for about 20-30 s before proceeding. This delay serves to assure that no aggregation happens before adding the agonists.

Note: As any difference in platelet number may have an effect on the measured OD, the 0% calibration step needs to be repeated for each individual measurement.

4.6) Add 20 µL of desired agonist, such as 15 µg/mL collagen (1 µg/mL final) or 1.125 mM arachidonic acid (75 µM final), into the cuvette. Immediately start the recording under continuous automatic stirring by pressing the corresponding button on the keyboard of the computer linked to the aggregometer.

Note: The addition of the agonist induces platelet activation. Platelet aggregates can clearly be distinguished in the glass cuvette at the end of the experiment (Figure 1A; right cuvette).

4.7) The recording automatically stops after 6 min. At this point, save the data by clicking on the save icon of the computer.

Note: The calculation of the rate of aggregation is performed by the computer, which expresses the end results of the aggregation process as a percentage.

4.8) Analyze the data.

4.8.1) For additional extensive information on protocols for the preparation of washed platelets suspensions and turbidimetric measurement of platelet aggregation, refer to other papers authored by experts in the field[12,13].

REPRESENTATIVE RESULTS:

The aggregometer software automatically produces the aggregation curves and gives the values for maximal aggregation in percentage. The values can be copied to a data analysis software in order to perform statistical analysis and visualize maximal aggregation values in form of bar charts. Optionally, each individual point of the aggregation curves can be exported successively into a spreadsheet software and then to statistical software (*e.g.* GraphPad) in order to visualize the curves. Some investigators use the maximal slope of the aggregation curve to calculate the velocity and the area under the curve to assess platelet activity. The lag time and shape change can also be visualized graphically.

A typical example of an aggregation curve of washed human platelets under control conditions (H_2O) is shown in Figure 2A. Addition of the agonist (collagen), induced (after a brief delay) a depression in the aggregation curve caused by the shape change of the platelets. Then, the percentage of aggregation gradually increased over time until a maximum value was reached at about 3-4 min. A slight decrease in the percentage of aggregation was observed towards the end of the 6 min recording, which reflects some disaggregation. As illustrated in Figure 2A-B, preincubating human washed platelets with the Panx1 channel inhibitor Brilliant Blue FCF, at a concentration of 1 mM, slowed down or totally abolished the initial platelet shape change and blocked collagen-induced aggregation of washed platelets obtained from 5 different unrelated healthy volunteers. When platelets where preincubated with a lower concentration (100 µM) of Brilliant Blue FCF, the inhibitory effect of the dye on collagen-induced responses and shape change were not observed anymore (see Figure 2A for an example). Quantification of the platelet aggregation responses induced by 1 µg/mL collagen revealed that 1 mM Brilliant Blue FCF significantly reduced maximal aggregation responses as compared to control conditions as well as to a lower concentration (100 μM) of Panx1 inhibitor (Figure 2C). Inhibition of platelet aggregation was specific for collagen-induced responses and not due to undesired side-effects of Brilliant Blue FCF (on cell viability, for example) as the same concentration of the dye (1 mM) did not affect the aggregation response induced by another agonist, i.e. 75 μM AA, as illustrated in Figure 2D. These results confirm the specific role of Panx1 channels in collagen-induced aggregation responses of human platelets that we and others have previously shown with other pharmacological inhibitors of Panx1 such as probenecid, mefloquine, and the specific ^{10}Panx1 peptides[7,8].

Figure 1: Aggregometry. A: Representative image of glass cuvettes (containing a stirring magnet) used for aggregometry. The cuvette on the left shows resting platelets while the cuvette on the right illustrates platelet aggregates after collagen-induced activation. **B:** Representative image of an 8-well aggregometer for turbidimetric measurements. The wells used (asterisk) to incubate platelet suspensions present in a glass cuvette at 37 °C, and those used for the measurements (white arrow) are indicated in **C**.

Figure 2: A high concentration of Brilliant Blue FCF blocks platelet activation in response to collagen. A: Representative traces of collagen-induced aggregation of human washed platelets obtained from the same healthy volunteer (V1) under control conditions (H_2O; dark blue) or after 7 min preincubation with the Panx1 inhibitor Brilliant Blue FCF at 1 mM (light blue) or at 100 μM (intermediate blue color). Aggregation traces were recorded for 6 min. **B**: Representative traces of collagen-induced aggregation of human washed platelets obtained from 4 healthy volunteers (V2-V5) after 7 min preincubation with the Panx1 inhibitor Brilliant Blue FCF (1 mM). **C**: Quantification of maximal aggregation responses of human washed platelets under control conditions (white bar) or after 7 min preincubation with Brilliant Blue FCF at 100 μM (grey bar) or at 1 mM (black bar). N=5. *****P* < 0.0001; ANOVA followed by Bonferroni's post-test for multiple comparison; Results are expressed as mean ± SEM. **D**: Representative traces of AA-induced aggregation of human washed platelets obtained from 5 healthy volunteers (V1-V5) after 7 min preincubation with the Panx1 inhibitor Brilliant Blue FCF (1 mM).

DISCUSSION:

There is great interest in finding new drugs capable of modulating platelet function in order to prevent thrombosis without enhancing the risk of bleeding. For this purpose, *in vitro* laboratory tests which can reliably and reproducibly monitor aggregation responses in human platelets are absolutely necessary. Turbidimetric aggregometry is an easy technique to perform. However, some precautions need to be kept in mind. The measurements need to be performed under continuous stirring as the aggregation process is largely dependent on stirring. It is also important to keep the platelets at a physiological temperature of 37 °C in order to avoid any type of premature activation. Preactivation of platelets can also occur during blood collection and washed platelets preparation, leading to spontaneous aggregation. Thus careful measures should be taken during the complete procedure of platelet preparation.

Turbidimetry is based on the measurement of the OD of the platelet suspension. This makes the technique unsuitable for aggregation measurement in whole blood due to the presence of red blood cells. Thus, turbidimetry can be performed only on PRP or washed platelets isolated from PRP. Although easy to deal with and rather inexpensive, turbidimetry has been recognized as insensitive to the presence of microaggregates[14,15]. When microaggregates are expected to be of critical importance, impedence aggregometry, which measures the variation in electrical resistance when platelets adhere to an electrode immersed in a citrated whole blood sample, is better suited[16]. The platelet aggregation process is also largely dependent on platelet count in the platelet suspension[17]; flow cytometry is used in patients suffering from thrombocytopenia[18], from whom the maximum number of platelets that can be obtained is insufficient for turbidimetry. However, the general advantage of turbidimetry is that it allows a detailed analysis of platelet reactivity, such as shape change and disaggregation, which cannot be assessed in whole blood samples.

It is important to realize that natural variation in human platelet aggregation exists due to differences in age, sex and life style as well as health status of the subject whose platelet function is challenged[19-21]. Such natural variation may be on the order of 10-20% in the turbidimetric outcomes, thus care should be taken, when performing and interpreting these experiments, in obtaining platelets from sufficient numbers of unrelated healthy volunteers to allow reliable statistics. This limitation is however counterbalanced by the fact that turbidimetric measurements are highly reproducible, at least when aggregation procedures are well standardized across laboratories. Standardization efforts have been made by the International Society of Thrombosis and Haemostasis[22]. For this reason, turbidimetry is still the most commonly encountered test for the measurement of platelet aggregation in both clinical and basic science laboratories and remains a technique of choice for studying the effect of drugs on platelet responses.

In the present study we have demonstrated, using turbidimetry to measure responses in human washed platelets, that a commonly-used food dye Brilliant Blue FCF affects platelet aggregation. Elegant electrophysiological studies have revealed a specific blockade of Panx1 channels by this dye[9,23]. Indeed, high concentrations (1 mM) of this dye showed inhibitory effects on both collagen-induced shape change and maximal aggregation but 10-fold lower concentrations (100 μM) of the dye did not affect the platelet aggregation response. According to the Scientific Opinion on the re-evaluation of Brilliant Blue FCF (E133) as afood additive, published by the European Food Safety Authority[24], the maximal allowable concentration of Brilliant Blue FCF (molecular weight = 792.84 g/mol) is 500 mg/kg of food. For H_2O, this would correspond to 0.63 mM, which is a 1.59-fold lower concentration than the one inhibiting collagen-induced platelet aggregation in our experiments. Assuming that only a small fraction of the dye will be absorbed in the intestines after oral ingestion, and that the dye will finally be diluted in the 5-L volume of blood of an adult person, the daily intake of this blue food dye likely has to largely exceed the normal levels before any side-effects on platelet aggregation may be anticipated. Possible side-effects of the highest concentration of the dye in our experiments on platelet viability, for example, were excluded by the presence of solid aggregation responses to another agonist (AA). These unaffected AA responses also confirmed that inhibitory effects of Brilliant Blue FCF seem to specifically involve the collagen signalling pathway. This is in-line with our earlier study[7] detailing the specific place for ATP release through Panx1 channels in the collagen-induced platelet aggregation response.

By adapting the protocol of washed platelet isolation to this study, we describe a simple and straightforward method for isolating platelets from human blood. After several washing steps performed essentially by centrifugation, platelets are resuspended in a medium that respects precise physiological conditions including pH, working temperature and concentration of divalent ions but assures the absence of plasma proteins. This is an advantage of this technique over alternative methods such as gel filtration in which elimination of plasma components does not occur[25].

All the steps of the washing procedure are of crucial importance to obtain reproducible results when using washed platelets. A plethora of drugs may influence platelet reactivity, thus it is essential that the healthy volunteers are asked to not take any medication for at least 10 days preceding blood collection. In addition, blood collection necessitates that attention is paid to avoid vein trauma or too slow flow as this may cause the generation of thrombin and subsequent platelet activation[22]. Along the same lines, potential platelet activation during the centrifugation and washing steps can be avoided by the repeated use of PGI_2. Due to its very low stability, PGI_2 should be added to each washing step immediately before each centrifugation. The resuspension of platelets in TA buffer (at a physiological pH) containing apyrase ensures that platelet responses remain intact. The presence of apyrase in the platelet suspension is essential to permit the degradation of ATP and ADP in order to preserve platelets from a desensitization of their purinergic receptors. As the Panx1 signaling pathway involves P2X1 receptor activation in human platelets upon collagen receptor activation, we added a relatively high concentration of apyrase (0.32 U/mL) to the platelet suspension in order to avoid P2X1 desensitization[7]. To avoid densensitization of other purinergic receptors in platelets, lower concentrations of apyrase would be sufficient[10].

Although the procedure of serial centrifugations and washings steps are labor intensive and require more time, the washed platelets obtained using this protocol offer the benefit of higher stability at 37 °C (5-8 h) as compared to platelets used directly from PRP (1-3 h). However, the choice of using PRP or washed platelets should be made cautiously and should include considerations such as the quantity of blood available for the experiment, the agonist to be used as well as the question that will be addressed. For example, as binding of fibrinogen to activated α2β3 integrins is the main mechanism mediating platelet aggregation, absence of fibrinogen in washed platelets might influence the reaction; in particular, when weak agonists (such as ADP) are used[13]. This problem is less critical when a powerful agonist is used, such as thrombin or collagen, which are by themselves able to induce the release of fibrinogen from α-granules. Still, we routinely add exogenous fibrinogen to the washed human platelet suspension in order to increase the amplitude of the aggregation response induced by collagen.
In conclusion, by using human washed platelets and turbidimetry, we found that the food dye Brilliant Blue FCF, through its inhibitory effects on Panx1, represents a potential inhibitor of platelet function.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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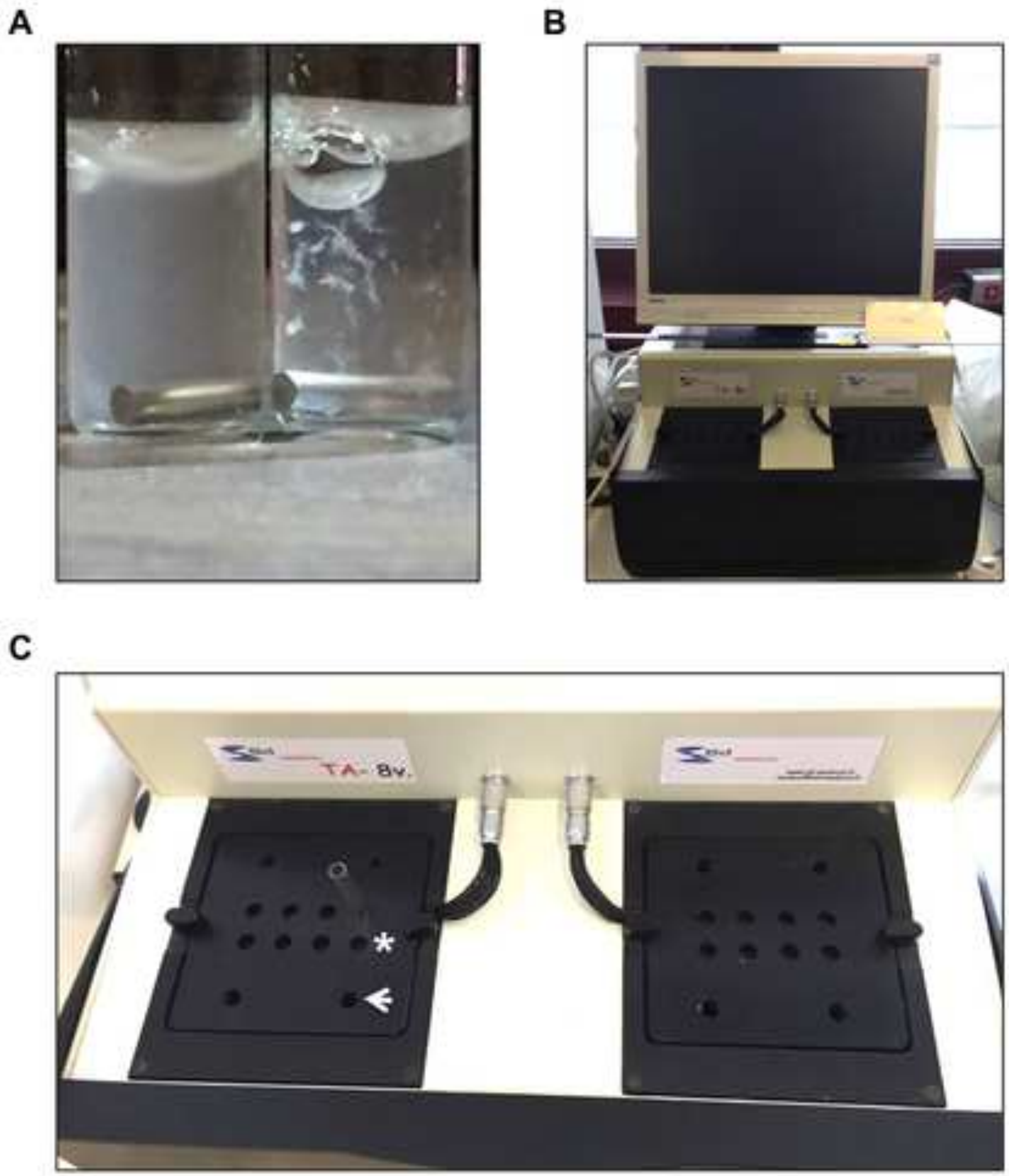
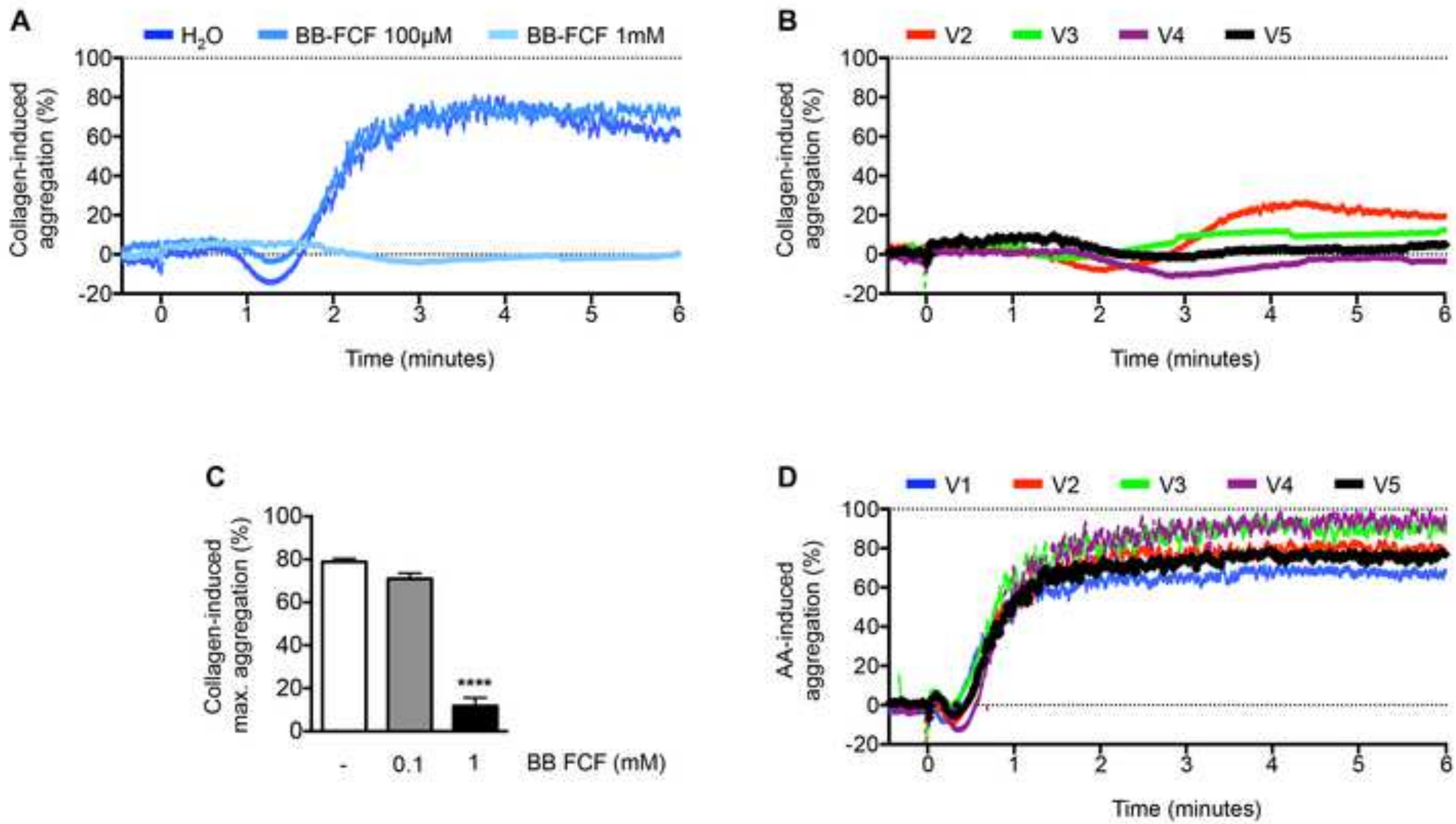


Figure 2


A
H2O
BB-FCF 100μM
BB-FCF 1mM
Collagen-induced aggregation (%)
Time (minutes)
B
V2
V3
V4
V5
Collagen-induced aggregation (%)
Time (minutes)
C
Collagen-induced max. aggregation (%)
****
-
0.1
1
BB FCF (mM)
D
V1
V2
V3
V4
V5
AA-induced aggregation (%)
Time (minutes)

Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
citric acid monohydrate ($C_6H_8O_7*H_2O$)	Roth	5949-29-1	danger of eye damage/irritation
trisodium citrate dihydrate ($Na_3C_6H_5O_7*2H_2O$)	Sigma-Aldrich	S1804	-
D(+)-glucose	Sigma-Aldrich	G8270	-
Sodium chloride (NaCl)	Sigma-Aldrich	S9888	-
Potassium chloride (KCl)	Sigma-Aldrich	P9541	-
Sodium bicarbonate ($NaHCO_3$)	Sigma-Aldrich	S6014	-
Sodium dihydrogenophosphate monohydrate ($NaH_2PO_4*H_2O$)	Sigma-Aldrich	S9638	-
Magnesium chloride hexahydrate ($MgCl_2*6H_2O$)	Sigma-Aldrich	M9272	-
Calcium chloride hexahydrate ($CaCl_2*6H_2O$)	Sigma-Aldrich	442909	danger of eye damage/irritation
N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (Hepes)	ThermoFisher Scientific	15630	-
human serum albumin	CSL Behring	00257/374	-
hydrochloric acid	Sigma-Aldrich	320331	Corrosive and irritative for the respiratory system. Can cause severe skin and eye damages.
Eppendorf 5810 R	Fisher Scientific	-	-
heparin	Drosspharm AG/SA	20810	
prostacyclin I2 (PGI2)	Cayman	18220	-
apyrase from potatoes	Sigma-Aldrich	A6535	-
fibrinogen (Haemocomplettan)	CSL Behring	HS 73466011	-
thrombo-aggragometer SD-Medical	SD-Innovation	TA8V	-
Brilliant blue FCF (Erioglaucine disodium salt)	Sigma-Aldrich	80717	Harmful to aquatic life with long lasting effects (Avoid release to the environnement)
collagen	Horm, Nycomed		-
arachidonic acid	Bio/Data corporation	C/N 101297	-
cell counter Sysmex KX-21N	Sysmex Digitana	-	-
HEPES	Gibco	15630-056	-
glass cuvettes	SD-Innovation	THCV1000	-
magnetic stirrers	SD-Innovation	THA100	-

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Author(s): Filippo Molica; Séverine Nolli; Pierre Fontana; Brenda Renata Kwak.

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CORRESPONDING AUTHOR:

Name: Filippo Molica

Department: Pathology and Immunology

Institution: University of Geneva

Article Title: Turbidimetry on human washed platelets: the effect of the Pannexin1 inhibitor Brilliant Blue FCF on collagen-induced aggregation

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Département de pathologie et immunologie

Dr. Alisha DSouza
Review Editor
JoVE

MS: JoVE55525

Title: Turbidimetry on human washed platelets: the effect of the Pannexin1 inhibitor Brilliant Blue FCF on collagen-induced aggregation.

Dear Dr. DSouza,

Thank you for your recent letter regarding the above-mentioned manuscript. We thank the editors and reviewers for their constructive comments and suggestions.

We thoroughly revised the manuscript to address the points raised by the editors and reviewers. In short, more details on the protocol steps are now given, figures have been modified following the suggestions of the reviewers and the discussion has been extended.

For your information, all the changes specified under Editorial comments have been applied to the references, keywords, figures and figure legends, table of essential supplies, reagents and equipment, protocol steps as well as to the discussion have been performed. Of note, the signed Article and Video License Agreement has been sent by Email to Teena Mehta on October 6th, 2016. We have now uploaded it again in the *JoVE* website.

We are looking forward to your response regarding the acceptability of our revised manuscript for publication in the *JoVE*.

Yours Sincerely,

Filippo Molica, PhD
Postdoctoral Assistant

Brenda R. Kwak, PhD
Associate professor

Dept. of Pathology and Immunology
Dept. of Medical Specializations – Cardiology
University of Geneva

Reviewer #1:

We thank the reviewer for his/her constructive suggestions that helped us to improve our manuscript. We respond to each of his/her remarks in detail below. *Comments of the reviewer are cited in italics.*

Major concerns:

*Protocol step 1.2.1 requires 80g NaCl, which results in a concentration of 2.7 molar, not millimolar, NaCl in stock solution 1. Also, by my calculations, 0.58g NaH2PO4*H2O (138g/mol) is 8.4mM, not 8.6mM. Additionally, given the complexity of the stock buffers being mixed to create final buffers for use, it would be helpful to list the final concentrations of each component of the actual buffers, not only the stocks to be further diluted. As multiple variants of Tyrode's Buffer are searchable online, it is important to clarify what final ionic content your protocol seeks. E.g. at the end of 1.3, "Final concentrations are (in mM) NaCl 136.5, KCl 2.7, NaHCO3 11.9, NaH2PO4 0.4..."*

We thank the reviewer for this thoughtful comment. All the concentrations have been recalculated and the correct values are now given in the text. As suggested, the final concentrations of each component of the solutions and buffers are now given in protocol steps 1.2.1, 1.3 and 1.4.

- Apyrase is added to "avoid [allowing] washed platelets to become resistant against the action of ADP," per the introduction. However, apyrase is added at step 3.10 in the protocol and appears to remain in the suspension of platelets throughout the experiment. As you are describing a response that you claim is based on an extracellular ATP/ADP-driven signaling pathway, the presence of "a relatively high concentration of" a potent ATP/ADP-hydrolyzing enzyme throughout the experiment is a major methodological concern

Apyrase is added to the platelet suspension in order to reduce the risk of potential activation of platelets by extracellular ATP/ADP, which will cause desensitization of purinergic receptors. In this study, we investigate aggregation responses to collagen, which critically depends on a fast activation of P2X1 receptors by ATP released from activated platelets. To further clarify this issue, we have now added a phrase in the note following protocol step 3.10.

- The overall conclusion exceeds the scope of the paper for two reasons:
1) No mention of Panx1 is made in this paper, beyond citations of previous work discussing Brilliant Blue and a role for Panx1 in platelet aggregation. To claim that Brilliant Blue has the claimed effects on platelets via action on Panx1, additional experiments would be needed. You currently offer as comparison the activation of a different pathway via arachidonic acid, showing that BB effects are specific to the collagen pathway, but don't show that the BB effects are due to inhibition of Panx1 in particular. Use of other pharmacologic inhibitors of Panx1 (e.g. Trovafloxacin, Probenecid, Carbenoxolone, 10Panx1) or genetic knockout of Panx1 to confirm the effects of BB as acting via Panx1 would solidify this claim.

Our manuscript has been written to join the overall goal of *JoVE*, *i.e.* providing the readers with written and visual detailed protocols of scientific experiments. As such the experiments involving Brilliant Blue were intended as a working example and justified by and asked for by *JoVE* on the basis of our earlier publication in *Thrombosis and Haemostasis*. We agree with the reviewer that investigating the effect of Brilliant Blue FCF on collagen responses of platelets extracted from Panx1$^{-/-}$ mice would confirm that the inhibitory action of the dye on platelet aggregation is due to its action on Panx1 channels. Given the short time given by *JoVE* for this revision (2 weeks), these experiments can unfortunately not be performed due to lack of availability of Panx1$^{-/-}$ mice. Moreover, the protocol to prepare washed platelets from mice differs considerably from the protocol for human washed platelets, and is beyond the scope of this manuscript.

2) BB is suggested here as a non-toxic potential inhibitor of platelet function, but earlier in the manuscript you note that intake of BB "likely has to largely exceed the normal levels before any side effects on platelet aggregation may be anticipated." Thus you have already stated that BB dye cannot be used as a potential platelet inhibitor at the concentrations known to be non-toxic, and no detail is given of the therapeutic window of BB or the extent of the gap between food-related concentrations and platelet-inhibiting concentrations of the dye. To quote the age-old toxicology adage, "the dose makes the poison." It is inappropriate to claim that BB dye may be expected simultaneously to be non-toxic while used at levels presumably far higher than for existing uses, unless evidence exists to support this claim. Unless data or citations exist to back up this conclusion, it should be revised to reflect more accurately the limited extent of the data contained in this paper.

The reviewer is right that it is inappropriate to claim that BB dye may be expected simultaneously to be non-toxic while used at levels presumably far higher than for existing uses in daily food practice. Our intentions with the word "toxic" were however different, i.e. we were not referring to toxicity for the human race but rather to side-effects in the experiments that could mask an anticipated experimental outcome. We have changed the paragraph in the discussion accordingly (p.9; 4th paragraph of the discussion section).

Minor concerns:

- The first paragraph of introduction should include a citation, e.g. to a review of platelet function. It should also be edited to read in complete sentences instead of as an unformatted list in paragraph form.

As suggested by the reviewer, we added two new references in the first paragraph of the introduction (Patel *et al. J Clin Invest.* **115** (12), 3348-3354, Andrews *et al. Thromb Res.* **114** (5-6), 447-453).

In protocol step 1.4, what is the osmolarity of the buffer? This should be calculable. Does an osmolarity adjustment always require the addition of 10% total volume of H2O at this step? If so, this volume should be included in the final volume of the previous sentence (110mL instead of 100mL diH2O). Or, simply instruct to test osmolarity and adjust to 295mOsm/L. How much H2O is acceptable to add for this purpose before significantly changing the ionic concentration of the buffer to an unacceptable extent? Please advise.

We provide the value of the desired osmolarity (295 mOsm/L) in case other investigators would like to measure it. In practice however it always appeared correct to simply add 10% of the total volume H_2O, which we now standardly do.

In protocol step 3.2, clarify what portion of the centrifuged blood to collect, e.g. "PRP will be the top layer of liquid in the tube, above the cell pellet of white and then red blood cells." Additionally, in 3.3, avoiding contamination should reference avoiding the entire cell pellet, including the buffy coat of white blood cells, not only the underlying red blood cell pellet.

Following the suggestion of the reviewer, we have now added the requested information in a note after protocol step 3.2. (page 5).

Protocol step 3.7 should read "TA buffer" per the previously noted abbreviation, not "TA."

The change has been made.

In the discussion, what are "normal levels' of blue food dye? Can you provide a rough estimate of order of magnitude of dietary levels versus that required for platelet aggregation?

We thank the reviewer for raising this interesting point. We give now information published by the European Food Safety Authority on the levels of Brilliant Blue FCF in food and compare them to the concentration used in our experiments. Moreover we state now that "assuming that only a small fraction of the dye will be absorbed in the intestines after oral ingestion and that the dye will finally be diluted in the 5 L blood of an adult person, the daily intake of this Blue food dye likely has to largely exceed the normal levels before any side-effects on platelet aggregation may be anticipated." (p.9; 4th paragraph of the discussion section).

In protocol step 3.8, give the parameters for identification of platelets using an automated cell counter - e.g. min/max size, roundness, etc. It should not be assumed that all counters use the same parameters, or have preset parameters by cell type.

The automatized cell counter used to determine the number of platelets detects the size of blood cells by measuring the changes in direct-current resistance. This information has been added to protocol step 3.8.

In the last sentence of the second-to-last paragraph of your discussion, why would a lower concentration of apyrase be sufficient to avoid desensitization of other PRs in platelets? If you have data or a citation for ATP levels that cause desensitization of different PRs in platelets, please back up this claim. Otherwise, it would not logically follow given the expression of other PRs in platelets, including P2YRs (including P2Y1 and P2Y12, both accepted to have roles in platelet aggregation) with ATP/ADP affinities more sensitive by an order of magnitude or greater, than the P2X1R that is your main concern.

As specified in the manuscript, we use a relatively high concentration of apyrase to avoid the desensitization of P2X1 receptors by spontaneous secretion of ATP in absence of agonists. However, when the studied platelet signaling pathway does not critically require preservation of P2X1 function, a final concentration 0.02 U/mL apyrase may be used. This statement is in accordance with many studies (reviewed in Mahaut-Smith *et al. Purinergic Signal.* **7** (3), 341-356) demonstrating that 0.02 U/mL apyrase avoids ADP receptor P2Y1 desensitization with negligible P2X1 responses. This information and reference is now added to the manuscript in a note after protocol step 3.10. (page 5/6)

Reviewer #2:

In the paragraph 1 in the Introduction, at platelet adhesion step, besides collagen, VWF is also involved. This part should be rewrote.

We thank the reviewer for this insightful remark. We now mention of Von Willebrand factor's role in platelet adhesion after injury in the first paragraph of the introduction. (page 2)

Some spelling error in the text," a-nuclear" --"anuclear","such a myocardial infarction"--"such as myocardial infarction","resupension"--"resuspension".Please check the whole manuscript carefully.

A careful check throughout the manuscript has been done to correct spelling errors.

Reviewer #3:

Major concerns:

Figure 1: instead of representative images of the aggregometer herein employed, which may vary depending on the manufacturer, authors could provide imagens of cuvettes containing washed platelets either in solution of after the aggregation process. These imagens easily apply to any device.

We thank the reviewer for this attractive suggestion. Accordingly, we modified Figure 1A into a glass cuvette containing resting platelets or activated platelets that formed aggregates after collagen activation.

Figure 2: Should we expect intermediary aggregation curves by using 250 or 500 µM Brilliant Blue FCF? Representative curves showing the maximum effect (i.e. no aggregation) vs no effect towards collagen-induced aggregation fall to exemplify a dose-response curve. In addition, it would be informative to make clear that other parameters could be extracted from the aggregation curves (slope, area etc.).

Our manuscript has been written to join the overall goal of *JoVE*, *i.e.* providing the readers with written and visual detailed protocols of scientific experiments. As such the experiments involving Brilliant Blue were intended as a working example and justified by and asked for by *JoVE* on the basis of our earlier publication in *Thrombosis and Haemostasis*. We completely agree with the reviewer that other parameters could be extracted from the aggregation curves (slope, area etc.) and have added this valuable information to the manuscript (point 4.8; page 7). Although we also agree that showing aggregation curves after pre-incubation of human washed platelets with intermediate concentrations of Brilliant Blue FCF might be informative, these additional experiments have not been performed given the short time given by *JoVE* for this revision (2 weeks).

Minor concerns:

On page 3, Introduction section, 1st paragraph: Here the authors provide the basic information concerning the role of platelets in the hemostatic system but no references are given.

As suggested by the reviewer, we added two new references in the first paragraph of the introduction (Patel *et al. J Clin Invest.* **115** (12), 3348-3354, Andrews *et al. Thromb Res.* **114** (5-6), 447-453).

On page 9, Discussion section, 2nd paragraph: authors discuss the possible sources of individual variability in the aggregation assays but, again, no references have been provided.

Some references (Fusegawa *et al. Thromb Res.* **93** (6), 271-278, Davis *et al. Med Sci Sports Exerc.* **22** (1), 49-53, Patel *et al. FEBS Lett.* **588** (8), 1372-1378) concerning the individual variability point have now been added to the manuscript.

It would be nice to expand the discussion by including other inhibitors of collagen-mediated platelet aggregation including synthetic compounds and naturally-occurring factors.

This *JoVE* manuscript focusses on the methodology of preparing washed human platelets and performing aggregation experiments. The effect of Brilliant Blue on collagen-induced platelet aggregation is only intended as an illustration. We therefore consider an extensive discussion on other inhibitors of collagen-mediated platelet aggregation beyond the scope of this manuscript.